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Short communication

Bioluminescent Assay of α-Oxidase from *Cucumis sativus* using Bacterial Luciferase-Coupled Reaction

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A new assay method of α-oxidase (fatty acid : oxygen dioxygenase, 1-decarboxylating) was developed using a bioluminescence reaction system of marine luminous bacterium, *Photobacterium phosphoreum*. α-Oxidase was isolated from a cucumber (*Cucumis sativus*). Pentadecanoic acid was used as a substrate, and the product, tetradecanal, was analyzed with a bacterial luciferase-coupled reaction. Initial light intensity was directly related to the concentration of tetradecanal in the range of 1 nM to 10 μM. Optimal pH and temperature were 7.5 and 25°C, respectively. Optimal pentadecanoic acid concentration in a standard assay of α-oxidase was 0.1 mM. The *Km* value of pentedecanoic acid was 85 μM. This method is straightforward, rapid, convenient, and easy. Its needs no treatment or extraction of reaction mixture.

Keywords: α-oxidase, Bacterial luciferase, Pentadecanoic acid, *Photobacterium phosphoreum*

The fatty acid α-oxidation reacts to from a one carbon shorter chain fatty aldehyde (alkanal) and carbon dioxide from fatty acid using molecular oxygen (Eq. 1).

$$R\text{-}CH_2\text{-}COOH + O_2 \text{ ------> } R\text{-}CHO + CO_2 \text{ by } \alpha\text{-oxidase} \quad (1)$$

The fatty acid α-oxidation system has been found in various kinds of higher plants sources, such as cucumber and young pea leaves, and even in some animal tissues (Baardseth *et al.*, 1987; Singh, *et al.*, 1993). For mammalian, α-oxidase is responsible for the oxidation of branched fatty acids, which would be impossible by the usual β-oxidation system. The deficiency of the α-oxidase system is known to cause Refsum's disease as there is an accumulation of phytanic acid in the serum (Stokke *et al.*, 1967; Herbert and Clayton, 1994).

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In plants the produced fatty aldehydes are responsible for the characteristic flavor and taste of their fruits. The flavor of seaweeds, such as *Ulva pertusa*, was also reported due to a series of unsaturated aliphatic aldehydes, such as (8Z)-heptadecenal, (8Z, 11Z)-heptadecadienal, (8Z, 11Z, 14Z)-heptadecatrienal. These were produced from oleic acid (C18 : 1, n-9), linoleic acid (C18 : 2, n-6), and α-linolenic acid (C18 : 3, n-3), respectively, probably by α-oxidase (Kajiwara *et al.*, 1989a). Many sex pheromones found in insects are known to be alkyl aldehydes, especially the odd numbered chain aldehydes such as undecanal. These seem to be produced from dodecanoic acid by the α-oxidase system (Morse *et al.*, 1986).

The usual assay method for this enzyme is either by following the consumption of molecular oxygen with oxygen electrode, or by measuring the produced carbon dioxide ($^{14}CO_2$) using a 1-^{14}C labeled fatty acid as a substrate (Galliard and Matthew, 1976; Baardseth *et al.*, 1987). Although the radioisotope method is so far the most sensitive and reliable, it requires a liquid scintillation counter and radioisotope-labeled substrate. This couold cause hazardous contamination, especially when the radioisotope labeled carbon dioxide evolution is not properly trapped. Using oxygen electrode for the measurment of oxygen consumption is not sensitive enough and needs intensive calibration before every assay. Thus a fast, reliable, and convenient assay method of α-oxidase is required.

Bacterial luciferase (BL'ase) catalyzes the oxidation of long chain fatty aldehydes to the corresponding fatty acids with concomitant emission of blue-green light (max about 490 nm) in the presence of reduced flavin mononucleotide and molecular oxygen (Hastings *et al.*, 1978). Because of its high sensitivity and easy operation, bacterial bioluminescence has gained many applications in the analysis of enzyme reactions or environments (Cho and Shim, 1993; Cho, 1993; 1994; 2000). Due to the relatively broad substrate specificity of α-oxidase, a series of fatty acids can be good substrates of α-oxidase, and the reaction products, long chain aliphatic

aldehydes can be readily assayed as a substrate of bacterial luciferase with a high sensitivity. Employing the luciferase-coupled reaction, a new method to assay the activity of α-oxidase was developed (Eq. 2).

$$\text{Pentadecanoic acid} \xrightarrow{\alpha\text{-oxidase}} \text{Tetradecanal} \xrightarrow{\text{BL'ase}} \text{Tetradecanoic acid} + \text{light} \quad (2)$$

In this report, this luminescence detection method of α-oxidase is demonstrated with cucumber α-oxidase and pentadecanoic acid as a substrate and bacterial luciferase purified from *Photobacterium phosphoreum.*

Materials and Methods

Materials Long chain aliphatic aldehydes, such as decanal and tetradecanal, fatty acids such as tetradecanoic acid, pentadecanoic acid, Triton X-100, flavin mononucleotide (FMN), EDTA, DEAE-Cellulose, DEAE-Sephadex A-25 were purchased from Sigma Chemical Co (St. Luois, USA). Sodium dithionite was a product of Aldrich Chemical Co (USA). All other solvents or chemicals were of analytical grade.

Preparation of α-Oxidase α-Oxidase was isolated from cucumber (*Cucumis sativus*) which was purchased from a local market in a fresh condition by the methods of Galliard and Matthew (1976). Only the fresh of the cucumber fruit was cut into small pieces and homogenized with 20 volume of cold acetone (-30°C) for 5 min. After vacuum filtration through a Whatmann #1 filter paper, the filter cake was washed with cold acetone and cold diethylether and then dried under vacuum. The resulting powder was collected and stored at -20°C until used (the activity was stable up to one month in this condition.). To prepare α-oxidase solution for each working day, the powder was added into 200 volume of a cold buffer (50 mM Na-phosphate, pH 7.0, containing 1.0 mM EDTA, 0.25 M sucrose and 0.005% Triton X-100) and stirred for 30 min at 4°C, then centrifuged at 7,000 rpm with a Sorval SS-34 rotor to remove undissolved particles and then it was used immediately.

Preparation of bacterial luciferase and dithionite assay
Bacterial luciferase was purified from *Photobacterium phosphoreum* by the method of Hastings *et al.*, (1978) to a purity of 95%. The specific activity was about 4×10^{14} quanta sec^{-1} mg^{-1} with an activity assay named "dithionite assay" with saturating tetradecanal as a substrate. Dithionite assay was performed as follows; To 1 ml of 50 μM FMN solution in phosphate buffer (50 mM, pH 7.0) in 20 ml capacity of scintillation vial, 10 μl of luciferase solution was added and then the whole mixture was reduced by the addition of a fresh sodium dithionite solution (about 10 μl of 1.5% W/V solution). The reduced mixture (solution A) was placed in the reaction chamber of photomultiplier photometer designed for 20 ml capacity of scintillation vial. Then 1 ml of the air-saturated long chain aliphatic aldehyde solution (or α-oxidase reaction mixture) was vigorously injected into this mixture by a 3 ml hypodermic syringe attached with a 20 gauge and 7.5 cm long needle. Tetradecanal and pentadecanoic acid stock solutions were prepared in absolute ethanol, and diluted with a buffer immediately prior to use. The amount of ethanol was kept under 1% in all cases.

An α-oxidase: bacterial luciferase-coupled reaction:
To 2 ml of 50 mM phosphate buffer (pH 7.5) in 20 ml scintillation vial, 10 μl of pentadecanoic acid solution (0.1% in ethanol to make a final solution of 0.2 mM) was added. The reaction was started by the addition of 10 μl of α-oxidase. The reaction mixture was incubated at 25°C with shaking, and at the given time, 10 μl aliquots were taken and diluted in 1 ml of 0.1 M phosphate buffer (pH 7.0). This solution (1 ml) was injected using a 3 ml hypodermic syringe into a premixed bacterial luciferase-reduced flavin mononucleotide ($FMNH_2$) mixture (solution A) that was already put in the chamber of the photometer. At low aldehyde concentration (below *Km*, the *Km* of tetradecanal is about 5 μM), the initial light intensity is proportional to the aldehyde concentration. The light intensity was measured by a photomultiplier photometer (Mitchell and Hastings, 1971) equipped with a Hamamatsu R447 photomultiplier tube and high voltage DC-power supply (GW-2500, Bioneer Co., Korea) and recorded with a strip chart recorder (Linear 1200). One light unit (LU) in this photometer corresponds to 7×10^8 quanta sec^{-1} calibrated by the standard of Hastings and Weber (1963).

Results and Discussion

Bacterial luciferase uses long chain aliphatic aldehydes of 7 carbons or longer as substrates. Also, tetradecanal shows optimal light production for the *P. phosphoreum* luciferase used in this work. Bacterial luciferase can be obtained from *P. phosphoreum, P. leiognathi, V. fischeris* or *V. harveyi.* Luciferase of *P. phosphoreum* was used, because it gives much less light response than the *V. harveyi* luciferase from

Table 1. Substrate specificity for long chain aliphatic aldehydes of bacterial luciferases from *Photobacterium phosphoreum* and *Vibrio harveyi.* The initial maximum light intensity was measured by dithionite assay at saturating aldehyde concentration.

Carbon numbers of aldehyde	Relative reactivities (%)	
	P. phosphoreum	*V. harveyi*
7	3	4
8	7	12
9	15	85
10	20	75
11	20	42
12	50	16
13	80	80
14	100	100
15	85	70
16	80	60
18	50	40

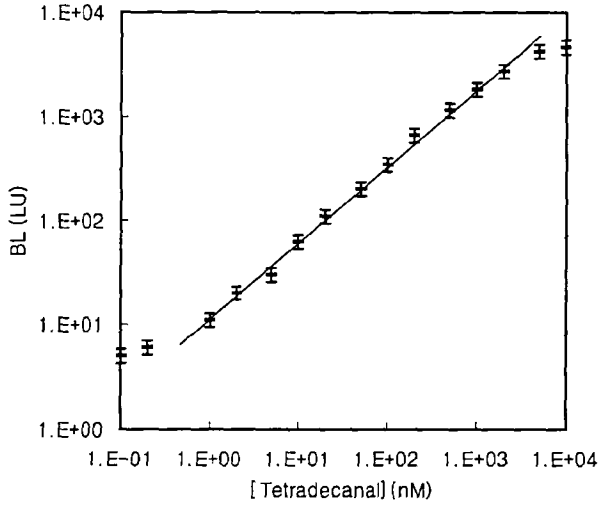

Fig. 1. Standard curve of tetradecanal concentration and initial light intensity (Io) using dithionite assay of bacterial luciferase. Various concentration of tetradecanal standard solution were prepared in absolute ethanol, and add 10 μl of these solution into 1 ml of 0.1 M phosphate buffer (pH 7) which was then injected into the mixture of luciferase and reduced FMN solution as described in Materials and Methods.

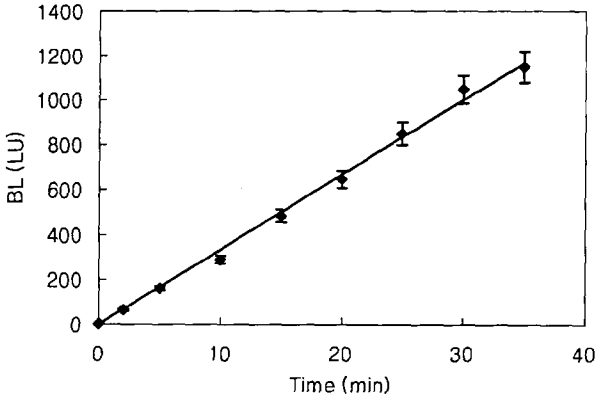

Fig. 2. Time-dependent tetradecanal production from pentadecanoic acid by α-oxidase assayed using luciferase reaction. To 1 ml of 30 mM phosphate buffer (pH 7.5), added were 10 μl of 5.2 mM ethanolic pentadecanoic acid solution. The reaction was then started by addition of about 1.5×10^{-3} unit of α-oxidase. The reaction mixture was incubated at 25°C with shaking under atmospheric condition. At every 5 min, 10 μl aliquots were taken, diluted in 1 ml of 0.1 M phosphate buffer (pH 7.0) and then assayed for produced tetradecanal using dithionite luminescence assay as described in Material and Methods.

decanal or nonanal. These are usually contained in most plant sources land ead to less interference from such shorter chain aldehydes (Table 1). Fatty acids with carbon chain of 7 to 18 were reported to be substrates of α-oxidase. Pentadecanoic acid was selected as the most suitable substrate for both α-oxidase and *P. phosphoreum* bacterial luciferase (Table 1).

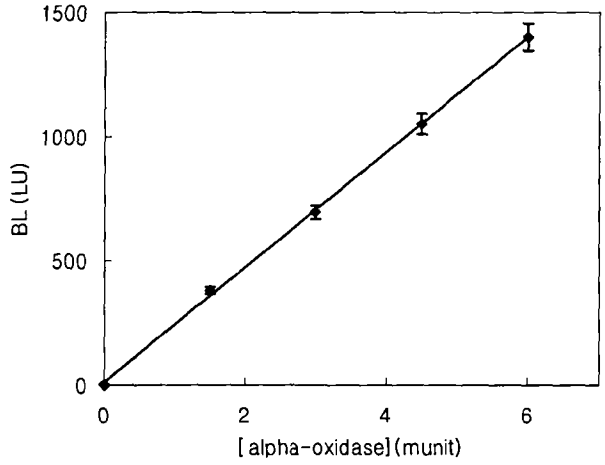

Fig. 3. Dependence of light intensity upon the concentration of α-oxidase. Various amount of α-oxidase activity was used in the presence of 204 μM of pentadecanoic acid in 30 mM phosphate buffer (pH 7.5 at 25°C), using 10 min fixed time assay with shaking procedure. One unit of α-oxidase was defined as the amount of enzyme in 1 ml of enzyme preparation showing A_{280}nm = 1.0 as described in Materials and Methods.

The *Km* value of tetradecanal in the luminescence reaction was found to be 5 μM. Below this concentration, the initial light intensity (Io) is linearly correlated with the concentration of tetradecanal. Using these results, a standard curve for the determination of tetradecanal (initial light intensity against the concentration of tetradecanal) was established (Fig. 1). The amount of tetradecanal from 1 pmol to 5 nmol in a 1 ml sample can be readily quantified with this standard curve.

The time-dependent production of tetradecanal from pentadecanoic acid by α-oxidase under atmospheric condition (shaking) was measured by the luciferase-coupled system (Fig. 2). It showed a linear pattern of tetradecanal production up to 20 min. A 10 min-fixed time assay was found to be most suitable for a large number of analyses, such as the chromatographic fraction assay.

Using the 10 min-fixed time assay, the plot of light intensity against the amount of α-oxidase showed a good linearity with this luminescent assay (Fig. 3). It is not really necessary to perform the reaction with shaking for the purpose of the oxygen supply, because the decreased oxygen concentration is equimolar with the decreased pentadecanoic acid, which is less than 10 μM. The participation of the hydrophobic substrate and product in the reaction did, however, require some degree of shaking for the best result. α-Oxidase activity, measured by the luminescence method with an increasing concentration of pentadecanoic acid, showed a typical hyperbolic saturation curve. From the double reciprocal plot, the *Km* of pentadecanoic acid for α-oxidase from *Cucumis satiba* was about 85 μM (Fig. 4). The apparent *Km* of pentadecanoic acid has not been reported, but that of palmitic acid using radioisotope assay is about 60 μM. This is

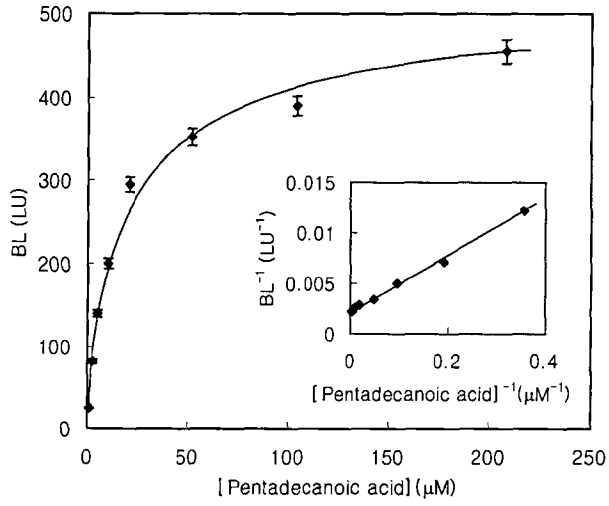

Fig. 4. Dependence of light intensity upon the concentration of pentadecanoic acid. Luciferase coupled α-oxidase assay was performed at various concentrations of pentadecanoic acid. 1.5×10^{-3} unit of α-oxidase was used in 30 mM phosphate buffer (pH 7.5 at 25°C), 10 min fixed time assay with shaking. Inset: the *Km* value of α-oxidase for pentadecanoic acid was determined with a double reciprocal plot.

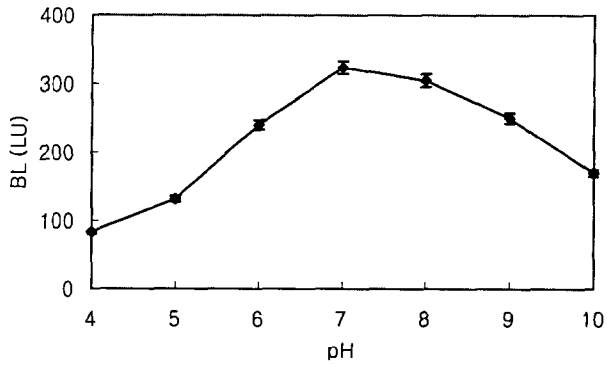

Fig. 5. pH-Dependence of α-oxidase activity. 7.5×10^{-4} unit of α-oxidase was used in the presence of 52 μM of pentadecanoic acid at 25°C using 10 min fixed time assay procedure with shaking. Buffers used were as follows, pH 4 and 5: sodium acetate buffer (50 mM), pH 6 and 7: sodium phosphate buffer (50 mM), pH 8 and 9: Tris-HCl buffer (50 mM), pH 10: carbonate buffer (50 mM).

reasonably close to the value obtained in the present assay considering a one carbon chain difference (Gilliard and Mattew, 1976). The pH-dependence of α-oxidase showed an optimal activity around pH 7.5 (Fig. 5).

Although pentadecanoic acid was used in this demonstration because of its high luminescence response, other fatty acids, such as palmitic acid, can be readily used in this assay system with some compromise in sensitivity. The product from palmitic acid is pentadecanal. This gives about 85% of light intensity compared with tetradecanal (Table 1).

α-Oxidase is a very important enzyme in lipid metabolism and agriculture. However, its exact function, other than the oxidation of the branched fatty acid or the reaction mechanism of the α-oxidase, has not yet been elucidated. Shine and Stumpf (1974) proposed 2-hydroperoxy fatty acid (R-CH(OOH)-COOH) as a probable intermediate in the α-oxidation process. Campa *et al.* (1989) proposed α-peroxylactone, a kind of dioxetanone intermediate on the basis that the α-oxidase system can produce enough energy to emit visible light in the presence of a proper sensitizer. This is probably through a similar mechanism of firefly bioluminescence or other dioxetane chemiluminescence (Campa *et al.*, 1989). The production of an artificial flavor of unsaturated long chain aldehydes from fatty acids can be an important application of α-oxidase (Kajiwara *et al.*, 1989b). The elucidation of this reaction system would give valuable information about the metabolism of fatty acid, as well as some insight into the nature of the chemiexitation process.

A number of assay methods for this enzyme have been developed, such as oxygen consumption measurement with an oxygen electrode, This is a quantitation of radioisotope-labeled CO_2 production that uses a liquid scintillation counter, organic solvent extraction, and GC analysis for the aldehyde (Kajiwara *et al.*, 1989a). The oxygen electrode method is not very sensitive in measuring a small change of oxygen concentration during the α-oxidase reaction. This requires intensive calibration of the electrode for each assay. The concentration of oxygen in the air saturated aqueous solution is 0.25 mM at 25°C, and a decrease of less than 0.1% oxygen concentration (0.25 μM) is hard to detect (De Groot, 1990). The detectablility obtained with this bioluminescence method is down to 1 nM. This is at least 100 fold higher sensitivity than the oxygen measurement methods using an oxygen electrode. Another draw-back of the oxygraph method is the concentration of oxygen is continously decreasing in the closed reaction system, while a decrease of concentration is hard to detect in the open system. Radioisotope methods require an expensive and hazardous radioisotope labelled substrate, and trapping the radioisotope labelled carbon dioxide has always been a potential risk for environmental contamination. GC analysis usually requires a lot of time for solvent extraction and concentration process and quantitation is not reliable between each assay without an internal standard.

The luminescence assay method described in this work is based on the measurement of light emission produced from the reaction. There are very few background signals leading to higher sensitivity and reproducibility. It is also a very fast method, finishing each assay in less than 1 min. The bacterial luciferase is relatively easy to prepare to high purity with little cost and labor. it also has a good thermal stability, which permits a long storage and operation life (Hastings *et al.*, 1978). Although pure luciferase was used in this work, the purity of luciferase was not that essential. It sufficient to use a desalted sample after DEAE-cellulose chromatography of the cell lysate and ammonium sulfate precipitation (35% to 70%).

This can be prepared from a frozen cell in a single day of work. The substrates are available with little cost. Reaction products of this assay are readily degradable aliphatic compounds, which give almost a negligible environmental contamination problem. This method is quite useful for the rapid assay of a large number of samples, such as the enzyme assay of fractions from the chromatographic process.

A luminometer can be made in the laboratory at little cost (Mitchell and Hastings, 1971). Alternatively, the spectrofluorometer or spectrophotometer in the transmission mode with the lamp turn-off or close can be used with some compromise in sensitivity. Nowadays, many kinds of well designed luminometers are available. Some models have autosampler and reagent injection pump to allow those types of luminescence analysis automatically.

Another important factor in this kind of bioluminescence coupled assay system, as well as other enzyme assay systems, is the interference caused by the bioluminescence inducing chemicals. The possible contamination of natural aldehydes in the α-oxidase sample, especially in the case of plant origin, can give a false signal or increase background. However, the enzyme is resistant to organic solvent, such as acetone and diethyl ether.Therefore, careful washing with these solvent during the preparation of α-oxidase can remove almost all of the natural aldehyde and other lipid compounds. The inclusion of 1 mM hydroxylamine in the buffer used in the preparation of α-oxidase can also trap contaminated natural aldehyde very efficiently (Cho, 1994). The reaction of α-oxidase is the oxidation of fatty acid to aldehyde, and in many biological samples, especially in the crude state some amount of fatty acid either in free form or ester form is present. This can be a substrate of α-oxidase that produces aldehyde to cause false luminescence signal. In the case, a control reaction without addition of a pentadecanoic acid should be run in parallel, and the difference in the bioluminescence intensity is the indication of enzyme activity. The most probable fatty acid that induces a false signal is palmitic acid. It is the most abundant fatty acid in biological samples. The product produced by α-oxidase is pentadecananal which gives about 85 % of light intensity of tetradecanal. However the concentration difference between added pentadecanoic acid (typically 52 μM) and endogeneous palmitic acid in the enzyme sample after acetone precipitation and diethyl ether washing, makes the interference negligible in most cases.

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