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Production of 8-hydroxy-9,12(*Z*,*Z*)-octadecadienoic acid from linoleic acid by recombinant cells expressing H1004A-C1006S variant of *Aspergillus nidulans* diol synthase

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ABSTRACT

An H1004A-C1006S variant of *Aspergillus nidulans* diol synthase was identified as an 8-dioxygenase. Whole recombinant *Escherichia coli* cells expressing the double-site variant showed the highest activity for converting linoleic acid to 8-hydroperoxy-9,12(*Z*,*Z*)-octadecadienoic acid (8-HPODE), which in turn was reduced to 8-hydroxy-9,12(*Z*,*Z*)-octadecadienoic acid (8-HODE) by the addition of a reducing agent. The optimization of 8-HPODE production was performed by response surface methodology, and the optimal conditions were pH 8.3, 27.2 °C, 22.7% dimethyl sulfoxide, 27.0 g l^{-1} cells, and 16.5 g l^{-1} linoleic acid in a 100 ml baffled flask containing a 10 ml reaction mixture with agitation at 236 rpm. The reduction of 8-HPODE to 8-HODE was highest when Tris(2-carboxyethyl)phosphine hydrochloride (TECP-HCl) was added at a final concentration of 25 mM. Under the optimized reaction and reduction conditions, whole cells expressing H1004A-C1006S variant of *A. nidulans* diol synthase produced 5.0 g l^{-1} 8-HODE for 1 h without the formation of di-hydroxy fatty acids. This is the first report of the biotechnological production of 8-HODE.

1. Introduction

Hydroxy fatty acids exist widely in nature as components of wax, triglycerols, cerebrosides, and other lipids in plants, animals, insects, and microorganisms [1–3]. Hydroxy fatty acids are important compounds, used as starting materials for waxes, nylons, resins, plastics, lubricants, and biopolymers, as additives in paintings and coatings, and as precursors of lactones [4–6] due to higher viscosity, reactivity, and solubility than non-hydroxylated fatty acids [7].

Hydroxy fatty acids are produced from fatty acids by microbial fatty acid oxygenases, including cytochrome P450, lipoxygenase, hydratase, 12-hydroxylase, and diol synthase [8]. Lipoxygenase catalyzes dioxygenation of unsaturated fatty acids with one or more (1*Z*,4*Z*)-pentadiene moieties to form (1*S*,2*E*,4*Z*)-hydroperoxy fatty acid [9]. Fatty acid hydratase catalyzes hydration for non-conjugated carbon double bonds in unsaturated fatty acids to produce 10-hydroxy fatty acids [10], while 12-hydroxylase catalyzes hydroxylation of unsaturated fatty acids to form 12-hydroxy unsaturated fatty acids [11]. When 12-hydroxylase catalyzes hydroxylation of linoleic acid, 12*Z* carbon double-bond in linoleic acid changes to 15*Z*. Cells containing lipoxygenase, hydratase, and 12-hydroxylase convert linoleic acid to mono-hydroxy fatty acids such as 9-hydroxy-10,12(*E*,*Z*)-octadecadienoic acid and 13-hydroxy-9,11(*Z*,*E*)-octadecadienoic acid [12,13], 10-hydroxy-12(*Z*)-octadecenoic acid and 13-hydroxy-9(*Z*)-octadecenoic acid [14,15], and 12-hydroxy-9,15(*Z*,*Z*)-octadecadienoic acid [11], respectively. These mono-hydroxy unsaturated fatty acids have antifungal [16,17], anti-inflammatory [18], and surface tension-reducing activities [19].

Diol synthase is a fusion protein of N-terminal fatty acid-heme peroxidase (dioxygenase) domain and C-terminal cytochrome P450-heme thiolate (hydroperoxide isomerase) domain. Fatty acid is converted to hydroperoxy fatty acid by dioxygenase activity in the peroxidase domain, which in turn is isomerized to di-hydroxy fatty acid by hydroperoxide isomerase activity in the P450 domain [20]. The intermediate product, 8-hydroperoxy-9,12(*Z*,*Z*)-octadecadienoic acid (8-HPODE) is easily reduced to 8-hydroxy-9,12(*Z*,*Z*)-octadecadienoic acid (8-HODE) by a reducing agent and has been produced in small quantities during the conversion of linoleic acid to 5,8-, 7,8-, and 8,11-dihydroxy-9,12(*Z*,*Z*)-octadecadienoic acid (diHODE) by 5,8-, 7,8-, and 8,11-diol

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synthases, respectively [20–23]. However, 8-HODE production using only dioxygenase activity in the peroxidase domain of diol synthase has not yet been attempted.

In this study, whole recombinant cells expressing H1004A-C1006S variant of *Aspergillus nidulans* diol synthase as 8-dioxygenase were used to convert linoleic acid to 8-HPODE, which was reduced to 8-HODE by a reducing agent. The reaction conditions such as pH, temperature, solvent, agitation speed, and the concentrations of cells and substrate were optimized. After the reaction, the type and concentration of reducing agent were optimized for 8-HODE production. Under the optimized reaction and reduction conditions, 8-HODE was produced without further hydroxylation to form 5,8-diHODE.

2. Materials and methods

2.1. Preparation of fatty acid standards

Fatty acid standards, including palmitoleic acid (C16:$1^{\Delta 9Z}$), stearic acid (18:0), oleic acid (C18:$1^{\Delta 9Z}$), elaidic acid (C18:$1^{\Delta 9E}$), linoleic acid (C18:$2^{\Delta 9Z,12Z}$), ricinoleic acid (18:$1^{\Delta 9Z,12R}$), α-linolenic acid (C18:$3^{\Delta 9Z,12Z,15Z}$), γ-linolenic acid (18:$3^{\Delta 6Z,9Z,12Z}$), arachidonic acid (20:$4^{\Delta 5Z,8Z,11Z,14Z}$), and erucic acid (C22:$1^{\Delta 13Z}$) were purchased from Sigma–Aldrich (St, Louis, MO, USA). Whole recombinant cells expressing H1004A-C1006S variant were used for the preparation of mono-hydroxy fatty acids, including 8-hydroxy-9(*Z*)-hexadecenoic acid, 8-hydroxy-9(*Z*)-octadecenoic acid, 8-HODE, and 8-hydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from palmitoleic acid, oleic acid, linoleic acid and α-linolenic acid, respectively. The reactions were performed at 35 °C in 50 mM Tris–HCl buffer (pH 8.0) containing 6 g l^{-1} unsaturated fatty acid and 15 g l^{-1} cells with shaking at 200 rpm for 1 h. After the reaction, purification of these compounds was performed with solvent fractional crystallization at low temperature [24] and semi-prep HPLC system as previously described [25].

2.2. Microorganisms, plasmids, and culture conditions

A. nidulans ATCC 10074, *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK), and pET-21a(+) plasmid (Novagen, Madison, WI, USA) were used as the sources of DNA template for the diol synthase gene, host cells, and expression vector, respectively. *A. nidulans* was incubated at 28 °C in a 500 ml baffled flask containing 100 ml potato dextrose broth (PDB) with shaking at 150 rpm for 5 days. For protein expression, recombinant *E. coli* cells expressing H1004A-C1006S variant were cultivated in a 2-l flask containing 500 ml of Luria–Bertani (LB) medium and 20 μg ml^{-1} ampicillin at 37 °C with shaking at 200 rpm. When the optical density of the bacterial culture reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce enzyme expression. The culture was incubated at 16 °C with shaking at 150 rpm for 16 h to express the enzyme.

2.3. Gene cloning and site directed mutagenesis

Gene cloning of diol synthase from *A. nidulans* was performed as previously described [25]. Cysteine at position 1006 and histidine at position 1004, catalytic residues, in the P450 domain of diol synthase from *A. nidulans* were replaced with serine and alanine by site-directed mutagenesis using a Muta-Direct kit (Intron, Seongnam, South Korea), respectively. DNA sequencing was performed at the Macrogen facility (Seoul, South Korea).

2.4. Determination of specific activity and kinetic parameters

The reactions were performed at 35 °C in 50 mM Tris–HCl (pH 8.0) containing 0.28 g l^{-1} linoleic acid and 0.044 g l^{-1} cells for 10 min or containing 0.14 g l^{-1} linoleic acid and 0.05 g l^{-1} enzyme for 5 min. Prior to the optimization for the reducing agent type and concentration, the reduction of 8-HPODE in the reaction solution to 8-HODE was performed by adding cysteine at a final concentration of 100 mM and incubating on ice for 20 min. The specific activity of the cells or enzyme was defined as an increase in the amount of product per amount of cells or enzyme per unit reaction time. To determine the kinetic parameters for the H1004A-C1006S variant, the reactions were performed at 35 °C in 50 mM Tris–HCl buffer (pH 8.0) containing various amount of substrates (62.5–500 μM) and 0.05 g l^{-1} enzyme. Palmitoleic acid (16:$1^{\Delta 9Z}$), oleic acid (18:$1^{\Delta 9Z}$), linoleic acid (18:$2^{\Delta 9Z,12Z}$), and α-linolenic acid (18:$3\Delta^{9Z,12Z,15Z}$) were used as substrates. Kinetic parameters were determined from the Hans–Woolf plot derived from the Michaelis–Menten equation.

2.5. Statistical analysis

The response surface methodology experiments using cells expressing H1004A-C1006S variant were designed and analyzed by MINITAB RELEASE 17. The three independent variables for 8-HODE production were labeled as X_1, X_2, and X_3, and are coded by pH (−1, 0, and 1 as 7, 8, and 9), temperature (−1, 0, and 1 as 25 °C, 35 °C, and 45 °C), dimethyl sulfoxide concentration (−1, 0, and 1 as 10%, 25%, and 40%), respectively. The reactions for 15 experimental runs of X_1, X_2, and X_3 were performed in 50 mM Tris–HCl buffer containing 6 g l^{-1} linoleic acid and 15 g l^{-1} cells with agitation at 200 rpm in a 100 ml baffled flask containing a 10 ml reaction mixture for 1 h. The another three independent variables were labeled as X_4, X_5, and X_6 and are coded by cell concentration (−1, 0, and 1 as 10 g l^{-1}, 25 g l^{-1}, and 40 g l^{-1}), substrate concentration (−1, 0, and 1 as 5 g l^{-1}, 12.5 g l^{-1}, and 20 g l^{-1}), and agitation speed (−1, 0, and 1 as 100 rpm, 185 rpm, and 270 rpm), respectively. The reactions for 15 experimental runs of X_4, X_5, and X_6 were performed at 27.2 °C in 50 mM Tris–HCl buffer (pH 8.3) in the presence of 22.7% (v/v) dimethyl sulfoxide in a 100 ml baffled flask containing a 10 ml reaction mixture for 1 h.

2.6. Reduction of 8-HPODE to 8-HODE by a reducing agent

The reactions using cells expressing H1004A-C1006S variant were performed in 50 mM Tris–HCl buffer (pH 8.0) containing 16.5 g l^{-1} linoleic acid, and 27.0 g l^{-1} cells in the presence of 22.7% (v/v) dimethyl sulfoxide at 27.2 °C with agitation at 236 rpm for 1 h. After the reaction, 8-HPODE in the reaction solution was reduced to 8-HODE with 50 or 100 mM reducing agent, including cysteine, Tris(2-carboxyethyl) phosphine hydrochloride (TECP-HCl), 2-mercatoethanol, $NaBH_4$ or KOH on ice for 20 min. The effect of TECP-HCl concentration on the transformation of 8-HPODE to 8-HODE was investigated by varying its concentration from 0 to 50 mM.

2.7. Production of 8-HODE from linoleic acid

The time-course reactions of 8-HODE production from linoleic acid by whole recombinant cells expressing H1004A-C1006S variant were conducted at 27.2 °C in 50 mM Tris–HCl buffer (pH 8.0) containing 16.5 g l^{-1} linoleic acid, 27.0 g l^{-1} cells, and 22.7% (v/v) dimethyl sulfoxide with shaking at 236 rpm for 3 h. After the reactions were completed, 8-HPODE in the reaction solutions was reduced to 8-HODE by adding TECP-HCl at a final concentration of 25 mM on ice for 20 min.