Study of an Enzyme Activity in Extracts of *Ginkgo biloba* Leaves

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Biocatalyst is a strong tool for preparation of enantiopure compounds through kinetic resolution or direct synthesis.[1] However, enzymes do not always satisfy the needs of researchers for this purpose because they often do not have enough activity or selectivity toward unnatural substrates. To solve the problems, it is important to find new enzymes from nature, although researchers have tried to change enzyme activity or selectivity by mutagenesis such as random mutagenesis and rational design.[2] Researchers have isolated a variety of enzymes from various sources such as bacteria, fungi, mammals, and plants. Diabetic enzymes such as lipases and proteases from bacteria and mammals are well characterized and utilized successfully in academic and industrial areas.[3] The enzymes involved in mechanisms for protecting plants from insect or fungal attack are also useful in organic synthesis.[4] Such enzymes generally catalyze reactions to produce toxic chemicals in the last step of secondary metabolism to kill predators. As an example, hydroxynitrilases from cyanogenic plants catalyze the biodegradation of cyanogenic glycosides to release hydrogen cyanide.[5] The reverse reaction is interesting in organic synthesis because hydroxynitrilases catalyze formation of enantiopure cyanohydrins from aldehydes or ketones and hydrogen cyanide.

Searching enzymes involved in the secondary metabolism of plants may provide useful biocatalysts in organic synthesis because the enzymes have a key role for synthesis of many biologically active compounds. *Ginkgo biloba* leaves have high anti-insect activity and biological activity.[6] Thus, they might contain an interesting enzyme involved in the secondary metabolism and the enzyme can be useful for organic synthesis. We have chosen *Ginkgo biloba* leaves for finding a new enzyme because *Ginkgo biloba* leaves potentially contain enzymes involved in the secondary metabolism and also the study of the enzymatic activity of extracts from *Ginkgo* leaves has not been focused[7] while the chemicals in them have been well characterized.[8] Herein, we represent an enzyme activity in *Ginkgo biloba* leaves.

Most enzymes are classified into six groups, such as oxidoreductase, transferases, hydrolases, lyases, isomerases, and ligases.[9] Activities for oxidoreductase, hydrolase, and lyase were chosen for screening enzyme activities in *Ginkgo biloba* leaves because these enzymes are important in organic synthesis.[10] We assayed the activities of peroxidase for oxidoreductase, β-amylase, nitrilase, and esterase for hydrolase, and hydroxynitrilase for lyase. *Ginkgo biloba* leaves were ground by a homogenizer and washed with ethyl acetate to remove organic components. *Ginkgo* powders were extracted with the reaction buffers for activity screening. Under the assay condition tested, *Ginkgo* extracts showed only the peroxidase activity (Table 1). In the assay condition, peroxidases oxidize pyrogallol to purpurogallin using hydrogenperoxide (eq. 1). The absorbance change can be monitored at 420 nm. The reaction coordinate showed clear difference between the reaction by *Ginkgo* extracts and the blank reaction (Figure 1).

Table 1. Enzyme activities in *Ginkgo* leaves[a]

tested enzyme activity	activity (units/mg)
Peroxidase activity[b]	6.4×10^{-2}
Hydroxynitrilase activity	n.d[c]
Nitrilase activity	n.d
Esterase activity (hydrolysis)	n.d
Esterase activity (transesterification)	n.d
β-Amylase activity	n.d

[a]Assay condition: see the experimental section. [b]One unit of peroxidase is defined as the amount of enzyme required to catalyze the production of 1 mg of purpurogallin from pyrogallol under the assay conditions described. [c]n.d: not detected.

$$2 \text{ pyrogallol} + 3H_2O_2 \rightarrow \text{purpurogallin} + 5H_2O + CO_2 \quad (1)$$

The absorbance change may occur by some non-catalytic proteins or metal ions. To see if non-catalytic proteins or metal ions perform the oxidation, BSA (bovine serum albumin) as well as iron(II) ion was tested under the same assay condition (Table 2, entries 3-4). The initial rates for

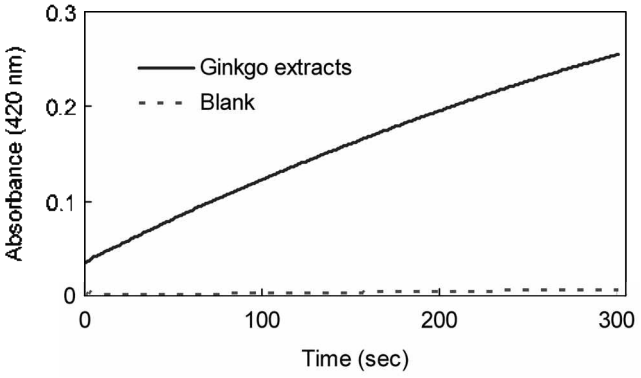

Figure 1. The time course of the oxidation of pyrogallol by *Ginkgo* extracts. *Ginkgo* extracts catalyze the oxidation of pyrogallol 100-fold faster than the blank reaction.

Table 2. Peroxidase activity of Ginkgo extracts and that with additives[a]

entry	additives	activity[b] (units[c]/mL)
1	Ginkgo extracts (36 μg/mL)	$2.3 \times 10^{-3} \pm 0.13 \times 10^{-3}$[d]
2	Blank	3.0×10^{-5}
3	BSA (1 mg/mL)	4.6×10^{-5}
4	1 mM $FeSO_4$	$1.1 \times 10^{-5} \pm 0.54 \times 10^{-5}$
5	Ginkgo extracts with NaN_3 (10 mM)	$1.4 \times 10^{-4} \pm 0.44 \times 10^{-4}$
6	Ginkgo extracts with EDTA (10 mM)	$2.2 \times 10^{-3} \pm 0.21 \times 10^{-3}$
7	Horseradish peroxidase (36 μg/mL)	4.3×10^{-1}

[a]The reaction condition: see the experimental section. For blank and other reactions, the same volume of buffer or the solution of additives, respectively, were added to the reaction mixture. For the reactions with sodium azide and EDTA, the same volume of the solutions (10 mM) was added to the Ginkgo extracts and incubated at r.t. for 1 hr before assayed. [b]The activity was calculated by the following equation: (units/mL) = [$\Delta A_{420\ nm}$ / time (sec) × reaction volume (mL)] / [molar extinction coefficient (12) × volume of enzyme solution (0.050 mL)]. [c]See the definition in the table 1. [d]Errors are standard deviations for at least three measurements; entries without errors are single measurements.

both cases were similar to that for the blank reaction. In addition, sodium azide was added in the reaction mixture of *Ginkgo* extracts if the reaction can be inhibited by a known inhibitor of peroxidases. Sodium azide reduced the initial reaction rate by a factor of 10 (Table 2, entry 6). For comparison with a commercial peroxidase, we have measured the activity of horseradish peroxidase with same protein concentration. The peroxidase activity in *Ginkgo* extracts was about 200 times lower than that of horseradish peroxidase (compare entry 1 with entry 7 in Table 2). *Ginkgo* extracts may contain many inactive proteins for peroxidase activity. Although the peroxidase activity in *Ginkgo* extracts is lower than a commercial purified peroxidase, these results show that *Ginkgo* extracts presumably contain a peroxidase.

It is important to find new enzyme to expand the scope of biocatalysis. *Ginkgo* extracts contain a lot of biologically active compounds. Several metabolisms and enzymes would be involved in the synthesis of these compounds. Thus, it is worth to searching new enzyme activities from *Ginkgo* extracts. *Ginkgo* extracts clearly showed peroxidase activity. The peroxidase can be useful in organic synthesis because oxidation is a key reaction to generate a chiral building block such as chiral epoxides.[11] At this stage, it is rather difficult to define the characteristics of peroxidase in *Ginkgo* extracts. For continuous research, we are planning to compare the distinct characteristics of peroxidase in *Ginkgo* extracts with peroxidases from other plants and apply the *Ginkgo* extracts to synthesis of chiral compounds.

Experimental Section

All chemicals, buffers, and horseradish peroxidase were purchased from Sigma-Aldrich Korea (Yongin, Korea). Protein assay kit (Bio-Rad Laboratories, Hercules, CA) was used for determining the amount of proteins in extracts of *Ginkgo biloba* leaves as the manufacturer's direction. The leaves were obtained from the *Ginkgo biloba* trees in the garden of the Sungshin Women's University. The leaves were ground by a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). UV/Vis (Varian technologies, Seoul, Korea) was used for determining the amount of proteins and for enzyme assay.

Treatment of *Ginkgo biloba* leaves with organic solvents. *Ginkgo biloba* leaves were frozen and ground by a homogenizer at 1500 rpm for 30 min with dry ice filled around it. The powders of *Ginkgo Biloba* leaves (60 g) were added into ethyl acetate (60 mL). The suspension was stirred and kept at 4 °C for 6 hours and filtered. This procedure was repeated until the green color disappeared (about 7 times). The pale green powders were air dried at room temperature. The dried powders were kept in a freezer (−25 °C) until use.

Preparation of extracts of *Ginkgo biloba* leaves. The *Ginkgo* powders (200 mg) were added to 1 mL of the buffer solutions (potassium phosphate, 100 mM, pH 6.0; MOPS, 5 mM, pH 7.2; sodium citrate, 50 mM, pH 5.5) and kept at 4 °C for 24 hours. After centrifuge (30 min, 13000 rpm, 4 °C), the supernatant was used for the further experiments. The amount of protein in the *Ginkgo* extracts was determined as 36 μg/mL.

Peroxidase activity.[12] The assay solution was prepared by mixing potassium phosphate buffer (160 μL, 100 mM, pH 6.0), hydrogen peroxide solution (80 μL, 0.5 wt%), pyrogallol solution (160 μL, 5 w/v%), and Milli-Q water (1,050 μL). The extracts of *Ginkgo Biloba* leaves (50 μL) were added to the above assay solution. The total volume was 1.5 mL. The reaction was monitored at 420 nm for 5 min.

Hydroxynitrilase activity. For the reaction, the powders of *Ginkgo Biloba* leaves (300 mg) were equilibrated by adding of sodium citrate buffer (120 μL, 20 mM, pH 5.5). Sodium cyanide (110 mg) was dissolved in milli-Q water (1.5 mL) and adjusted pH to 5.5 by addition of acetic acid. Isopropyl ether (2.5 mL) was added to the solution. The organic layer containing hydrogen cyanide was separated and added to the vial containing the powders of *Ginkgo Biloba* leaves. To start the reactions, benzaldehyde (100 μL) was added. And the reaction was monitored by TLC (ethyl acetate : hexane = 1 : 5).

Nitrilase activity. To a suspension of the ground *Ginkgo* leaves (100 mg) in a potassium phosphate buffer solution (5 mL, pH 7.0, 100 mM) was added 1,4-dicyanobenzene (300 mg). The mixture was shaken at 30 °C. The reaction was monitored by TLC (ethyl acetate : hexane = 1 : 1).

Acylation of 1-phenylethanol. To a solution of vinyl acetate (92 μL) and 1-phenylethanol (120 μL) in diethyl ether (2 mL) was added the ground *Ginkgo* leaves (30 mg). The reaction mixture was stirred at room temperature and the reaction was monitored by TLC (ethyl acetate : hexane = 1 : 5).

Hydrolysis of *p*-nitrophenyl acetate. An assay solution was prepared by mixing *p*-nitrophenyl acetate solution (20 μL, 200 mM in acetonitrile), acetonitrile (870 μL), and MOPS buffer (11,110 μL, 5 mM, pH 7.2). To the assay solution (3 mL) was added the *Ginkgo Biloba* extracts (150 μL, in MOPS, pH 7.2, 5 mM). The reaction was monitored

by UV/Vis spectra at 404 nm.

***β*-Amylase activity.** Color reagent solution was prepared by mixing sodium potassium tartrate tetrahydrate (12.0 g), NaOH solution (8 mL, 2 M), 3,5-dinitrosalicylic acid solution (20 mL, 96 mM), and adding ddH_2O up to 40 mL. To a starch solution (1.0 w/v %, 1 mL) was added the ground powders of the ground *Ginkgo* leaves (100 mg). The suspension was stirred at 20 °C for 20 min. The color reagent solution (1 mL) was added to the suspension and heated at 100 °C for 15 min and cooled to room temperature. After the solution was diluted with ddH_2O (9 mL), the absorbance was measured at 540 nm.

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