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Enzymatic Production of 15-Hydroxyeicosatetraenoic Acid from Arachidonic Acid by Using Soybean Lipoxygenase

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15-Hydroxyeicosatetraenoic acid (HETE), as a mammalian biologically active metabolite, has anticarcinogenic effect. The conditions of producing 15-HETE from arachidonic acid by using soybean lipoxygenase were optimal at pH 8.5 and 20°C with 9 g/l arachidonic acid, 54.4 U/ml soybean lipoxygenase, and 4% methanol. Under these optimized conditions, the enzyme produced 9.5 g/l 15-HETE after 25 min, with a molar conversion yield of 99% and a productivity of 22.8 g l^{-1} h^{-1}. To the best of our knowledge, this is the first biotechnological production of 15-HETE.

Keywords: 15-Hydroxyeicosatetraenoic acid, arachidonic acid, soybean lipoxygenase, enzymatic production

Hydroxyeicosatetraenoic acids (HETEs) as hydroxy fatty acids, including 5-HETE, 12-HETE, and 15-HETE, are biologically active metabolites that influence cell signaling, immunity, structure, and metabolism [2]. In particular, 15-HETE has anticarcinogenic effects by inhibiting cancer cell proliferation and inducing their apoptosis [3, 13]. All of these HETEs can be synthesized from arachidonic acid by the action of lipoxygenase enzymes.

Lipoxygenases (E.C. 1.13.11.12) are a family of dioxygenases that catalyze regiospecific dioxygenation of polyunsaturated fatty acids with *cis*,*cis*-1,4-pentadiene moieties, to form hydroperoxy fatty acids, which can then be reduced to hydroxy fatty acids [11]. Soybean lipoxygenase was the first reported lipoxygenase and therefore has been extensively studied. This enzyme has activity not only for linoleic acid but also for arachidonic acid [12]. Although the qualitative conversion of arachidonic acid to 15-HETE by lipoxygenase has been reported [10], the quantitative production of HETE has not yet been attempted.

In this study, the reaction conditions for the production of 15-HETE from arachidonic acid were optimized by using soybean lipoxygenase. Under the optimized conditions, the enhanced production of 15-HETE was achieved.

To prepare the arachidonic acid substrate, the emulsifier Tween 40 at 1 mM was added to 20 mM arachidonic acid in distilled water. After the mixture was homogenized by sonication, the homogenized solution was used as the substrate [15]. The reactions for the effects of pH, temperature, and solvent were done in a Beckman DU-700 spectrophotometer. The blank contained reaction mixture without soybean lipoxygenase. The lipoxygenase activity was determined at 20°C by the increase in absorbance at 234 nm. The extinction coefficient of 23,000 M^{-1} cm^{-1} for 15-HETE was used in calculating the activity of the enzyme [6, 7]. The reactions for the effects of enzyme and substrate concentration and the production of 15-HETE were performed in a 50 ml falcon tube containing 10 ml of reaction mixture. One unit of activity was defined as enzyme required to produce 1 μmol 15-HETE per minute at 20°C. The concentrations of arachidonic acid and 15-HETE were determined using HPLC with a UV detector at 202 nm and a phenomenex nucleosil C18 column [1]. 15-HETE was identified based on retention time, which was the same as that of the standard 15-HETE (Supplemental Fig. S1).

The effect of pH on the activity of soybean lipoxygenase for arachidonic acid was measured at 20°C for 5 min using 50 mM HEPES buffer (pH 7.5–8.0), 50 mM EPPS buffer (pH 8.0–8.5), and 50 mM CHES buffer (pH 8.5–9.5) containing 0.2 mM arachidonic acid and 0.014 U/ml lipoxygenase. To determine the effect of temperature on the activity, the reactions were performed in 50 mM EPPS buffer (pH 8.5) containing 0.2 mM arachidonic acid and 0.014 U/ml lipoxygenase for 5 min by varying the temperature from 10°C to 40°C. The activity of soybean lipoxygenase for arachidonic acid to produce 15-HETE was found to be maximal at pH 8.5 and 20°C (Supplemental Fig. S2). The reaction pH and temperature of soybean lipoxygenase for linoleic acid [9], α-linolenic acid [5], and γ-linolenic acid [8] were 9.0 and 4°C; 9.0 and 20°C; and 9.5 and 25°C, respectively. The activity of soybean lipoxygenase was maximal in the conditions of relatively basic pH and low temperatures because its fatty acid substrates are more soluble in alkaline conditions, and also because soybean lipoxygenase is stable at low temperatures.

The effect of solvent type and concentration on the activity were evaluated using various solvents, including ethanol, methanol, isooctane, hexane, dimethyl sulfoxide, chloroform, toluene, and dichloromethane, at the concentrations of 5% and 10%. The effects of methanol concentration were assessed by varying the concentration from 0 to 10%. The production of 15-HETE from arachidonic acid was highest when methanol was used as the solvent (Fig. 1a), and its optimal concentration was 4% (v/v) (Fig. 1b). Thus, 4% (v/v) methanol was used for 15-HETE production. However, in the reactions of soybean lipoxygenase for linoleic acid, γ-linolenic acid, and linoleic acid as substrates, octane [4], hexane [8], and isooctane [12], respectively, were used as solvents.

To determine the enzyme concentration for maximum 15-HETE production, the enzyme activity was tested at concentrations of 3–65 U/ml. The reactions were performed in 50 mM EPPS buffer (pH 8.5) containing 9 g/l arachidonic acid and 4% methanol at 20°C for 20 min (Fig. 3a). 15-HETE production increased with increasing enzyme concentration and reached a plateau above 54.4 U/ml soybean lipoxygenase. The effect of substrate concentration for 15-HETE production was investigated by varying the concentration of arachidonic acid. The reactions were performed in 50 mM EPPS buffer (pH 8.5) containing 54.4 U/ml soybean lipoxygenase and 4% methanol at 20°C for 20 min (Fig. 2b). At up to 9 g/l arachidonic acid, increases in substrate concentration led to proportional increases in 15-HETE production and the molar conversion yield was constant, at approximately 99%. However, above 9 g/l arachidonic acid, the molar conversion yield decreased with increasing the concentration of arachidonic acid. Therefore, the optimum concentrations of substrate and enzyme for 15-HETE production were determined to be 9 g/l arachidonic acid and 54.4 U/ml soybean lipoxygenase, respectively.

Under the optimized conditions, time-course reactions for 15-HETE production were performed (Fig. 3). 15-HETE concentration increased with reaction time, reaching a maximum value of 9.5 g/l 15-HETE at 20 min. After 20 min, the molar conversion yield and productivity of 15-HETE from arachidonic acid by the enzyme were 99%

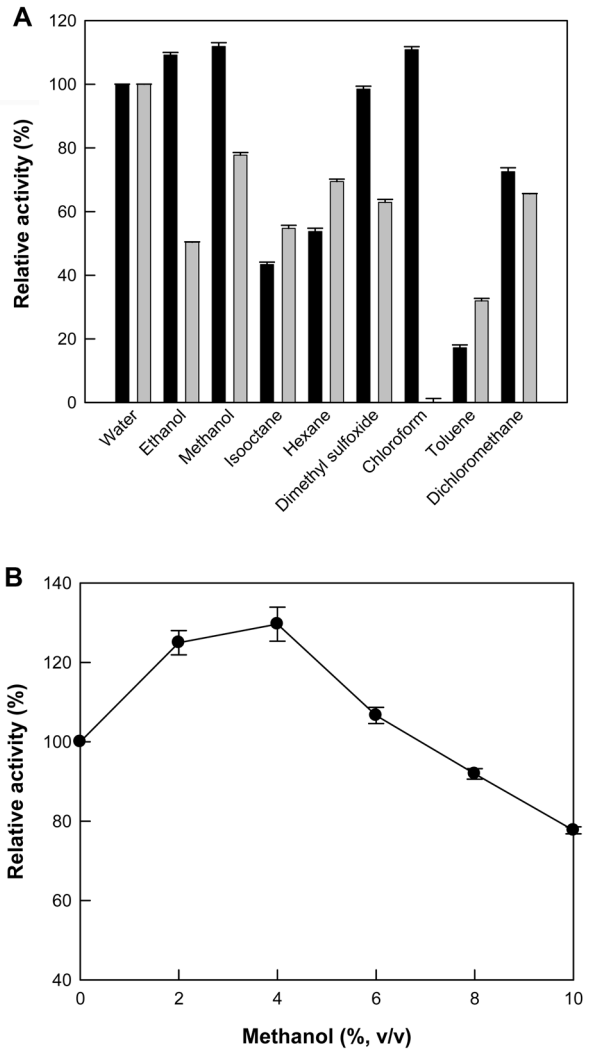

Fig. 1. Effect of solvent on the activity of soybean lipoxygenase for arachidonic acid.

(**A**) Effect of solvent type at 5% (black bar) or 10% (v/v) (gray bar) solvent. (**B**) Effect of methanol concentration. 100% activity was 5.44 U/mg for arachidonic acid. Data represent the means of three experiments and error bars represent standard deviation.

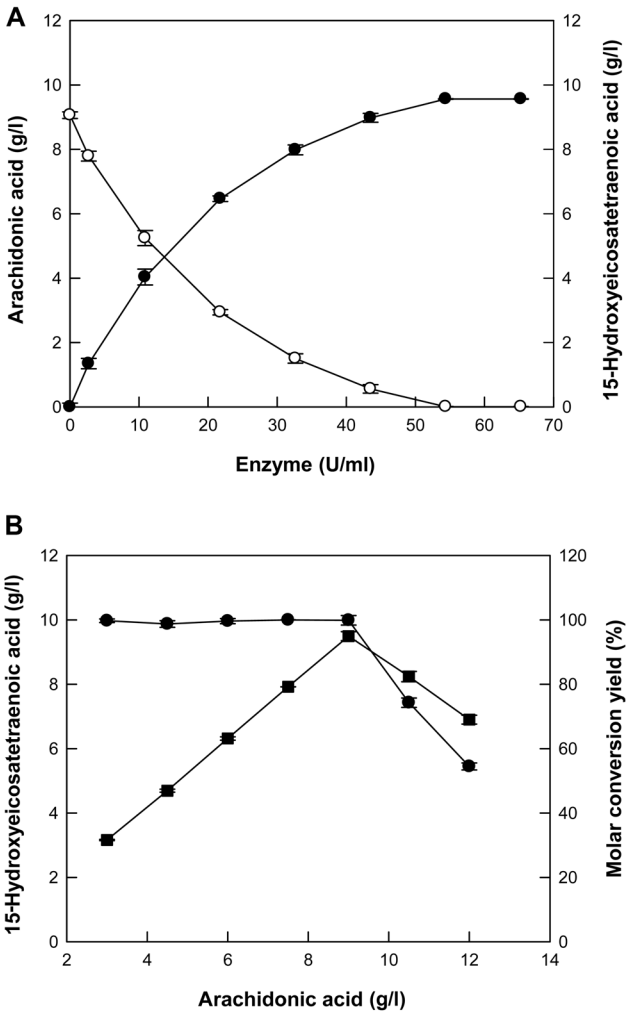

Fig. 2. Effects of enzyme and substrate concentrations on the production of 15-HETE from arachidonic acid.
(**A**) Effect of enzyme concentration. Arachidonic acid (open circle) and 15-HETE (filled circle). (**B**) Effect of substrate concentration. Conversion yield (filled circle) and 15-HETE production (filled square). Data represent the means of three experiments and error bars represent standard deviation.

(mol/mol, corresponding to 105% (w/w)) and 22.8 g l^{-1} h^{-1}, respectively.

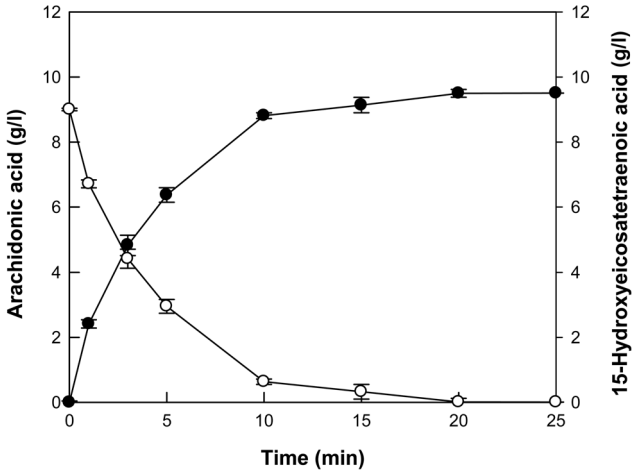

Fig. 3. Time-course reactions for the production of 15-HETE (filled circle) from arachidonic acid (open circle) by soybean lipoxygenase under the optimized reaction conditions.
Data represent the means of three separate experiments, and error bars represent the standard deviation.

The enzymatic conversion of fatty acids to hydroperoxy and hydroxy fatty acids by lipoxygenases is summarized in Table 1. Soybean lipoxygenase produced 25 g/l 13-hydroperoxyoctadecadienoic acid (HPOD) from 28 g/l linoleic acid after 1 h with a molar conversion yield of 80% and a productivity of 22 g l^{-1} h^{-1} [9], 57 g/l 13-hydroperoxyoctadecatrienoic acid (HPOT) from 54 g/l α-linolenic acid after 1.5 h with a molar conversion yield of 94% and a productivity of 60 g l^{-1} h^{-1} [5], and 0.4 g/l 13-HPOT from 1.3 g/l γ-linolenic acid after 0.3 h with a molar conversion yield of 30% and a productivity of 1.8 g l^{-1} h^{-1} [8]. The productivity of soybean lipoxygenase was highest when α-linolenic acid was used as a substrate. However, the quantitative production of 15-HETE from arachidonic acid by lipoxygenase has not been reported to date.

In summary, soybean lipoxygenase produced 9.5 g/l 15-HETE from 9 g/l arachidonic acid, with a molar conversion yield of 99% and a productivity 22.8 g l^{-1} h^{-1}. This study

Table 1. Production of hydroperoxy and hydroxy fatty acids by lipoxygenases.

Substrate	Organism	Product	pH	Temperature (°C)	Productivity (g l^{-1} h^{-1})	Molar yield (%)	Reference
Linoleic acid	Soybean	13-HPOD	9.0	4	22	80	[9]
Linoleic acid	*Gaeumannomyces graminis tritici*	13-HPOD	7.0	24	3.6	40	[14]
α-Linolenic acid	Soybean	13-HPOT	9.0	20	60	94	[5]
γ-Linolenic acid	Soybean	13-HPOT	9.5	25	1.8	30	[8]
Arachidonic acid	Soybean	15-HETE	8.5	20	23	99	This study

HPOD: hydroperoxyoctadecadieonic acid; HPOT: hydroperoxyoctadecatrienoic acid; HETE: hydroxyeicosatetraenoic acids.

provides the first evidence of the biotechnological production of 15-HETE from arachidonic acid, and our findings will contribute to the industrial production of mammalian biologically active molecules such as 15-HETE.

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