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Bioluminescent Assay of Bovine Liver Riboflavin Kinase Using a Bacterial Luciferase Coupled Reaction

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For the demonstration of a novel riboflavin kinase assay method based on the bacterial bioluminescence, partially purified riboflavin kinase was prepared from bovine liver through ammonium sulfate precipitation and DEAE-cellulose ion exchange chromatography. Using bacterial luciferase from *Photobacterium phosphoreum* and the dithionite reduction method, an easy, safe, and fast assay method was established. The optimal temperature, pH, Km values for riboflavin and ATP of bovine liver riboflavin kinase determined with this luminescence method were 35°C, pH 7, 15.3 μM and 8.3 μM, respectively. The detection limit of FMN produced by riboflavin kinase was in the range of 200 pM to 4 μM which is comparable to the HPLC-fluorescence detection method, while the detection time for each assay was less than 15 sec compared to the HPLC method which requires at least 10 min for completion.

Key words: Riboflavin kinase, bioluminescence

Riboflavin has been known as an essential nutrient (vitamin B_2) and exists in the cell in the form of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). These flavin compounds are very important cofactors in the electron transport pathway as well as in many enzymes, especially oxidases and monooxygenases, due to the unique nature of the flavin system mediating one or two electron transports. The conversion of riboflavin into FMN is a critical step in the uptake of riboflavin into the cell and catalyzed by riboflavin kinase (flavokinase, ATP : riboflavin 5'-phosphotransferase, EC 2.7.1.26) which introduces the phosphate group in the ribosyl 5'-hydroxyl group. This phosphorylation greatly increases the solubility of flavin compounds in aqueous cytosolic solution and also prevents outbound diffusion from the cell through the cell membrane (16). Riboflavin kinases have been found in many plants and animals, especially in the liver and small intestine (1, 9, 16), as well as in bacteria, such as *Bacillus subtilis* (10, 12, 19) and some yeasts (17).

Riboflavin and FMN are spectrophotometrically almost identical. But it is quite difficult to follow the conversion of riboflavin into FMN using a spectrophotometric or fluorometric method (13). Differences in water solubility and ionic character due to the presence or absence of the phosphate group were used in the main methods for analysis and separation. The assay method for the reaction of riboflavin kinase is usually performed using a chromatographic method, such as paper chromatography or HPLC (11, 12). In some cases, [γ-^{32}P] was used as a phosphate donor and the incorporated phosphate group in FMN was quantified after separation using TLC or HPLC (10, 11). However, these methods require lots of time and cost, thus development of an easy and reliable method for the assay is necessary.

Recently chemiluminescence and bioluminescence assay methods are being used increasingly for the measurement or environmental toxicity mainly because of their low hazard and high sensitivity (2, 3, 4). One of these systems is bacterial luciferase from marine luminous bacteria, such as *Vibrio harveyi, V. fischeri,* and *Photobacterium phosphoreum*, producing blue-green light (λmax=490 nm) with reduced flavin mononucleotide ($FMNH_2$), long chain aliphatic aldehydes, and molecular oxygen as substrates (6, 7). The overall reaction is :

$$FMNH_2+R\text{-}CHO+O_2 \rightarrow FMN+RCO_2H+H_2O+light$$

In this research, a new bioluminescent assay method of riboflavin kinase was developed using bacterial luciferase and riboflavin kinase partially purified from bovine liver.

Materials and Methods

Chemicals

Riboflavin, flavin mononucleotide (FMN), ATP, DEAE-cellulose and long chain aliphatic aldehydes such as deca-

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nal and tetradecanal were purchased from Sigma Chemical Co. (USA). Commercial FMN sample was usually contaminated with small amounts of riboflavin and was purified by DEAE-cellulose column chromatography (1 cm × 5 cm). FMN and riboflavin solution (about 5 mM) was loaded onto the column and unbound riboflavin was washed out with 20 mM phosphate buffer (pH 7.0) and then FMN was eluted with 0.1 M phosphate buffer (pH 7.0). The concentration of purified FMN was determined by spectrophotometry with an extinction coefficient of 12,200 at 445 nm (7) using a Milton-Roy MR-3000 spectrophotometer and stored frozen in aliquots. Sodium dithionite was a product of Aldrich Chemical Co. Solvents used for HPLC and TLC were of HPLC grade purchased from Merck Co (Germany).

Enzymes

Bacterial luciferase was purified from *Photobacterium phosphoreum* by the method of Hastings *et al.*, (8) to 95 % purity and the specific activity was about 4×10^{14} quanta·$sec^{-1}mg^{-1}$ with an activity assay named "dithionite assay" with saturating tetradecanal as substrate.

Riboflavin kinase was isolated and partially purified from bovine liver by the method of Bandyopadhyay *et al.* (1). First, fresh bovine liver obtained from a local slaughterhouse was homogenized with 4 volumes of cold 0.1 M phosphate buffer (pH 7.2) containing 0.25 M sucrose. After filtration, the clear filtrate was saturated with ammonium sulfate and the precipitant collected between 35~75 % saturation. Salts and low molecular weight materials were removed using an Econo 10 DG desalting column (BioRad Co., USA). Ion exchange chromatography using a DEAE-cellulose A-25 column (2.5 × 20 cm) in a low temperature chromatographic system (UA-6, ISCO, USA) with 0.1~0.8 M NaCl was used and the combined active fractions were concentrated by ultrafiltration with a PM10 membrane (molecular weight cut off : 10,000, Amicon Co., USA) and used in this experiment.

Dithionite assay of bacterial luciferase

To 1 ml of 50 μM FMN solution in phosphate buffer (50 mM, pH 7.0) in a 20 ml scintillation vial, 10 μl of luciferase solution was added and then the whole mixture was reduced by addition of fresh sodium dithionite solution (about 10 μl of 1.5%, W/V solution) using a microsyringe. A little excess dithionite keeps this reduced mixture from reoxidizing by diffused oxygen from the atmosphere. Put this reduced mixture (solution A) into the reaction chamber of a photomultiplier photometer designed for a 20 ml scintillation vial, then 1 ml of buffer saturated with air and tetradecanal (solution B) was injected into this mixture vigorously using a 3 ml hypodermic syringe attached with a 20 gauge 7.5 cm long needle. Mixing of these two solutions was completed with this injection, and the maximum light intensity (Io) was obtained in less than 1 sec.

A riboflavin kinase-bacterial luciferase coupled reaction

To 1.0 ml of 50 mM phosphate buffer (pH 7.0) containing 20 μM riboflavin, 20 mM $MgCl_2$ and 2 mM ATP, the reaction was initiated by the addition of 10 μl of riboflavin kinase solution. The reaction mixture was incubated at 25°C by shaking in the dark, and at every 5 min, 100 μl aliquots were taken and diluted with 0.9 ml of 0.1 M phosphate buffer (pH 7.0). This solution (1 ml) was used as the flavin solution (solution A) in the dithionite assay. At low $FMNH_2$ concentration (below Km, the Km of $FMNH_2$ is about 0.5 μM (7)), the initial maximum light intensity (Io) is almost linearly proportional to the FMN concentration.

The maximum light intensity (Io) was measured using a photomultiplier photometer equipped with a R-447 phototube (Hamamatsu, Japan) with a Mitchell-Hasting type pre-amplifier assembled in this laboratory (14) and a high voltage DC-power supply (GW-2500, Bioneer Co., Korea) and recorded with a strip chart recorder (Linear-1200, USA). Calibration was performed using the continuous light source of Hastings and Weber (8). One light unit (LU) was defined as 2×10^9 quanta·sec^{-1}.

Results and Discussion

Preparation of riboflavin kinase from bovine liver

As riboflavin kinase is not commercially available, some riboflavin kinase was prepared from bovine liver to demonstrate the application of the bioluminescence assay in riboflavin kinase activity analysis. In Table 1, the partial purification of riboflavin kinase is summarized. Because the purpose of this work was to obtain enzyme material to use for the demonstration, purification was not intensely performed. The crude enzyme used in this work was pre-

Table 1. Preparation of partially purified riboflavin kinase from bovine liver

Steps	Total protein (A_{280nm})	Total activity* (unit)	Specific activity (mUnit/mg)	Purification fold
Crude extract	1,426	84	58.9	1
35-75 % AS ppt and desalting	634	39	61.5	1.04
DEAE-cellulose column	230	22	95.7	1.62
Ultrafiltration (MWCO : 10,000)	180	20	111.1	1.89

*Determined with bioluminescence method. One unit was defined as the activity which produces 1 mol of FMN per 10 min at 25°C.

Table 2. Initial light intensity of bioluminescence reaction with dithionite assay using riboflavin, FMN and FAD in the presence of saturated tetradecanal.

Compound	Concentration (μM)	Initial light intensity (Io) (quanta·sec^{-1}mg^{-1})	Ratio (%)
Riboflavin	75	5×10^{11}	0.1
FMN	50	4×10^{14}	100.0
FAD	50	7×10^{12}	1.8

pared as a flavin free form by illuminating with an UV lamp for 5 min, then gel filtration chromatography with a Bio-Rad 10 DG desalting column and intensive dialysis. The absence of flavin compound in this enzyme sample was confirmed with a spectrophotometric assay and fluorescence assay (data not shown). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmole of FMN for 10 min at 25°C.

Standard curve for the concentration of FMN in bacterial luciferase reaction

Because the activity of reduced riboflavin as a substrate of bacterial luciferase is about 0.1% of reduced FMN ($FMNH_2$) due to lower solubility in aqueous buffer than FMN and lack of a phosphate group (7, Table 2), the reaction mixture afterwards including FMN produced by riboflavin kinase as well as the unreacted riboflavin were reduced together with sodium dithionite, and used as a substrate of bacterial luciferase. Due to the activity difference of riboflavin and FMN to bacterial luciferase, the amount of light produced in the luciferase reaction is generated almost exclusively by FMN and the contribution of riboflavin is negligible. The standard curve for the concentration of FMN in the mixture of FMN and riboflavin was obtained in which the total flavin concentration was fixed at 5 μM. The standard curve showed good linearity in the range of 0.2 nM to 0.4 μM of FMN concentration which corresponds to 4 μM of FMN before dilution (Fig. 1). The lower detection limit of FMN (0.2 nM) is comparable to a HPLC-fluorescence detector and not very low for other luminescence detection methods which had a detection limit usually in the range of pM or even fM because of the contribution of reduced riboflavin to light production.

Time dependent FMN production by riboflavin kinase

To 0.5 ml of 50 mM phosphate buffer (pH 7.0) containing 20 μM riboflavin, 20 mM $MgCl_2$ and 2 mM ATP, the reaction was started by adding 10 μl of riboflavin kinase solution. The reaction mixture was incubated at 25°C with shaking, and at every 5 min, 100 μl aliquots were taken and assayed for the production of FMN (Fig. 2). In this reaction, the production of FMN was linearly dependent on the incubation time up to 20 min, and thereafter a little decline was found in the curve. Thus a 10 min incubation period was selected in a fixed time assay for temperature or pH dependence analysis. To terminate the reaction at 10 min, the best way was to immerse the vial or test tube including the reaction mixture into ice-water. Addition of acid, such as trichloroacetic acid or HCl, or boiling can degrade the produced FMN and give false results.

Temperature and pH dependence

In Fig. 3, the dependence of riboflavin kinase activity upon various temperatures was determined. The optimal temperature was around 35°C and at a higher temperature the activity steeply declined. The optimal pH measured with bioluminescence methods in various buffer system

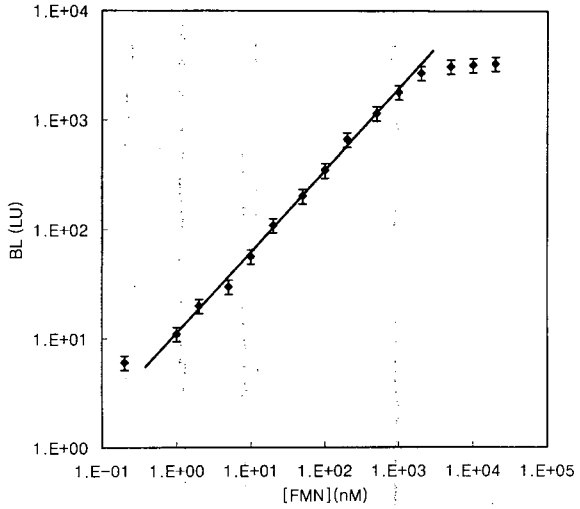

Fig. 1. The standard curve of initial light intensity (Io) of bioluminescence (BL in light unit, LU) against the concentration of FMN using the dithionite assay. The sum of FMN and riboflavin was kept constant at 20 μM as the riboflavin kinase-luciferase coupled reaction.

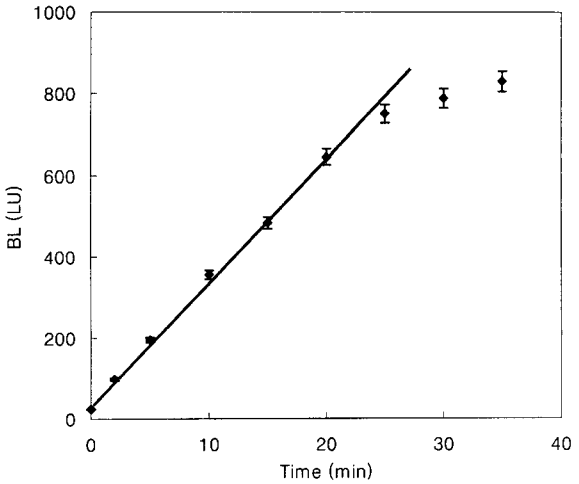

Fig. 2. The time dependent production of FMN in a riboflavin kinase reaction using a bioluminescence assay. Incubation temperature at 25°C with shaking, and at every 5 min, 100 μl aliquots were taken and assayed as described in Materials and Method.

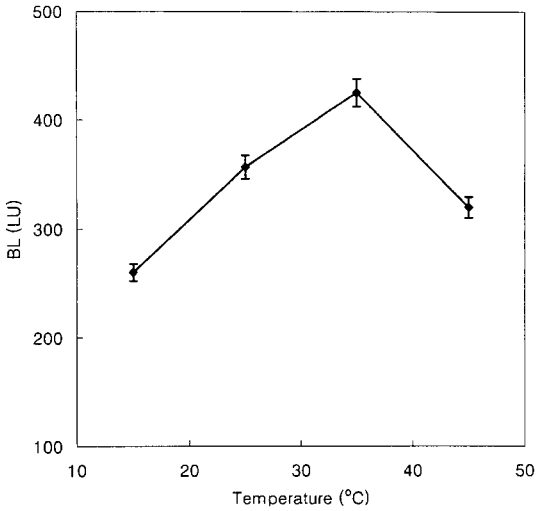

Fig. 3. The dependence of riboflavin kinase activity upon different temperatures using bioluminescence assay. A fixed time assay for 10 min incubation at 25°C with shaking was employed.

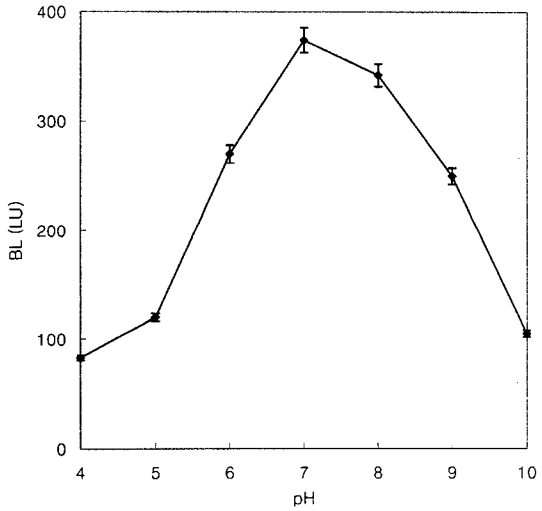

Fig. 4. Determination of the dependence of riboflavin kinase activity at different pH using bioluminescence assay. A fixed time assay for 10 min incubation at 25°C with shaking was employed. Buffers used in the pH dependent assay were as follows: pH 4 and 5, sodium acetate buffer (50 mM); pH 6 and 7, sodium phosphate buffer (50 mM); pH 8 and 9, Tris-HCl buffer (50 mM); pH 10, carbonate buffer (50 mM).

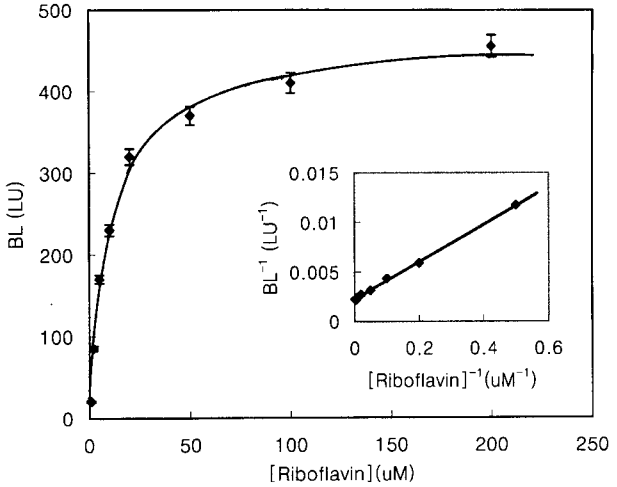

Fig. 5. The dependence of riboflavin kinase activity upon the concentration of riboflavin using bioluminescence assay. A fixed time assay for 10 min incubation at pH 7.0 and 25°C with shaking was employed. Inset: the Km value of riboflavin kinase for riboflavin was determined with a double reciprocal plot.

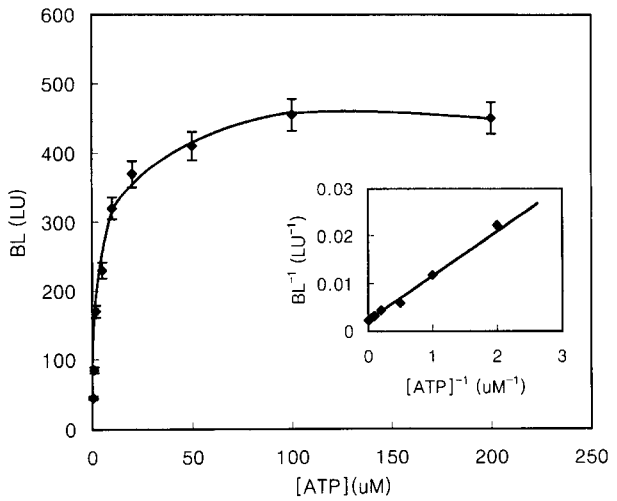

Fig. 6. The dependence of riboflavin kinase activity upon the concentration of ATP using the bioluminescence assay. A fixed time assay for 10 min incubation at pH 7.0 and 25°C with shaking was employed. Inset: the Km value of riboflavin kinase for ATP was determined with a double reciprocal plot.

was around pH 7.0 (Fig. 4). Although most studies about riboflavin kinase were derived from rat or human and previous results about bovine liver riboflavin kinase are not available, these results were in the similar range for the riboflavin kinase of rat liver (20).

Dependence of riboflavin kinase activity on substrate concentration

To remove any contaminated FMN in the riboflavin, 5 ml of saturated riboflavin solution was passed through a DEAE-cellulose column, and the unbound riboflavin solution was collected and the concentration was measured with a spectrophotometer at 445 nm. The Km value of riboflavin kinase for bovine liver riboflavin kinase was determined with a double reciprocal plot to be 15.3 μM which is a little higher value obtained for rat liver riboflavin kinase (11 μM, 20) by the radioisotope assay (Fig. 5). The Km value of riboflavin kinase for ATP in the presence of 20 mM $MgCl_2$ was determined with a double reciprocal plot to be 8.3 μM which is about a 2.5 fold higher value obtained for rat liver riboflavin kinase (3.7 μM, 20) by the radioisotope assay method (Fig. 6).

The application of bacterial bioluminescence reaction for the assay of riboflavin kinase was demonstrated on the

basis that bacterial luciferase used reduced FMN as substrates. In previous works, the production of aldehyde (4) or free fatty acid (2, 3) were demonstrated to be assayed with bacterial bioluminescence. The reaction of riboflavin kinase is the phosphorylation of riboflavin to FMN. In some cases, the phosphorylation and dephosphorylation can change the spectral properties of the compounds greatly, such as p-nitrophenol and p-nitrophenyl phosphate, in which the electrons of the phosphorylating hydroxyl group are delocalized in the aromatic ring moiety. However in riboflavin and riboflavin-5-phosphate (FMN), the phosphorylating hydroxyl group is separated from the aromatic ring moiety (isoalloxazine) by a linear ribosyl group and does not affect the spectral properties. The only difference caused by phosphorylation is the change in polarity, and many analytical methods have been developed based on this difference. Riboflavin kinase can be assayed with HPLC using a reverse phase or an anion exchange type analytical column and UV-visible detector (11). Although this process gives quite reliable results, it takes lots of time (at least 10 minutes per assay) and only one assay at a time can be performed with one HPLC. Preparation of chromatograph solvent is also cost and time consuming. Assay with TLC or paper chromatography has some advantage in that many samples can be analyzed at a time, but it is not sensitive enough and the accurate quantization of the result is quite difficult and in many cases, not so reliable even though some kinds of TLC densitometer or TLC-FID (Iatroscanner) are used. The separation and quantization of FMN from riboflavin based on solvent partition was found to be erroneous because the efficiency of partition is dependent upon the concentration of each flavin compound (6).

The advantage of the bioluminescent assay is its high sensitivity and reproducibility using relatively inexpensive equipment. The time for each assay is less than 15 seconds, and a number of assays, such as chromatographic fractions, can be performed very quickly. Although, it cannot give continuous result, this bioluminescent method to determine riboflavin kinase is quite specific, rapid, and easy to do. In some cases, especially in crude enzyme preparation, tightly bound flavin compounds are difficult to remove completely with gel filtration or dialysis. In these cases, illumination of the enzyme preparation with a conventional UV-lamp for 5 min usually solve the problem by photochemical degradation of flavin into lumiflavin or other nonreactive compounds. There are no interference to bacterial luciferase reaction due to the covalently bound flavin compounds or pyridine nucleotide (7).

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