Oxyluciferin, a Luminescence Product of Firefly Luciferase, Is Enzymatically Regenerated into Luciferin

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Abstract

The activity regenerating luciferin from the luminescent product oxyluciferin was found in the protein fraction of a lantern extract from Photinus pyralis. The protein, luciferin-regenerating enzyme (LRE), was purified by homogeneity by ammonium sulfate precipitation followed by successive column chromatography on Ultrogel AcA34, S-Sepharose FF, Q-Sepharose FF, TSKgel super Q 5pw and TSKgel G3000 SWXL. This enzyme was a single polypeptide with a molecular mass of 38 kDa. LRE converted oxyluciferin to 2-cyano-6-hydroxybenzothiazole and thioglycolic acid. In the presence of D-cysteine, 2-cyano-6-hydroxybenzothiazole was turned over into luciferin. The same activities were detected in the extracts from two Japanese fireflies, Luciola cruciata and Luciola lateralis. We have cloned a cDNA encoding LRE from poly(A)+ RNA of the lantern of Photinus pyralis using reverse transcription-polymerase chain reaction, 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE. The primary structure of LRE from P. pyralis deduced from the nucleotide sequence was shown to consist of 308 amino acids with a molecular weight of 33,619. The cDNA was successfully expressed under the control of the tac promoter in Escherichia coli.

Firefly luciferase (Luc, EC 1.13.12.7) catalyzes the oxidative decarboxylation of luciferin (LH$_2$) in the presence of ATP, O$_2$, and Mg$^{2+}$, producing yellow-green light ($\lambda_{max}$ = 560 nm) as described in the following reaction sequence:

$$\text{ATP} + \text{LH}_2 + \text{Luc} \rightarrow \text{Lac} + \text{LH}_2\text{AMP} + \text{PP}_i$$

$E \cdot \text{LH}_2\text{AMP} + \text{O}_2 \rightarrow \text{Luc} + \text{AMP} + \text{CO}_2 + \text{Oxyluciferin} + \text{light}$

Photinus pyralis luciferase is an enzyme that appears to have no prosthetic groups or tightly bound metal ions and is comprised of 550 amino acids and has a molecular mass of about 60 kDa (1, 2). Studies of the molecular components in the bioluminescence mechanism of fireflies have shown that the substrate of luciferase is firefly luciferin, a polyheterocyclic organic acid, 2-(6-hydroxybenzothiazol-2-yl)-2-thiazoline-4-carboxylic acid (3). Oxyluciferin, which is the product of the luciferase reaction, has a strong inhibitory effect on the firefly luciferase (4, 5).

Suzuki and Goto injected [$^{14}$C]oxyluciferin and 2-[$^{14}$C]cyano-6-hydroxybenzothiazole into live fireflies and detected [$^{14}$C]luciferin after several hours (6). They concluded that the luminescence product, oxyluciferin, is supplied as the substrate luciferin for next light emission. Their results may be explained by the following two-step reaction: 1) transformation of oxyluciferin to 2-cyano-6-hydroxybenzothiazole, and 2) condensation of 2-cyano-6-hydroxybenzothiazole with D-cysteine to yield luciferin. The second step can occur under non-enzymatic conditions (Ref. 6, Fig. 1). They also suggested that this transformation (the first step) might not be catalyzed by an enzyme because 2-cyano-6-hydroxybenzothiazole was produced from oxyluciferin without firefly extract for several hours in vitro. We also detected luciferin regeneration from oxyluciferin under non-enzymatic conditions, although this was a very slow reaction.

We found an activity that enhanced luciferin regeneration from oxyluciferin in the protein fraction of some firefly extracts (P. pyralis, Luciola cruciata and Luciola lateralis). In this study, we purified the proteins from these fireflies to homogeneity and compared some of their properties. Moreover, we cloned the LRE cDNA from P. pyralis and confirmed expression the cDNA in Escherichia coli.

**EXPERIMENTAL PROCEDURES**

**Assay of LRE Activity**

Activity measurement of LRE was carried out by the following three steps: 1) the fraction was mixed with oxyluciferin, 2) the generated substance, 2-cyano-6-hydroxybenzothiazole, was reacted with D-cysteine, 3) and the amount of the generated substance, D-luciferin, was measured by reacting with luciferase. Oxyluciferin was synthesized by Prof. Isobe of Nagoya Univ. The protein fraction was incubated with 25 mM glycyglycine (pH 7.8) containing 0.1 mM oxyluciferin and 5.4 mM MgSO$_4$ at 37°C for 5 h in the dark. Next, 10 µl of the reaction solution was added to 100 µl of 0.01 mM D-cysteine. After 5 min, 100 µl of 10 mM ATP...
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Desiccated extract from 100 mg of firefly lanterns (Sigma) was dissolved in 1 ml of Buffer A (50 mMTris, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 100 mM NaCl). After centrifugation (12000 rpm, 10 min), 100 µl of supernatant was injected into a TSKgel G3000 SWXL column (0.78 × 60 cm, Tosoh, Tokyo, Japan). HPLC conditions were as follows: solution, Buffer A; flow rate, 1 ml/min; detector, 280 nm. The eluate was fractionated every 30 s and was analyzed for luciferase activity, amount of luciferin, and luciferin regenerating activity.

**Purification of A-LRE from Firefly Lanterns of P. pyralis**

Desiccated extract from 4 g of dried firefly lanterns (Sigma) was dissolved in 200 ml of Buffer A containing 10% saturation ammonium sulfate. After centrifugation, ammonium sulfate was added to the supernatant up to a concentration of 39% saturation and kept on ice for 2 h. After a second centrifugation, ammonium sulfate was added to the supernatant up to a concentration of 62% saturation and kept on ice overnight. The precipitate was collected and dissolved in 6 ml of Buffer A. The dissolved sample was loaded onto a gel filtration column (Ultrogel Aca34) equilibrated with Buffer A. A-LRE was eluted at 300 ml. The active fractions were combined and concentrated with Centriprep 10 (Amicon) and the buffer was replaced with Buffer B (5 mM Tris-HCl, pH 8.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 mM NaCl). The sample was loaded onto an S-Sepharose FF column. A-LRE was eluted at 100 mM NaCl using a NaCl concentration gradient of 1–200 mM in 500 ml of Buffer B. The active fractions were combined and concentrated with Centriprep 10, and the buffer was replaced with Buffer C (5 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 mM NaCl). The sample was loaded onto a Q-Sepharose FF column. A-LRE was eluted at 100 mM NaCl using a NaCl concentration gradient of 1–200 mM in 500 ml of Buffer C. The active fractions were combined and concentrated with Centriprep 10 to a volume of 2 ml. The concentrated sample was loaded onto a TSKgel super Q 5PW column and subjected to HPLC. A-LRE was eluted at 70 mM NaCl in 50 ml of a 0–200 mM NaCl gradient. The active fractions were pooled and concentrated with Centriprep 10 to 2 ml. The concentrated sample was loaded onto a TSKgel G3000 SWXL column equilibrated with Buffer A and subjected to HPLC under the same conditions as in Fig. 3. A-LRE was eluted at 21 ml.

**Purification of H-LRE**

Lanterns (wet weight, 2 g) of frozen *L. lateralis* were homogenized in 15 ml of 25 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol using Ace homogenizer AM-3 (Nihon Seiki, Tokyo, Japan). The supernatant of the homogenate was used for purification according the same procedure as described for H-LRE except for 30–60% saturated ammonium sulfate precipitation. G-LRE was purified from lanterns of *L. cruciata* according the same procedure as described for H-LRE.

**SDS-PAGE and Determination of Amino Acid Sequences of A-LRE**

Some fractions containing A-LRE were electrophoresed in 4–20% gradient polyacrylamide gels with SDS. The N-terminal amino acid sequence of A-LRE was determined using a peptide sequencer (ABI 492; PerkinElmer Life Sciences). To analyze internal amino acid sequences, LRE was digested by trypsin (7). Purified LRE (0.5 mg) was separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G-250 for 5 min and destained with 30% methanol for 5 min, followed by water until the protein band was visible. The band of LRE was cut out and destained with 30% methanol for 5 min. The band of LRE was dissolved in 6 ml of Buffer A. After drying, the gel was stored at −80 °C, a supernatant was removed after centrifugation, ammonium sulfate was added to the supernatant up to a concentration of 39% saturation and kept on ice for 2 h. After a second centrifugation, ammonium sulfate was added to the supernatant up to a concentration of 62% saturation and kept on ice overnight. The precipitate was collected and dissolved in 6 ml of Buffer A. The dissolved sample was loaded onto a gel filtration column (Ultrogel Aca34) equilibrated with Buffer A. A-LRE was eluted at 300 ml. The active fractions were combined and concentrated with Centriprep 10, and the buffer was replaced with Buffer B (5 mM Tris-HCl, pH 8.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 mM NaCl). The sample was loaded onto an S-Sepharose FF column. A-LRE was eluted at 100 mM NaCl using a NaCl concentration gradient of 1–200 mM in 500 ml of Buffer B. The active fractions were combined and concentrated with Centriprep 10, and the buffer was replaced with Buffer C (5 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 mM NaCl). The sample was loaded onto a Q-Sepharose FF column. A-LRE was eluted at 100 mM NaCl using a NaCl concentration gradient of 1–200 mM in 500 ml of Buffer C. The active fractions were combined and concentrated with Centriprep 10 to a volume of 2 ml. The concentrated sample was loaded onto a TSKgel super Q 5PW column and subjected to HPLC. A-LRE was eluted at 70 mM NaCl in 50 ml of a 0–200 mM NaCl gradient. The active fractions were pooled and concentrated with Centriprep 10 to 2 ml. The concentrated sample was loaded onto a TSKgel G3000 SWXL column equilibrated with Buffer A and subjected to HPLC under the same conditions as in Fig. 3. A-LRE was eluted at 21 ml.

**Isolation of mRNA from P. pyralis**

Desiccated firefly tails (Sigma) were ground with a mortar and suspended to 10 ml of ISOGEN (WAKO, Osaka, Japan). After centrifugation, total RNA was precipitated with isopropanol from the solution of upper layer. Finally, poly(A)+ RNA was isolated using an Oligotex-dT30 (Super) (Takara, Kyoto, Japan).
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A-LRE, luciferin-regenerating enzyme, was purified from firefly lantern extract of P. pyralis and was separated from luciferase and luciferin (Fig. 1). The A-LRE gene was sequenced using a dye terminator sequencing kit (PerkinElmer Life Sciences) running at 94 °C, 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were subjected to electrophoresis, and the amplified DNA band (0.7 kbp) was recovered from the gel.

3'-RACE was carried out under the same conditions as RT-PCR except for the primers used. Oligo(dT)-adapter primer (Takara) was used for reverse transcription, and oligonucleotide primers KN4 and M13-M4 (Takara) were used for PCR.

Culture and Extraction of LRE Expressed in E. coli

The full-length LRE cDNA was amplified by RT-PCR. Reverse transcription of 1 µg of poly(A)+RNA using 2.5 pmol of oligo(dT) primer KC9, 0.6 µg of P. pyralis mRNA, and 1 mM of each of the four deoxynucleoside triphosphates and 5 units of reverse transcriptase at 30 °C for 10 min, 55 °C for 45 min, 80 °C for 2 min, and 5 °C for 5 min. PCR was carried out in a final volume of 100 µl containing 20 µl each of the oligonucleotide primers (KN3, KC6), 20 µl of RT solution and 2.5 units of Taq polymerase. Amplification was carried out by an initial denaturation step at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min. Reaction products were subjected to electrophoresis, and the amplified DNA band (0.4 kbp) was recovered from the gel.

Nucleotide Sequencing Procedure and Analysis of Reaction Products by HPLC

cDNA fragments of the A-LRE gene were sequenced using a dye terminator sequencing kit (PerkinElmer Life Sciences) running on an ABI 373A. Amplification was carried out in a final volume of 50 µl containing 1 µl of the first reaction solution and 60 units of RNaseH at 30 °C for 60 min. After the reaction, single-stranded DNA was recovered by ethanol precipitation. The third reaction (single-stranded DNA ligation) was carried out in a final volume of 41 µl containing single-stranded DNA from the second reaction, 20 µl of 40% PEG 6000 and 40 units of T4 RNA ligase, at 16 °C overnight. The fourth reaction (first PCR) was carried out in a final volume of 50 µl containing 1 µl of 1/10 diluted third reaction solution, 0.4 mM of each dNTP, 10 pmol of each primer (KC10, KN4) and 2.5 units of Ex-Taq polymerase at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The fifth reaction (nested PCR) was carried out in a final volume of 50 µl containing 1 µl of the fourth reaction solution, 0.4 mM of each dNTP, 10 pmol of each primer (KC11, KN6) and 2.5 units of Ex-Tag polymerase by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were subjected to electrophoresis, and the amplified DNA band (0.4 kbp) was recovered from the gel.

Effects of LRE on Luciferin-Luciferase Reaction

The effects of LRE on the luciferase assay were determined using a luminometer (BLR-201, Aloka) at 25 °C with readings taken every 30 s. The reaction mixture contained 190 µl of 25 mM glycyglycine buffer (pH 7.8), 5 mM MgSO4, 0.25 mM luciferin, 2.5 × 10^-6 mg/ml luciferase, 5 mM d-cysteine, and 10 µl of purified LRE (13.8 µg) or Buffer A.

RESULTS

The resolved firefly lantern extract of P. pyralis (Sigma) was fractionated by HPLC with TSKgel G3000 SWXL. Each fraction was assayed with oxyluciferin and d-cysteine and the amount of regenerated luciferin was measured by luminescence of luciferase and ATP. LRE activity was eluted from the column in the ~40-kDa protein fraction and was separated from luciferase and luciferin (Fig. 3). The activity in the protein fraction suggested that oxyluciferin could be enzymatically regenerated into luciferin in the firefly lantern.

A-LRE, luciferin-regenerating enzyme, was purified from firefly lantern extract of P. pyralis by ammonium sulfate precipitation followed by successive column chromatography on Ultrogel AcA43, S-Sepharose FF, Q-Sepharose FF, TSKgel super Q 5pw and TSKgel G3000 SWXL. The purified A-LRE had a molecular mass of ~38 kDa (Fig. 4, lane 3) and showed maximum activity at pH 7–8 and 40 °C (data not shown).

Figure 4

SDS-PAGE. Some fractions containing LRE were electrophoresed in a 4–20% gradient polyacrylamide gel with SDS. Lane 1, 4 and 6, markers; Lane 2,
firefly lantern extract; lane 3, purified LRE; lane 5, lysate of transformant. Markers were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), rabbit muscle aldolase (42.4 kDa), bovine carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).

LRE activities were also found in the extracts of two Japanese fireflies (L. cruciata and L. lateralis). Both LREs were purified according to the purification procedure described for P. pyralis except for 30–60% saturated ammonium sulfate precipitation. LREs from three fireflies, A-LRE (P. pyralis), G-LRE (L. cruciata), and H-LRE (L. lateralis) were characterized together.

As shown in Fig. 5, the stability of the LREs at different temperatures was investigated. LREs were found to be relatively stable up to 50 °C. However, A-LRE and G-LRE were unstable at temperatures of 60 °C or more. On the other hand, the remaining activity of H-LRE was about 50% after heat treatment at 70 °C for 30 min. H-LRE was found to be most stable among these LREs. The optimum pH value for the reaction was 7.0 for A-LRE and G-LRE and 8.0 for H-LRE (data not shown).

The N-terminal amino acid sequence of the purified protein was determined to be GPVVEKIAELGKYTVGEGP by Edman degradation with an automated protein sequencer (model 492, PerkinElmer Life Sciences). A homology search for this sequence in the Swiss-Protein sequence data base revealed no matches. This indicated that LRE was a novel protein. LRE was the only enzyme found that acted on oxyluciferin.

To analyze the internal amino acid sequence, purified LRE was hydrolyzed by trypsin in gel. An ABI 492 peptide sequencer was used to analyze some peptides separated using the C18 column of the µ-blotter system (PerkinElmer Life Sciences), and the following sequences were determined: IPDPQVTSVAFGGPNLDELH (seq. 1), LDGDADPGLNLWTGMAIDAGLPVPVTG (seq. 2), YTVGEGPHWDHETQTLYFVDT (seq. 3), MHESNIAANGLAWSDLK (seq. 4), FTVSLER (seq. 5), VDEYDYDASTLSISNORPLTTFEK (seq. 6). The N-terminal seven residues of sequence 3 matched the N-terminal region amino acid sequence of LRE, which had already been determined.

To obtain a part of the cDNA of A-LRE, we used RT-PCR amplification with oligo(dT) primer and oligonucleotide primers KN3 and KN6. KN3, a sense primer, was designed and synthesized on the basis of the N-terminal amino acid sequence deduced from the 0.7-kbp DNA fragment. An ABI 492 peptide sequencer (model 492, PerkinElmer Life Sciences) was used for the internal amino acid sequence of the purified LRE. The resultant single-stranded DNA was ligated with T4 RNA ligase and used for first PCR amplification with KC10 and KN6. A DNA fragment of 0.53 kbp was amplified and sequenced. The N-terminal region amino acid sequence and several amino acid sequences of internal peptides were determined from the amino acid sequence deduced from the 0.7-kbp DNA fragment.

A new primer, KN4 was synthesized on the basis of the 0.7 kbp DNA fragment and was used for 3’-RACE (11). A DNA fragment of 0.53 kbp was amplified and sequenced. The amino acid sequence of an internal peptide was found in the amino acid sequence deduced from this fragment.

To obtain the 5’-terminal DNA sequence of the cDNA encoding LRE by 5’-RACE, four new primers, KC9, KC10, KC11, and KN6, were synthesized on the basis of the DNA sequence of a 0.7-kbp fragment (8) (Fig. 2). KC9 primer was phosphorylated by T4 kinase and used for reverse transcriptase reaction with avian myeloblastosis virus RT-XL. The resultant single-stranded DNA was ligated with T4 RNA ligase and used for first PCR amplification with KC10 and KN4 primers. Diluted first PCR solution was used for nested PCR amplification with KC11 and KN6 primers. A DNA fragment of 0.4 kbp was amplified and sequenced. The N-terminal amino acid sequence was found in the amino acid sequence deduced from the 0.4-kbp fragment.

New primers for the full-length gene, KN7 and KC12, were synthesized, and were used for RT-PCR with oligo(dT) primer for reverse transcription. A DNA fragment of 0.9 kbp was amplified and inserted into the pKK223–3 vector. E. coli JM109 was transformed with the ligated plasmid. Plasmid DNA (pLRE) from the transformant, E. coli JM109 (pLRE), was isolated and sequenced. Fig. 6 shows the nucleotide sequence of the A-LRE cDNA consisting of 927 bp. The nucleotide sequences determined by RT-PCR, 3’-RACE, and 5’-RACE existed in this full sequence. The identified N-terminal amino acid sequence was GPVVEKIAELGKYTVGEGP. Although no residue could be assigned to the first position, the remaining sequence is consistent with the ATG codon being used as
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The initiator methionine codon. The A-LRE polypeptide consists of 308 amino acids with a predicted molecular mass of 33619 Da. This is in agreement with the observed molecular mass of LRE (38 kDa) determined by SDS-polyacrylamide gel electrophoresis. Sequence comparison at the amino acid level (deduced from the nucleotide sequence) indicated low levels of similarity to some genes. The alignments revealed 33% similarity to Bacillus hypothetical 33.2-kDa protein in the gerac-fhuc intergenic region and 23% to yeast PCF11 protein.

Recombinant E. coli cells, JM109 (pLRE) and JM109 (pKK223–3), were cultured with the addition of 1 mM isoproopyl-1-thio-β-D-galactopyranoside at the Klett 100, and cell extracts were tested for LRE activity. As shown in Table I, LRE activity was detected in the extract of JM109 (pLRE).

Table I  
LRE activity in E. coli JM 109 containing pLRE and pKK223–3

R-LRE was purified from E. coli (pLRE) cells according to the method for purification of native A-LRE from P. pyralis. Purified R-LRE was assayed with oxyluciferin, and products of the reaction were analyzed by reverse phase chromatography with HPLC (Fig. 7). When LRE and D-cysteine were not contained in the reaction mixtures, two major peaks of 2-cyano-6-hydroxybenzothiazole and oxyluciferin and a minor peak of luciferin were observed (Fig. 7 a). When LRE was added to this reaction mixture, the peak of oxyluciferin was lost, and the peak of 2-cyano-6-hydroxybenzothiazole increased markedly (Fig. 7 b). Furthermore, after adding D-cysteine, the peak of 2-cyano-6-hydroxybenzothiazole decreased, and the peak of luciferin became the major peak (Fig. 7 c). Luminescence of each reaction mixture was measured by adding luciferase and ATP. Luminescence of the reaction mixture containing LRE and D-cysteine was increased to a greater extent than the others (data not shown).

Fig. 8 shows the effects of R-LRE on luciferin-luciferase reaction. Luminescence of luciferase and luciferin with or without R-LRE was monitored with a luminometer. The peak of luminescence with LRE doubled without LRE, and it decreased more slowly than that without LRE.
**DISCUSSION**

In this paper, we have described the detection, purification, and characterization of a novel enzyme, LRE, from three fireflies. Moreover, we have succeeded in cloning, sequence analysis, and expression in E. coli of A-LRE cDNA isolated from P. pyralis.

We found the activity regenerating oxyluciferin into luciferin in the extract of the firefly P. pyralis. The activity was eluted in the protein fractions after gel filtration (Fig. 3), which indicated that the regeneration reaction was enzymatic in contrast to Suzuki and Goto's suggestion (6). They suggested that D-luciferin was regenerated by a two-step reaction, which was non-enzymatic because D-luciferin was regenerated from oxyluciferin in buffer containing only cysteine. Our results indicated, however, that oxyluciferin was efficiently regenerated from oxyluciferin by an enzyme, LRE, in the firefly.

The molecular size of the purified A-LRE was 38 kDa as determined from its mobility on SDS-PAGE (Fig. 4) and 40 kDa as judged from the retention time on the gel filtration column. The value obtained by SDS-PAGE was slightly smaller than that determined by gel filtration, but A-LRE seems to be comprised of a single polypeptide. Intact A-LRE was not modified because R-LRE and A-LRE showed the same mobility on SDS-PAGE.

We also purified LREs from two Japanese fireflies (L. cruciata and L. lateralis). Molecular sizes of both LREs were ~35 kDa as judged from their mobility on SDS-PAGE (data not shown). These two Japanese fireflies are expected to have the same luciferin-regenerating system as P. pyralis, despite the distance between their habitat. Adult fireflies do not eat, drink only water, and therefore must make luciferin without intake of organic compounds. Thus, a system for recycling of luciferin would be necessary for fireflies. The purified LREs were characterized together. Especially, H-LRE was found to be the most stable among these LREs (Fig. 5). Previously, we reported purification and characterization of luciferases from three fireflies (12). Luciferase of L. lateralis was the most stable, similar to LRE. The stability of enzymes originating from insects may be influenced by environmental factors such as the temperature during the growth season because L. lateralis is active at a warmer time of the year than L. cruciata.

The cDNA sequence showed that the open reading frame consists of 927 bp, which encodes 308 amino acids. A homology search for amino acid sequence deduced from the cloned LRE cDNA in the Swiss-Prot protein sequence data base revealed some sequences with low levels of homology. This indicated that LRE was a novel protein.

Purified recombinant LRE was assayed with oxyluciferin, and products of the reaction were analyzed by HPLC (Fig. 7). When oxyluciferin was assayed with LRE, the peak of 2-cyano-6-hydroxybenzothiazole became high. When oxyluciferin was assayed with LRE and cysteine, however, a peak of luciferin was found, and the peak of 2-cyano-6-hydroxybenzothiazole had disappeared. These results suggested that LRE catalyzed the conversion from oxyluciferin to 2-cyano-6-hydroxybenzothiazole and 2-cyano-6-hydroxybenzothiazole produced by LRE was non-enzymatically converted to luciferin with cysteine. These observations agreed with those reported by Suzuki and Goto that condensation of 2-cyano-6-hydroxybenzothiazole with cysteine yielded luciferin (6). However, they also concluded that oxyluciferin was non-enzymatically converted into 2-cyano-6-hydroxybenzothiazole. Although luciferin can be reproduced non-enzymatically, LRE markedly increased the efficiency of the reaction. Despite the absence of cysteine, very small peaks of luciferin were detected (Fig. 7, a and b). These peaks may have been seen due to contamination by luciferin or cysteine in the reaction mixture. Oxyluciferin has a strong inhibitory effect on luciferase in a manner competitive with luciferin (4, 5). Based on these observations, conversion of oxyluciferin by LRE appears to result in the rapid turnover of luciferase for the next light emission.

To confirm this, purified recombinant LRE was assayed with luciferin and luciferase (Fig. 8). LRE showed some effects on the reaction. Luminescence without LRE was almost zero at 9 min under these conditions. Interestingly, the amount of luminescence integration for 20 min was increased by 25-fold in the presence of LRE as compared with that in its absence. Under these conditions, luminescence was not maintained at the same level as the peak of the reaction. Luciferin is thought to be efficiently recycled in the body of the firefly. Our data are still inadequate, and the reaction system still requires further optimization or other factors may be found. Other factors may be found by the same means as used in the present study; that is, incubation of all gel filtration fractions with oxyluciferin and LRE and analysis of the amount of luciferin regenerated by LRE and cysteine by monitoring the luminescence of luciferin. Fractions containing the factors will affect the luciferin regeneration reaction. Luciferase assays have been used to determine the amounts of ATP in the various biological samples, and biotinylated luciferase has been used for immunological detection of various compounds and microorganisms (13).

Eisaki et al. have reported a system for recycling of ATP by pyruvate orthophosphate dikinase (PPDK, E.C. 2.7.9.1) (14, 15). In this system, AMP and pyrophosphate are produced from ATP by luciferase were converted back into ATP by PPDK. The ATP generated by PPDK was used by luciferase, and luminescence of luciferase was amplified. Recycling of ATP is useful to increase the sensitivity of luciferase assays. The system with LRE is also expected to increase the sensitivity of luciferase assays to detect target compounds. Studies are currently underway in our laboratory to determine the optimum conditions for luminescence assays with LRE.

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**Footnotes**

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s).

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LRE luciferin-regenerating enzyme
HPLC high pressure liquid chromatography
A-LRE luciferin-regenerating enzyme from the American firefly (P. pyralis)
G-LRE luciferin-regenerating enzyme from the Japanese firefly, Genji (L. cruciata)
H-LRE luciferin-regenerating enzyme from the Japanese firefly, Heike (L. lateralis)
R-LRE recombinant A-LRE
RACE rapid amplification of cDNA ends
PAGE polyacrylamide gel electrophoresis
RT-PCR reverse transcription-polymerase chain reaction
kbp kilobase pair(s)
PPDK pyruvate orthophosphate dikinase

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