博士論文

Development of salt-resistant luciferase and its application to endotoxin measurement

（耐塩性ルシフェラーゼの開発とエンドトキシン測定法への応用）

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(2) Improved bioluminescence-based endotoxin measurement method using a salt-resistant luciferase mutant
Satoshi Yawata, Kenichi Noda, Ai Shimomura, Akio Kuroda
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Chapter 1: Introduction

1.1. Importance of endotoxin measurement

Endotoxin is a lipopolysaccharide in the cell membrane of Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* [1]. It is named endotoxin because it is a toxin released by the death of Gram-negative bacteria. Structurally, the endotoxin has both hydrophilic and hydrophobic moieties which form a micellar structure in an aqueous solution with a molecular weight of hundreds of thousands to millions. The toxicity of endotoxin differs depending on changes in the micelle structure and the bacteria type. When mixed with the blood in the human body, endotoxin causes fever and shock symptoms called endotoxic shock. Since injections and dialysis come into contact with human blood, the standards for endotoxin concentration in these pharmaceuticals are stipulated in the pharmacopeia and other guidelines [2, 3, 4]. Hemodialysis patients are exposed to a relatively 350–500 L of dialysis every week. Endotoxin in the dialysis can pass through the dialysis membrane into the patient's blood stream. The endotoxin concentration in the dialysis must be controlled below 0.001 EU/mL (EU: Endotoxin Unit) as per the Japanese Society for Dialysis Therapy (JSDT), which is lower than the injectables. The lowest mortality rate for patients was reported when treated with the dialysis controlled below 0.001 EU/mL [5]. Additionally, endotoxin has high heat resistance and is not

completely inactivated by autoclaving [6]. Dry heat sterilization at 250 °C for 30 minutes is required for complete inactivation. Therefore, it is necessary to measure and control the endotoxin concentration to prevent medical accidents. ISO and JSDT require pre-dialysis endotoxin measurement [3,4].

1.2. Endotoxin measurement method

In earlier times, a rabbit fever test had been used, wherein the sample is injected into a rabbit and the endotoxin concentration is calculated from the increase in body temperature. However, there are problems, such as that it takes a long time to obtain a result and numerous animals must be used for complicated operations and inspections [2]. Bang *et al.* discovered that horseshoe crab blood was coagulated by infection with *Vibrio* in the United States [7]. Levin *et al.* discovered that endotoxin caused coagulation of horseshoe crab blood cell extract (Limulus amebocyte lysate (LAL) reagent) [8]. Then, it was reported that the reaction of the LAL reagent showed a correlation with the fever test [9]. In the 1980s, measurement methods using LAL reagents were listed in the pharmacopeia of each country as endotoxin test methods. In 1992, it was discovered that the activation of the LAL reagent was a cascade reaction wherein the blood components factor C, factor B, pro-clotting enzyme, and coagulogen were sequentially activated [10]. In the reaction of the LAL reagent, the endotoxin cleaves factor C and activates factor C. This fully activated

factor C cleaves and activates factor B. Activated factor B then cleaves the pro-clotting enzyme to become the clotting enzyme. This clotting enzyme cleaves coagulogen and then form the gel (Fig. 1-1, coagulation).

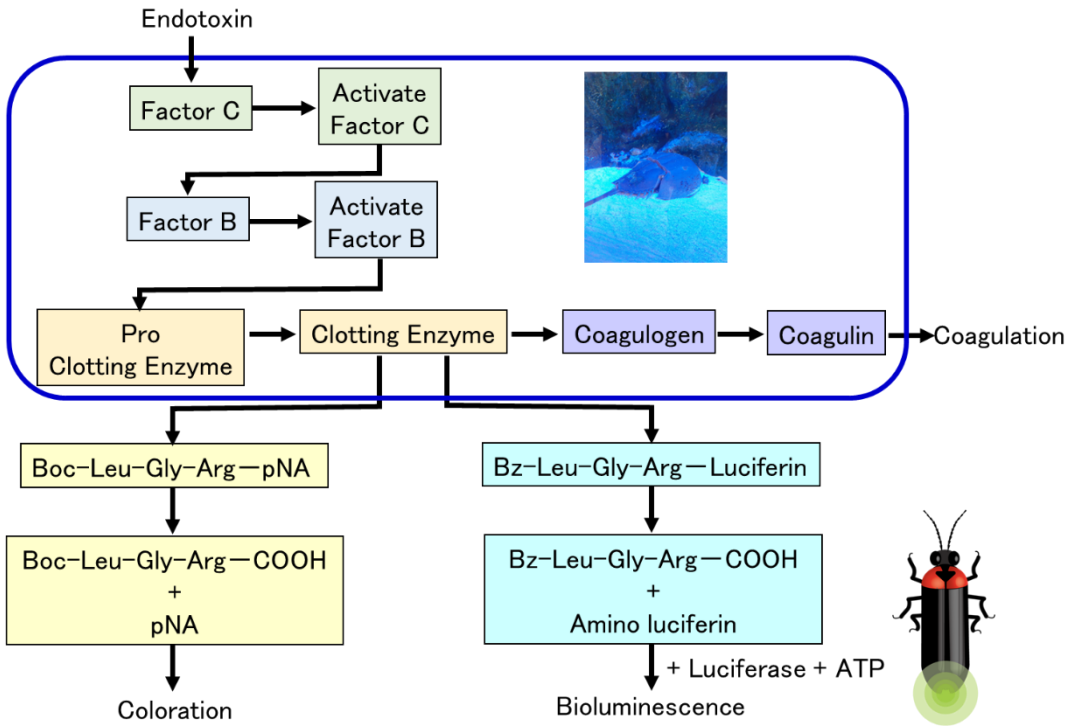

Fig. 1-1 Principle of endotoxin measurement using LAL reagent (encircled in blue). Coagulation (turbidimetric method): the amount of gel resulted from the cleavage of coagulogen is optically measured. Coloration (colorimetric method): pNA produced from the cleavage of substrate by clotting enzyme is optically measured. Luminescence (bioluminescence-based method): D-luciferin produced from the cleavage of substrate by clotting enzyme is measured using a photon counter.

Several methods were published using LAL reagent to measure the endotoxin levels. Initially, a gel-clotting method was developed wherein the gel was aggregated in a test tube and then overturned to check the state of the gel; however, this method required visual judgment of the

state of the gel and had been limited for quantification. Therefore, a turbidimetric method was developed wherein the amount of gel produced was optically measured and the endotoxin concentration was calculated from the amount of change in turbidity [11]. On the other hand, the coloring substrate (*tert*-Butoxycarbonyl-Leu-Gly-Arg-p-nitroaniline (pNA)) was added to the LAL reagent. In this test, the clotting enzyme cleaved the peptide bond between Arg and pNA to release pNA, and the color was developed. A colorimetric method was developed to optically measure this color change and calculate the endotoxin concentration from the amount of color change (Fig. 1-1, coloration) [12, 13].

1.3. Bioluminescence-based endotoxin measurement method

Chromogenic and turbidimetric methods are commonly used to measure endotoxin. However, chromogenic methods show low sensitivities (detection limit of approximately 0.01–0.1 EU/mL) and turbidimetric methods are time-consuming (over 60 min). In 2010, Noda *et al.*, developed a bioluminescence-based method that combines the LAL reaction and the luciferase luminescence reaction [14]. In the bioluminescence-based method, the luminescent substrate (benzoyl (Bz) -Leu-Gly-Arg-aminoluciferin) is added to the LAL reagent, and the clotting enzyme cleaves the peptide bond between Arg and aminoluciferin to release D-luciferin. Luciferase and adenosine triphosphate (ATP) are added to this reaction. The endotoxin concentration is calculated from the amount of light emission. The bioluminescence-based method using luciferase can

measure endotoxin in water rapidly (15 min) and with high sensitivity (0.0005 EU/mL) (Fig. 1-1, Bioluminescence) [14]. However, in the case of dialysis samples containing sodium chloride, bioluminescence-based method was not available due to the inhibition of luciferase by sodium chloride [15]. The guidelines for measuring endotoxin in dialysis indicate that the measured values of endotoxin in medical dialysis must be 75–125% of those tested in water [16, 17]. Therefore, the use of bioluminescence has been limited for endotoxin measurement in dialysis.

1.4. Evolutionary engineering modifications of firefly luciferase

The North American firefly luciferase is a 62 kDa enzyme that oxidizes D-luciferin in the presence of ATP and magnesium. It emits a yellow-green luminescence with a maximum wavelength of 562 nm [18]. Luciferase is used in many applications; however, there are limitations, such as low thermal stability and a small amount of luminescence for intracellular use. Replacing an amino acid with another amino acid by introducing a mutation into a luciferase gene can improve the properties of wild-type luciferase (Table 1-1).

Table 1-1 Mutation of firefly luciferase.

Mutation phenotype	Amino acid substitution	Characterization	Ref.
Thermostability	T214A, A215AL, I232A, F295L, and E345K	27-fold more thermally stable than wild type	21,22,23
	F14R, L35Q, V182K, I232K, and F465R	Enhanced thermostability up to 45 ℃ with higher pH-tolerance	25
	F14R, L35Q, A105V, V182K, T214C, I232K, D234G, F295L, E354R, D357Y, S420T, and F465R	Enhanced thermostability: Half-life of 15 min at 55 ℃	26
Catalytic activity	I423L, D436G, and L530R	20-fold lower Km for ATP and D-luciferin, and 4-fold higher k_{cat} than wild type	28
	T214A, A215L, I232A, F295L, E354K, I423L, D436G, and L530R	14-fold higher k_{cat} than wild type	22

	F14R, L35Q, V182K, I232K, V241I, G246A, F250S, and F465R	3.69-fold higher than luminescence intensity than the wild type	27
Maximum emission wavelength	R218K, R218Q, R218A R337K, R337Q	Shifted form (wild type:562nm) to 572nm, 608nm, 611nm, 595nm, 594nm, respectively	32
	H245A, G315A, T343A, A348V	Shifted form (wild type:562 nm) to 604 nm, 607 nm, 617 nm, 610 nm, respectively	33
	S284T	Shifted form (wild type:562 nm) to 615 nm	34

For example, the half-life of wild-type luciferase activity is less than 10 minutes at 35 °C. The crystal structure of North American firefly luciferase is composed of N domain (1-436) and C domain (440-544) [19]. The N domain consists of subdomain A (1-190) and subdomain B (191-436). Central subdomain B is less stable and therefore determines the overall stability of luciferase [20]. Various mutations in subdomain B, such as Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu, and Glu354lys, improve the thermostability of luciferase [21, 22, 23]. The half-life of mutant luciferase (mutant E) produced by the combination of the five mutations was improved to 11.5 hours at 37 °C [21, 22, 23]. Improvements to the protein surface increase protein stability [24]. Luciferase (x5 luciferase; Phe14Arg, Leu35Gln, Val182Lys, Ile232Lys, and Phe465Arg) wherein the hydrophobic amino acid that exists on the surface of luciferase is replaced with a hydrophilic amino acid showed higher pH resistance than the wild type and increased thermal stability to 45 ℃ [25]. Furthermore, 12 mutant-introduced x12 luciferases (Phe14Arg, Leu35Gln, Ala105Val, Val182Lys, Thr214Cys, Ile232Lys, Asp234Gly, Phe295Leu, Glu354Arg, Asp357Tyr, Ser420Thr, and Phe465Arg) had improved thermal stability with a half-life of 15 minutes at 55 °C [26]. The improved thermostability of x5g luciferase (Phe14Arg, Leu35Gln, Val182Lys, Ile232Lys, and Phe465Arg) also increased luminescence intensity, allowing *in vivo* imaging of the mouse brain [27].

Luciferase FM (Ile423Leu, Asp436Gly, and Leu530Arg), which introduced three mutations,

improved affinity and turnover rates with ATP and D-luciferin, expanding its potential for intracellular use [28]. Substitution of Ile423 with Leu and Met increased turnover rates. Mutations in Ile423 may affect the hydrogen bonding between the carboxyl group of Asp422 and the hydroxyl group of ATP [29]. Substitution of Asp436 with Gly increased the substrate affinity of ATP and D-luciferin. Since carboxy group of Asp436 may be electrostatically repulsive with ATP, the substitution with small amino acids could enhance ATP entry to the catalytic center [30]. Substitution of Leu530 with Arg increased the substrate affinity of ATP and D-luciferin. Substitution of nonpolar Leu530 with a cationic side-chain group may also enhance binding to phosphate groups of ATP [31]. Thr527 and Lys529 were thought to form active sites and form hydrogen bonds with the phosphate groups of ATP; therefore, the mutation of Leu530Arg replaced with a cationic side chain (Arg) may increase the affinity for ATP [29, 30, 31]. The combination of heat-stabilizing mutations (Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu, and Glu354lys) and activation mutations (LGR; Ile423Leu, Asp436Gly, and Leu530Arg) produced a novel mutant named YY5 of which k_{cat} is 14 times higher than wild-type luciferase [22].

The emission wavelength is red-shifted by luciferase mutation. The wavelength is red-shifted by introducing a mutation into the active center Arg218. Mutants harboring Arg218Lys (572 nm), Arg218Gln (608 nm), and Arg218Ala (611 nm) were developed [32]. Arg337 is located near the active center, whose mutation induced a red-shift in the emission spectra (Arg337Lys [595 nm]

and Arg337Gln [594 nm]). Mutants harboring His245Ala (604 nm), Gly315Ala (607 nm), Thr343Ala (617 nm), and Ala348Val (610 nm) also showed the red-shift. Most of these mutations are localized near the active center [33]. However, Ser284Thr (615 nm), which is a mutation of an amino acid distant from the active center, also underwent the red-shift [34].

1.5. Purpose of this study

The purpose of this research is to enable highly sensitive and rapid measurement of endotoxin in dialysis using the bioluminescence-based method. Earlier research has shown that properties of firefly luciferase can be improved by mutations. However, no studies have been conducted so far in order to improve the salt sensitivity of luciferase. Therefore, in Chapter 2, I created a mutant luciferase enzyme that is less susceptible to sodium chloride inhibition by introducing random mutations into firefly luciferase. In Chapter 3, I developed the bioluminescence-based method for measuring endotoxin in dialysis using the salt-tolerant luciferase. Therefore, endotoxin in dialysis can be measured rapidly and with enhanced sensitivity, which is expected to improve the safety of patients undergoing dialysis.

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Chapter 2: Mutant firefly luciferase enzymes resistant to the inhibition by sodium chloride

2.1. Introduction

Firefly luciferase, which generates bioluminescence during the oxidative decarboxylation of D-luciferin in the presence of ATP, has been used for numerous applications such as measuring biomass and cellular conditions [1] and protein-protein interaction [2,3], assaying ATP-related enzymes [4, 5], examining D-luciferin-generating enzymes using peptide-modified luciferin or luciferin derivatives [6, 7, 8, 9], and performing real-time ATP imaging [10]. The firefly luciferase gene, *luc*, has been used as a reporter gene [11] as well as for bioimaging [12, 13]. In the food industry, the bioluminescence-based ATP assay has received considerable attention, mainly because as a rapid monitoring system, it can ensure the safety of food products and contact surfaces at critical control points during food processing [14, 15, 16, 17].

Although the bioluminescence-based methods have numerous applications, their sensitivity is reduced by the presence of various salts. For example, sodium chloride, a commonly used reagent, is known to inhibit luciferase activity [18]. The considerable loss in the sensitivity of the bioluminescence-based method limits the applications in the presence of salts.

Mutations in the *luc* gene have been known to produce mutant luciferase enzymes with

properties significantly different from those of the wild-type gene. For example, some mutant luciferase enzymes have different bioluminescence spectra [19, 20, 21, 22, 23], increased enzyme stability [24, 25, 26, 27, 28, 29], or different catalytic activity [30, 31, 32]. Previously, I and colleagues developed the mutant luciferase FM (Ile423Leu, Asp436Gly, Leu530Arg) [31], later named LGR by another group [29], whose higher catalytic activity resulted in a luminescence intensity 10-fold higher than that of the wild type.

However, mutant luciferase that alleviates inhibition by salt has not been reported. From a random mutant library, this study identified two novel *Photinus pyralis* mutant luciferase enzymes that were found to retain their activities under medical dialysis condition (hereafter simply described as "dialysis condition"). Site-directed mutagenesis of the *luc* gene was conducted in these mutations to investigate the effect of substitution with other amino acids. Eventually, by combining the optimized mutations in the *luc* gene, I generated a mutant luciferase enzyme that retains its activity in the presence of various concentrations of sodium chloride.

2.2. Methods

2.2.1. Measurement of luciferase activity

Luminescence intensity, expressed as the generated light count per second, was measured in 96-well plates (Thermo Fisher, Massachusetts, USA) using a Microplate reader SH-9000 (Corona

Electric, Ibaraki, Japan). The reaction was initiated by the addition of 50 µl of 1 µM ATP and 0.1 µM D-luciferin in Tris–HCl buffer (50 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$) to 50 µl of 0.1 mg/ml luciferase. Luminescence was measured for 5 s after injection at 25°C. Luciferase protein concentrations were determined by measuring the absorbance at 280 nm.

2.2.2. The measurement of the inhibition of luciferase activity in the presence of sodium chloride and other salts

Luciferase reactions were performed in the absence or presence of various concentrations of sodium chloride, potassium chloride, calcium chloride, ammonium chloride, and sodium acetate. To test whether the inhibition is reversible or irreversible, luciferase was dissolved in the Tris–HCl buffer in 140 mM sodium chloride. After 120 min, luciferase was diluted 10-fold with the Tris–HCl buffer, mixed with 50 µl of 10 µM ATP and 1 µM D-luciferin in the Tris–HCl buffer to initiate the reaction, and measured. Each experiment was performed with three replicates.

2.2.3. Random mutagenesis and screening

The PCR Random Mutagenesis Kit (Takara, Shiga, Japan) was used to generate a random mutant library of the *Photinus pyralis luc* genes. The whole luciferase gene was used to generate random mutants. The error-prone PCR conditions were set to the rate of approximately two

mutations per 1000 bp, according to the manufacturer's instructions. PCR was conducted using the primers 5′-GACTCCATGGAAGACGCCAAAAAC-3′ and 5′-GACACTCGAGCAATTTGGACTTTCCGCC-3′ to generate mutant insert containing restriction sites *Nco*I and *Xho*I. All mutant genes were cloned into the pET-28a vector (Merck, Darmstadt, Germany) to generate recombinant luciferases containing a C-terminal His-tag.

The vectors containing the mutant *luc* genes were introduced into *Escherichia coli* HMS174 (DE3) (Merck), spread onto Luria–Bertani agar plates containing kanamycin (50 μg/ml), and incubated at 37°C. The colonies were inoculated into a 2YT liquid medium containing kanamycin (50 μg/ml) in deep-well plates (Watson, Tokyo, Japan). After incubation for 2 h at 25°C, 0.1 mM isopropyl-ß-D-thiogalactoside (IPTG) was added and luciferase proteins were expressed during further incubation for 65 h at 25°C.

Crude extracts containing mutant luciferase proteins were prepared by freezing at −80°C for 4 h and thawing the recombinant bacteria at 25°C for 1 h. Each extract was divided into two portions to measure the luminescence intensities in the absence or presence of 140 mM sodium chloride in a reaction mixture containing 50 μl each of 1 μM ATP and 0.1 μM D-luciferin in the Tris–HCl buffer.

2.2.4. Site-directed mutagenesis

The mutagenesis of the wild-type *luc* was conducted with primers, designed using the manufacturer's specification, and the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) to generate 19 substitutions of Val288 and Glu488.

2.2.5. Expression and purification of luciferase

The recombinant *luc* was expressed in *E. coli* HMS174 (DE3). The cells were cultured at 37°C in 2YT medium containing 50 μg/ml kanamycin. IPTG was added to the medium for incubation at 25°C for 65 h. The induced cells were harvested by centrifugation and stored at −20°C. The cell pellets were resuspended in a bacterial cell lysis reagent (B-PER, Thermo Fisher, Massachusetts, USA). After incubation at 25°C for 15 min, the whole-cell extracts were isolated by centrifugation at 16,000 *g* for 10 min at 4°C. The His-tagged recombinant luciferase was purified using the Ni Sepharose 6 Fast Flow column (GE Healthcare, Illinois, USA) according to the manufacturer's instructions. Whole-cell extracts were applied to the columns and washed with a binding buffer of 20 mM NaH_2PO_4, pH 7.4, 500 mM sodium chloride, and 20 mM imidazole. The recombinant luciferase was eluted with an elution buffer (20 mM NaH_2PO_4, pH 7.4, 500 mM sodium chloride, and 500 mM imidazole). The elution buffer was exchanged for the Tris–HCl buffer using a PD-10 Desalting Column (GE Healthcare).

2.2.6. Bioluminescence emission spectra

The bioluminescence emission spectra of the luciferase were measured using a Microplate reader SH-9000. The data were collected between 450 and 700 nm. The reactions were initiated by adding 1.8 ml of 100 μM ATP and 10 μM D-luciferin into 200 μl of 0.1 mg/ml luciferase in the Tris–HCl buffer.

2.3. Results

2.3.1. Inhibition of luciferase activity by sodium chloride

Luciferase is known to be inhibited by sodium chloride [18]. Indeed, in the presence of 200 mM sodium chloride, *P. pyralis* luciferase activity was decreased to 28% of the control (Fig. 2-1). Under dialysis condition (140 mM sodium chloride), luciferase activity was reduced to 44% of the control. Since luciferase was inhibited by potassium chloride, calcium chloride, and ammonium chloride, but not sodium acetate (Fig. 2-1), the inhibition was likely due to the chloride ion.

Next, I tested whether the inhibition of luciferase activity by sodium chloride was reversible. The luciferase was dialyzed in the 140 mM sodium chloride for 120 min and then diluted 10-fold; its final luciferase activity was compared with that of the luciferase directly diluted in 14 mM sodium chloride. Since there was no significant difference (within 1% difference in triplicated

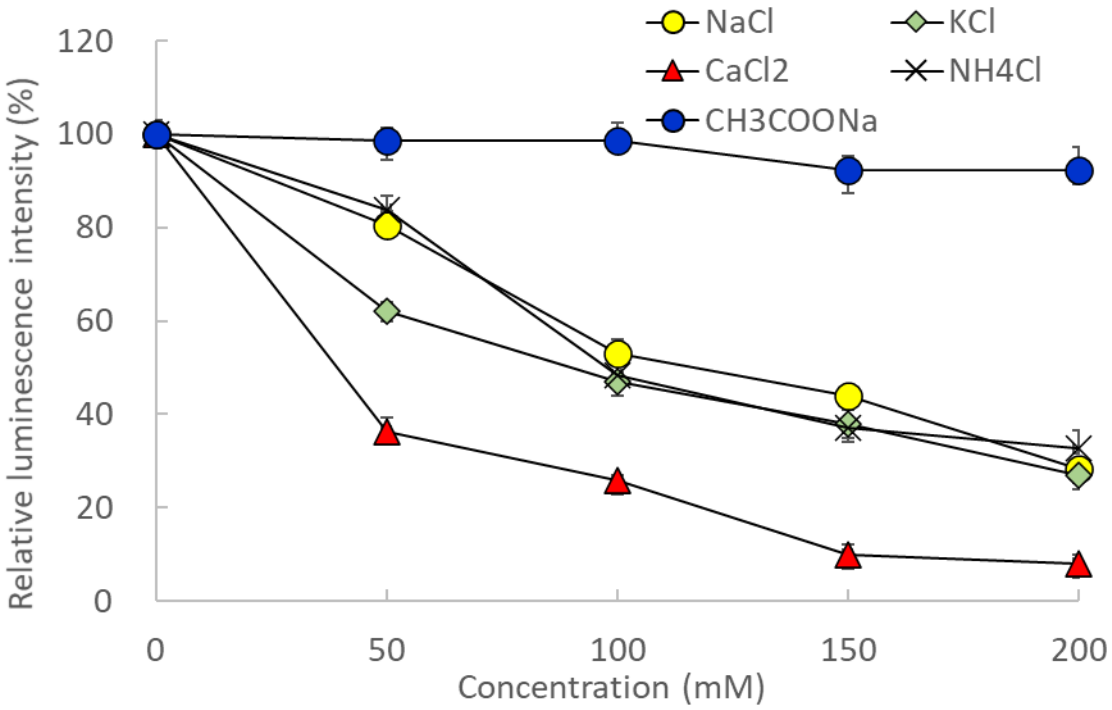

Fig. 2-1 The effects of various concentrations of sodium chloride, potassium chloride, calcium chloride, ammonium chloride, and sodium acetate on the activity of the wild-type luciferase. The relative luminescence intensity (%) was calculated by dividing the recorded intensity of luminescence in the presence of an indicated salt by that in the absence of a salt. The values are represented as means ± SD (n = 3).

experiments) between the condition with or without dialysis, the inhibition of luciferase activity by sodium chloride was found to be reversible.

2.3.2. Isolation of mutant luciferase enzymes retaining their activities under dialysis condition

Mutations were randomly introduced into *P. pyralis luc* and then mutant luciferase enzymes that retained more than 60% of their activities under dialysis condition were selected. Hence, two mutant luciferase enzymes were obtained from approximately twenty thousand transformants. DNA sequencing revealed that the mutations were localized at Val288, changing to Ile (Val288Ile) and at Glu488, changing to Val (Glu488Val), respectively. Luciferase activities of Val288Ile and Glu488Val mutants under dialysis condition were 67% and 79% of those in the absence of sodium chloride, respectively.

Site-directed mutagenesis was conducted to investigate the effect of substitution with other amino acids at positions 288 and 488 of luciferase (Tables 2-1 and 2-2). The substitution of Val288 with nonpolar amino acids, especially Ile, Leu, Met, and Phe, alleviated the inhibition under dialysis condition (Table 2-1). In contrast, the replacement with polar, positively charged, or negatively charged amino acids, except cysteine, failed to lessen the inhibition under dialysis condition (Table 2-1).

Alternatively, the substitution of Glu488 with nonpolar amino acids, except Ala and Trp,

mostly improved the inhibition (Table 2-2). Also, the replacement of Glu488 with Ser, Thr, Cys, and Asp also alleviated the inhibition. By contrast, the substitution with positively charged amino acids failed to ease the inhibition (Table 2-2). Finally, the best substitution at Val288 and Glu488 was found to be Ile and Val, respectively.

Table 2-1 The effect of Val288 substitutions on luciferase activity in the presence of 140 mM sodium chloride.

Luciferase	Substituted amino acid	Molecular weight (g/mol)	Polarity and charge	Relative luminescence intensity (%) [a]
Wild type	Val	117.15	Nonpolar	44.0 ± 0.6
Mutant	Gly	75.07	Nonpolar	16.3 ± 0.1
	Ala	89.09	Nonpolar	21.9 ± 1.3
	Pro	115.13	Nonpolar	19.6 ± 0.4
	Ile	131.17	Nonpolar	67.2 ± 2.9
	Leu	131.17	Nonpolar	46.8 ± 0.6

Met	149.21	Nonpolar	47.3 ± 0.7
Phe	165.19	Nonpolar	46.2 ± 0.8
Trp	204.23	Nonpolar	28.9 ± 0.9
Ser	105.09	Polar	16.3 ± 0.1
Thr	119.12	Polar	25.6 ± 0.5
Cys	121.16	Polar	46.5 ± 1.3
Asn	132.12	Polar	29.3 ± 0.5
Gln	146.15	Polar	32.8 ± 0.3
Tyr	181.19	Polar	38.3 ± 0.9
Lys	146.19	Positive	23.8 ± 1.8
His	155.15	Positive	31.1 ± 1.4
Arg	174.20	Positive	40.8 ± 0.8
Asp	133.10	Negative	16.1 ± 0.4
Glu	147.13	Negative	25.1 ± 1.0

[a] The relative luminescence intensity (%) was calculated by dividing the intensity in the presence of 140 mM sodium chloride by that in the absence of sodium chloride. The values are represented as means ± SD (n = 3).

Table 2-2 The effect of Glu488 substitutions on luciferase activity in the presence of 140 mM sodium chloride.

Luciferase	Substituted amino acid	Molecular weight (g/mol)	Polarity and charge	Relative luminescence intensity (%) [a]
wild type	Glu	147.13	Negative	44.0 ± 0.6
Mutant	Gly	75.07	Nonpolar	50.2 ± 0.2
	Ala	89.09	Nonpolar	43.4 ± 1.1
	Pro	115.13	Nonpolar	54.5 ± 1.2
	Val	117.15	Nonpolar	78.7 ± 1.3
	Ile	131.17	Nonpolar	58.0 ± 0.5
	Leu	131.17	Nonpolar	62.0 ± 0.5
	Met	149.21	Nonpolar	73.1 ± 0.7
	Phe	165.19	Nonpolar	56.6 ± 1.1
	Trp	204.23	Nonpolar	40.0 ± 1.2
	Ser	105.09	Polar	50.9 ± 1.0

Thr	119.12	Polar	66.8 ± 1.7
Cys	121.16	Polar	69.5 ± 1.1
Asn	132.12	Polar	30.9 ± 1.1
Gln	146.15	Polar	34.8 ± 1.4
Tyr	181.19	Polar	40.2 ± 0.7
Lys	146.19	Positive	22.9 ± 1.3
His	155.15	Positive	32.8 ± 0.5
Arg	174.20	Positive	43.1 ± 1.0
Asp	133.10	Negative	68.1 ± 1.2

[a] Relative luminescence intensity (%) was calculated by dividing the intensity in the presence of 140 mM sodium chloride by that in the absence of sodium chloride. The values are represented as means ± SD (n = 3).

2.3.3. The characterization of a Val288Ile and Glu488Val double mutant luciferase enzyme

A *luc* gene encoding the Val288Ile and Glu488Val double mutant luciferase, tentatively named CR for chloride ion resistance, was constructed with site-directed mutagenesis. Bioluminescence activities of the wild-type, single mutants Val288Ile and Glu488Val, the double mutant CR, and the FM mutant that I and my colleagues previously developed, were tested under dialysis condition and in the absence of sodium chloride (Fig. 2-2).

The luminescence intensity of the CR double mutant luciferase under dialysis condition retained more than 95% of its activity compared to that in the absence of sodium chloride. The inhibition of CR under dialysis condition was more alleviated than either Val288Ile or Glu488Val alone, suggesting that the effect of the double mutation was cumulative. Unexpectedly, the luminescence intensity of the double mutant luciferase was found to be 460% higher than that of the wild type (Fig. 2-2). Meanwhile, the luminescent intensities of Val288Ile and Glu488Val were 310% and 280% higher than that of the wild type, respectively, indicating that these mutations not only alleviated the inhibition by sodium chloride but also increased the luciferase activity. Alternatively, the luminescence intensity of the FM mutant (Fujii *et al.* 2007) increased 500% higher than that of the wild type in the absence of sodium chloride but failed to alleviate the inhibition by sodium chloride (Fig. 2-2), indicating that mutations increasing the luminescence intensity do not always lessen the inhibition by sodium chloride.

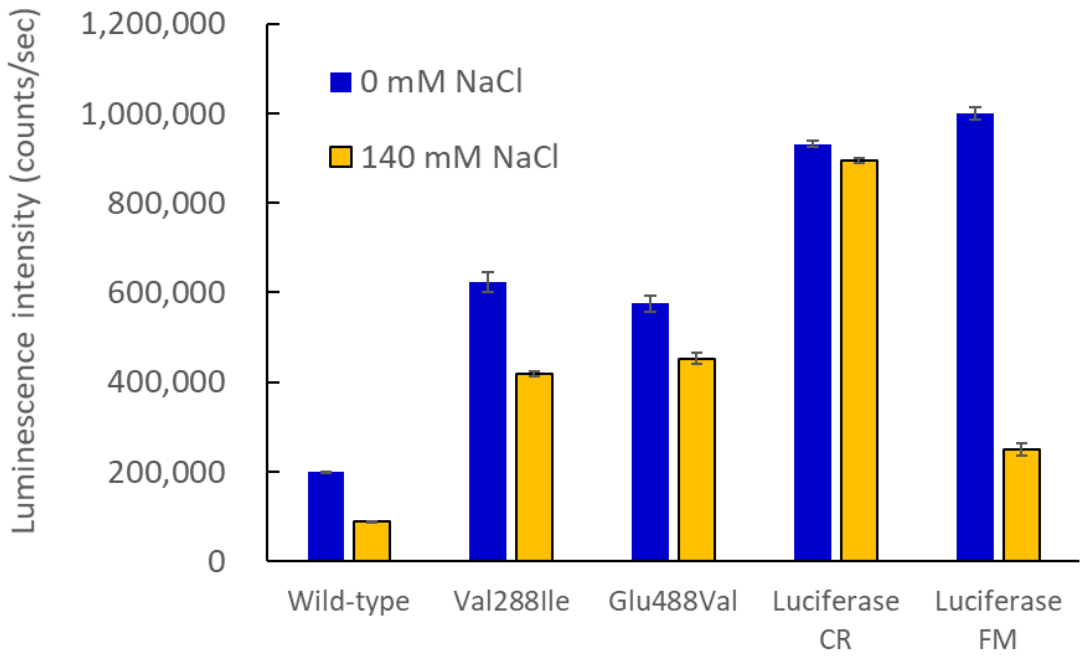

Fig. 2-2 The inhibition of the activity of the wild-type and mutant luciferases by sodium chloride.

The luminescence intensity of the luciferase reactions in the absence or presence of 140 mM sodium chloride was measured. The values are represented as means ± SD (n = 3).

Next, I evaluated the inhibition of wild type, the double mutant CR, and the FM mutant by various concentrations of sodium chloride (Fig. 2-3). Compared to the wild type, the double mutant CR retained its activity in various concentrations of sodium chloride. In contrast, the FM mutant was more susceptible to sodium chloride inhibition than the wild type. I concluded the double mutant CR as a new type of mutant luciferase resistant to the inhibition by sodium chloride.

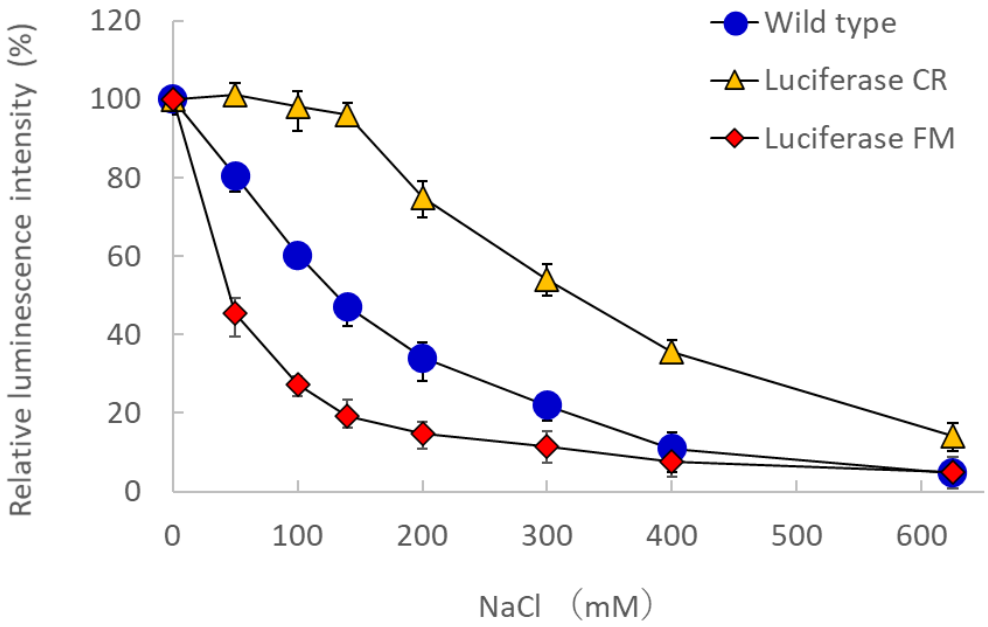

Fig. 2-3 The effect of sodium chloride concentration on the activity of the wild-type, luciferase CR, and FM.

The luminescence intensity of the luciferase reactions in various concentrations of sodium chloride was measured. The relative luminescence intensity (%) was calculated by dividing the recorded intensity of luminescence in the presence of sodium chloride by that in the absence of sodium chloride. The values are represented as means ± SD (n = 3).

Next, I evaluated the wavelength of bioluminescence emission by the wild type and CR. The patterns of bioluminescence wavelength were not significantly different between the enzymes (Fig. 2-4). The maximum wavelength emitted by luciferase CR was 556 nm.

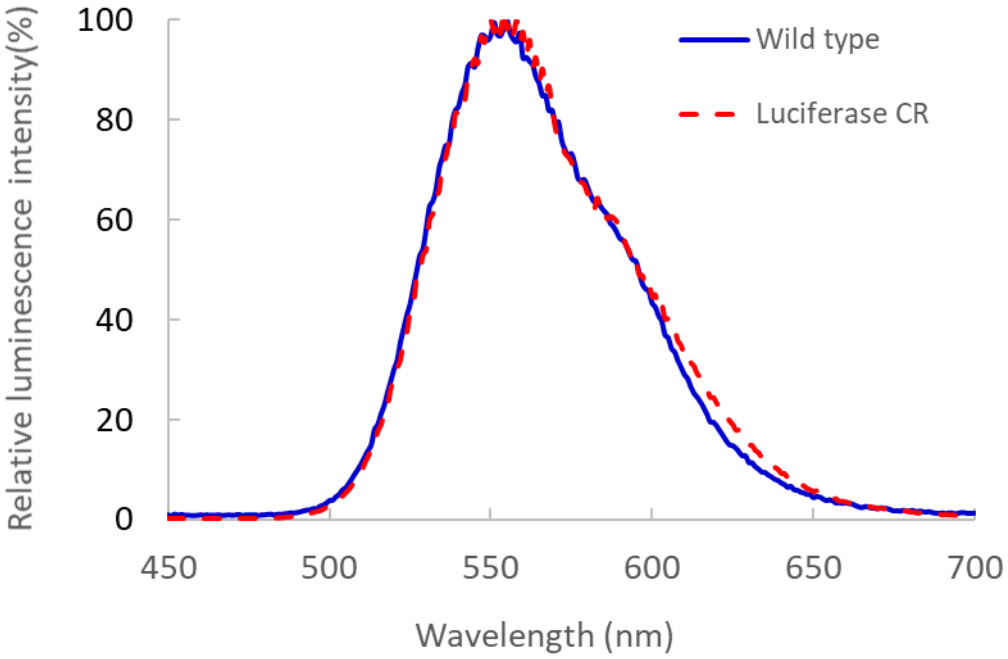

Fig. 2-4 The bioluminescence emission spectra of the wild-type and luciferase CR.

The relative luminescence intensity (%) was calculated by dividing the recorded intensity of luminescence at each wavelength by that recorded at 555 nm.

2.3.4. ATP assay using luciferase CR

I and my colleagues previously developed the higher catalytic mutant luciferase FM [31] and applied this enzyme for efficient assays for ATP as well as D-luciferin-generating enzymes using peptide-modified luciferin [8, 33, 34]. Here I found that luciferase CR showed higher luminescence than FM within various ATP concentrations in the presence of 140 mM sodium chloride (Fig. 2-5). Therefore, the mutant luciferase CR would expand the applications of luciferase in the presence of sodium chloride.

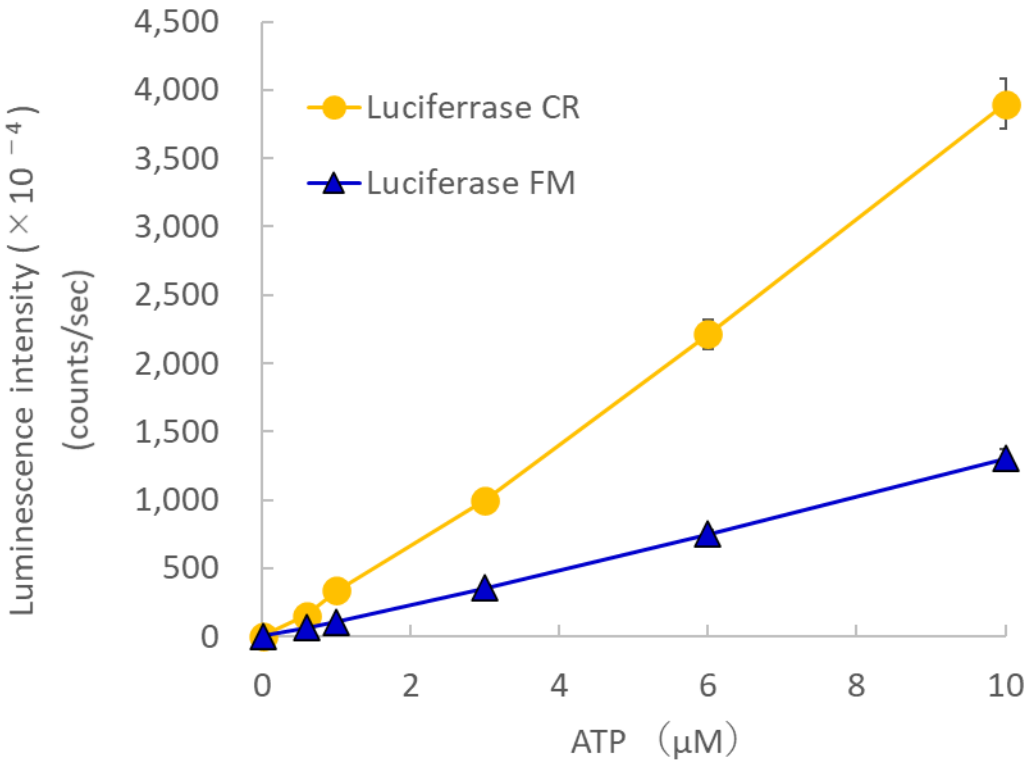

Fig. 2-5 The luminescence intensity of the luciferase reactions at various concentrations of ATP. The luminescence intensity of the luciferase reactions in various concentrations of ATP was measured. The values are represented as means ± SD (n = 3).

2.4. Discussion

In this study, two mutant luciferase enzymes that retained more than 60% of their original activity under dialysis condition, were obtained from approximately 20,000 candidates; the mutations were located at Val288 and Glu488. The double mutant luciferase CR (Val288Ile and Glu488Val) retained more than 95% of its original activity under dialysis condition. Recently, Davis *et al.* have found several mutations involved in the functional expression of the luciferase gene in an extreme halophile which grows under immensely high-salt conditions [35]. Because the luciferase activity (or its expression) was very low in the halophile, they selected mutants from a random mutation library which showed a higher luminescent intensity. From the bioengineering point of view, it seems very reasonable to use halophiles for laboratory-directed evolution of salt-tolerant enzymes. However, the authors have never performed an *in vitro* experiment to prove that the mutant luciferase enzymes were in fact salt-tolerant. Instead, they have concluded that the mutant luciferase gene could be used as an *in vivo* reporter in halophiles. The localization of mutations is also different from that in the mutant luciferase reported in our study. CR mutant luciferase, whose activity was less inhibited by salt, may be extensively useful in any bioassay which includes firefly luciferase and is employed in the presence of sodium chloride.

Electrostatic interaction is a significant force in mediating intramolecular and intermolecular

interactions for determining the structure, dynamics, and function of biomolecules [36]. In an aqueous solution, ions and small molecules of opposite charges accumulate around a highly charged biomolecule, resulting in electrical neutralization of the biomolecule, or the shielding effect. In the screening or shielding effect, salt molecules shield the long-range electrostatic repulsive forces between the intramolecular charges, decrease the repulsive interactions, and enhance the hydrophobic effects, thus increasing enzyme stability [37, 38]. On the other hand, certain types of salt ions could also shield enzyme-substrate electrostatic interactions, thus inhibiting enzyme activities. The salt inhibition of luciferase might indicate that the positive effect of salt ions could not overcome the negative effect of chloride ions.

The firefly luciferase enzyme, consisting of a large ~440 amino acid N-terminal domain and a small ~110 amino acid C-terminal domain connected by a short hinge (Fig. 2-6), catalyzes a sequence of reactions, i.e., adenylation and oxidation, that convert luciferin into an electronically excited state, oxyluciferin, that emits light [39]. The interface between the two domains contains the active site, with the N-terminal domain mainly contributing to substrate binding, whereas the C-terminal domain introduces Lys529 and Lys443 that participate in adenylation and oxidation reactions, respectively [40]. The Val288 residue is localized within 5 Å apart from Thr527 that interacts with the active site Lys529 (Fig. 2-6). Therefore, the mutation of Val288 to Ile might have changed the localization of Thr527 and strengthened the enzyme-substrate electrostatic

interactions, resulting in the alleviation of the inhibition.

On the contrary, the Glu488 residue is localized in the flexible region away from the active sites in the N-terminal domain (Fig. 2-6). It was recently reported that the substitution of histidine in the same flexible region by aspartate (His461Asp) decreased ATP binding affinity and shifted its optimum temperature of activity [41]. It was also reported that the substitution of His489 by aspartate (His489Asp) increased protein rigidity but only slightly improved its thermal stability [41]. Therefore, the finding that Glu488 in the flexible region contributes in alleviating the inhibition by chloride ion is novel.

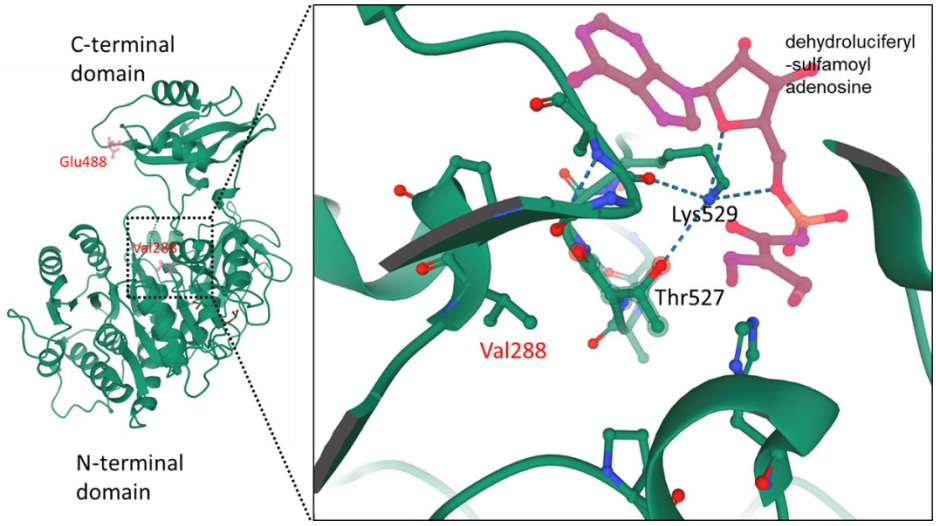

Fig. 2-6 The three-dimensional structure of *Photinus pyralis* luciferase and the locations of mutations in the luciferase CR. The active sites with a substrate analog (dehydroluciferyl-sulfamoyl adenosine) are indicated in the right box. The amino acid substitutions (red) in the luciferase CR were indicated. This figure was created based on the crystal structure (Protein Data Bank ID code 4G36) of *Photinus pyralis* luciferase.

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Chapter 3: Improved bioluminescence-based endotoxin measurement method using a salt-resistant luciferase mutant

3.1. Introduction

Endotoxin are present in the cell wall of gram-negative bacteria, which are known to cause a host immune response [1]. Endotoxin in water for pharmaceuticals and dialysis must be measured preoperationally [2, 3, 4]. The allowable endotoxin levels set by the ISO (23500) [3] and Japanese Society of Dialysis Medicine [4] are 0.03 and 0.001 EU/mL, respectively. Limulus amebocyte lysate (LAL) reagent is an aqueous extract of blood cells obtained from the horseshoe crab and reacts with endotoxin [5, 6, 7]. For endotoxin measurement, various LAL tests based on gel formation, increased turbidity after coagulant formation, or chromogenic substrates are used [8, 9]. However, these methods are time-consuming or show low sensitivity [10]. Noda *et al*. developed a bioluminescence-based LAL test using a peptide-modified D-luciferin substrate [11]. In this method, D-luciferin released by the LAL reaction was quantitatively measured using firefly luciferase in the presence of ATP. Use of a higher catalytic mutant luciferase enzyme (Ile423Leu + Asp436Gly + Leu530Arg) (luciferase FM) showed 10-fold higher luminescence intensity compared to the wild-type enzyme [12], increasing the sensitivity of endotoxin measurement. The bioluminescence-based LAL test using luciferase FM achieved 200-fold higher sensitivity

compared to the chromogenic method [11]. However, application of this method was restricted because luciferase is inhibited under dialysis conditions containing 140 mM sodium chloride (referred to as “medical dialysis”) [13, 14, 15]. According to the guidelines on validation of the LAL test, the measured values of endotoxin in medical dialysis must be 75–125% of those tested in water [16, 17]. Recently, I obtained mutant luciferase enzymes for which salt inhibition was alleviated and identified the mutations as Val288Ile and Glu488Val [18]. The double mutant, luciferase CR (Val288Ile + Glu488Val), showed higher luminescence than luciferase FM in a wide range of ATP concentrations and in the presence of sodium chloride [18]. In this study, I applied the salt-resistant luciferase mutants in the bioluminescence-based LAL test to determine whether this improvement meets the validation guidelines.

3.2. Methods

3.2.1. Measurement of luciferase activity

The luciferase mutants were prepared as described in reference [18]. Luciferase activity in the presence of various concentrations of D-luciferin was measured at 25 °C. The luminescence intensity was measured in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) using a microplate reader SH-9000 (Corona Electric, Ibaraki, Japan). The luminescence intensity is expressed as the generated light count per second. The reaction was initiated by adding 50 μL of

substrate (1 μM ATP and various concentrations of D-luciferin) in Tris–HCl buffer (50 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$) to 50 μL of 0.1 mg/mL luciferase. Luminescence was measured for 5 s after injection at 25 °C. The K_m and V_{max} values were calculated using Lineweaver–Burk plots, and k_{cat} values were obtained by dividing the V_{max} values by the amount of luciferase.

3.2.2. Measurement of endotoxin

The amounts of endotoxin in water and dialysis (FUSO Pharmaceutical Industries, Osaka, Japan) were measured using luciferase FM and luciferase CR. A peptide-modified D-luciferin substrate, benzoyl-Leu-Gly-Arg-aminoluciferin (synthesized by ABD Bioquest, Inc., Sunnyvale, CA, USA) [11], was dissolved in 95% dimethyl sulfoxide. Then the substrate concentration was adjusted to 75 μM in Tris–HCl buffer. The final concentration of dimethyl sulfoxide was 0.12%. A reaction mixture containing 50 μL of endotoxin and 50 μL of LAL (Limulus ES-II; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was incubated at 37 °C for 15 min. Next, 50 μL of peptide-modified D-luciferin was added, and the mixture was incubated at 37 °C for 5 min; 25 μL of 20 μM ATP and 25 μL of 250 μg/mL luciferase were added and mixed immediately, and the luminescence intensity was measured using a Luminutes-ET (DKK-TOA Corporation, Saitama, Japan).

3.3. Results

3.3.1. Luciferin assay using luciferase CR

During bioluminescence-based measurement of endotoxin, D-luciferin released by the LAL reaction is quantitatively measured using luciferase [11]. Therefore, I first determined the kinetic parameters of the salt-resistant luciferase mutants for D-luciferin. The kinetic parameters of the wild-type and mutant luciferase enzymes are listed in Table 3-1. All mutant luciferase enzymes showed lower K_m values for D-luciferin compared to the wild-type enzyme. The k_{cat} values of all mutant luciferase enzymes were significantly higher than those of the wild-type enzyme. Thus, the increase in k_{cat} values was likely the main contributor to the increased luciferase activity (Table 3-1). In the presence of 140 mM sodium chloride, the *kcat/Km* values of the mutants (Val288Ile), (Glu488Val), and luciferase CR were 62%, 77%, and 97% of the values observed in the absence of sodium chloride, respectively, whereas those of the wild-type and luciferase FMs were 42% and 20% of the wild-type enzyme activity, respectively (Table 3-1). Therefore, the mutant luciferase CR is least susceptible to sodium chloride inhibition in D-luciferin measurement and may meet the validation guidelines.

Table 3-1 Kinetic parameters of wild-type and mutant luciferase.

Luciferase	Sodium chloride	Kinetic parameters [a]		
		D-Luciferin		
		K_m (μM)	k_{cat} (×10^5 cps/μg)	k_{cat}/K_m (×10^4)
Wild-type	0 mM	12.7 ± 0.5	3.3 ± 0.2	2.6 ± 0.6
	140 mM	18.4 ± 0.6	2.0 ± 0.2	1.1 ± 0.6
Val288Ile	0 mM	9.2 ± 0.3	25.7 ± 0.3	28.1 ± 0.6
	140 mM	10.7 ± 0.5	18.7 ± 0.2	17.5 ± 0.5
Glu488Val	0 mM	9.2 ± 0.5	24.3 ± 0.4	26.4 ± 0.7
	140 mM	9.9 ± 0.1	20.2 ± 0.4	20.5 ± 0.5
Luciferase CR	0 mM	9.7 ± 0.4	27.0 ± 0.5	28.0 ± 0.8
	140 mM	9.8 ± 0.3	26.8 ± 0.2	27.3 ± 0.5
Luciferase FM	0 mM	8.4 ± 0.2	28.1 ± 0.3	33.4 ± 0.6
	140 mM	14.9 ± 0.5	10.0 ± 0.4	6.7 ± 0.8

[a] Values are means ± SD (n = 3)

Next, I tested whether luciferase CR could be used to measure a wide range of D-luciferin without significant inhibition by sodium chloride. Although a linear relationship was obtained for the luciferase FM, the luminescence intensities decreased in the presence of 140 mM sodium chloride (Fig. 3-1 a). In contrast, when luciferase CR was used, there were no significant differences between either the intensity or correlation in the absence and presence of 140 mM sodium chloride (Fig. 3-1 b).

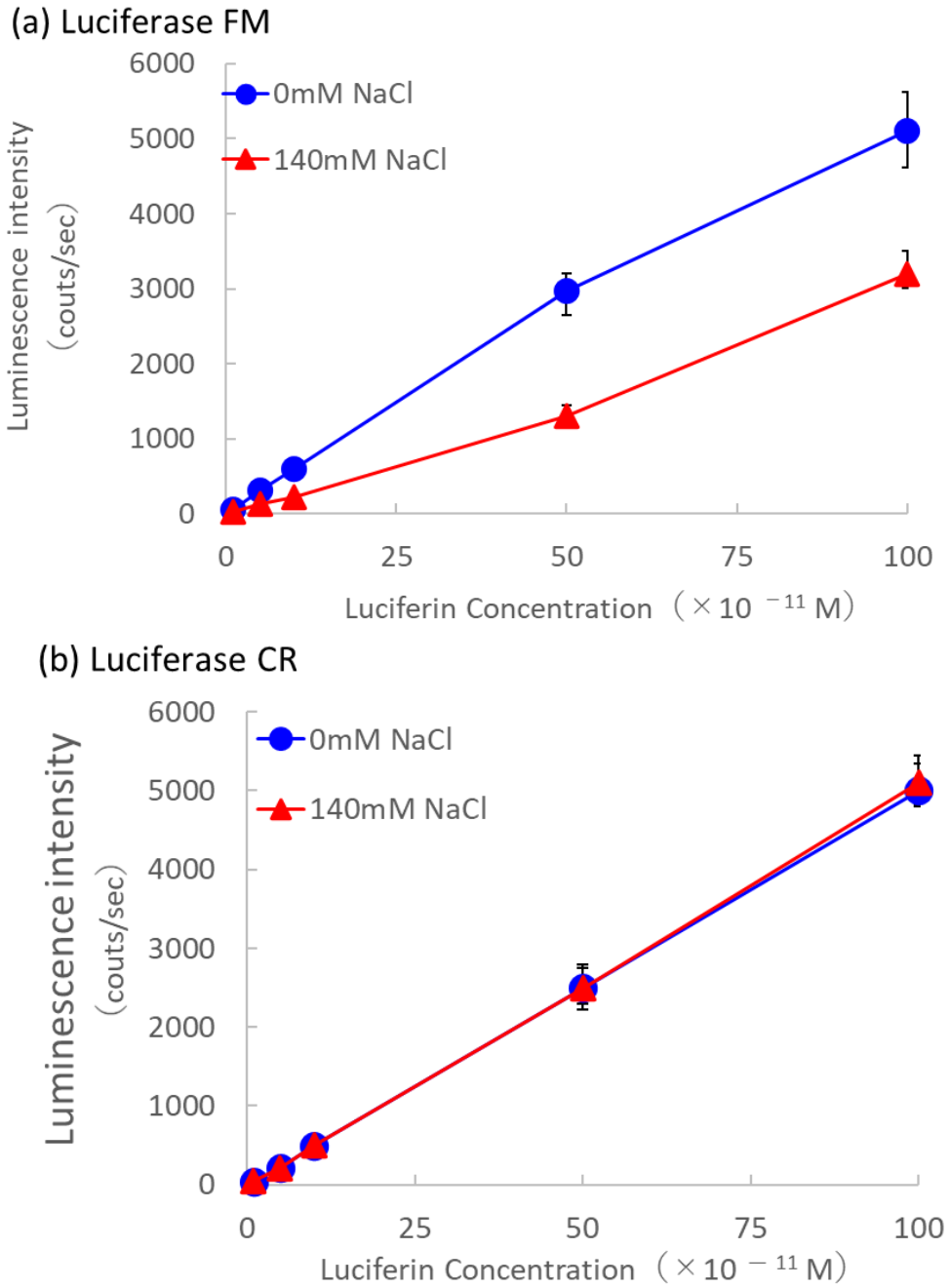

Fig. 3-1 Relationship of luminescence intensity versus D-luciferin. Luminescence intensities of the luciferase reaction were measured at various concentrations of D-luciferin using luciferase FM (a) and luciferase CR (b). These experiments were performed in the absence and presence of sodium chloride. Values shown are the means ± SD (n = 3).

3.3.2. Measurement of endotoxin using luciferase CR

I examined whether endotoxin measurement using luciferase CR could meet the validation guidelines. A linear relationship was observed between the luminescence intensities and endotoxin concentrations. The luminescence intensities of luciferase FM were decreased by 67% in dialysis (Fig. 3-2 a), whereas those of luciferase CR were retained more than 95% of that in water (Fig. 3-2 b). Therefore, the new method using luciferase CR meets the validation guidelines for endotoxin measurement in dialysis.

3.4. Discussion

Chromogenic and turbidimetric methods are commonly used to measure endotoxin. However, chromogenic methods show low sensitivities (detection limit of approximately 0.01–0.1 EU/mL) and turbidimetric methods are time-consuming (over 60 min) [10]. The lowest mortality rate for patients was reported when treated with dialysis controlled below 0.001 EU/mL [19]. The detection limit for endotoxin in the new bioluminescence-based LAL test at a measurement time of 20 min was 0.00025 EU/mL according to the ISO11843 method [20]. The recovery rate was 98 ± 2% (in triplicated experiments) when 0.001 EU/mL was spiked into the dialysis, indicating that this new method would accurately measure endotoxin levels that are clinically significant. On the other hand, the upper limit of detection was 0.5 EU/mL when the

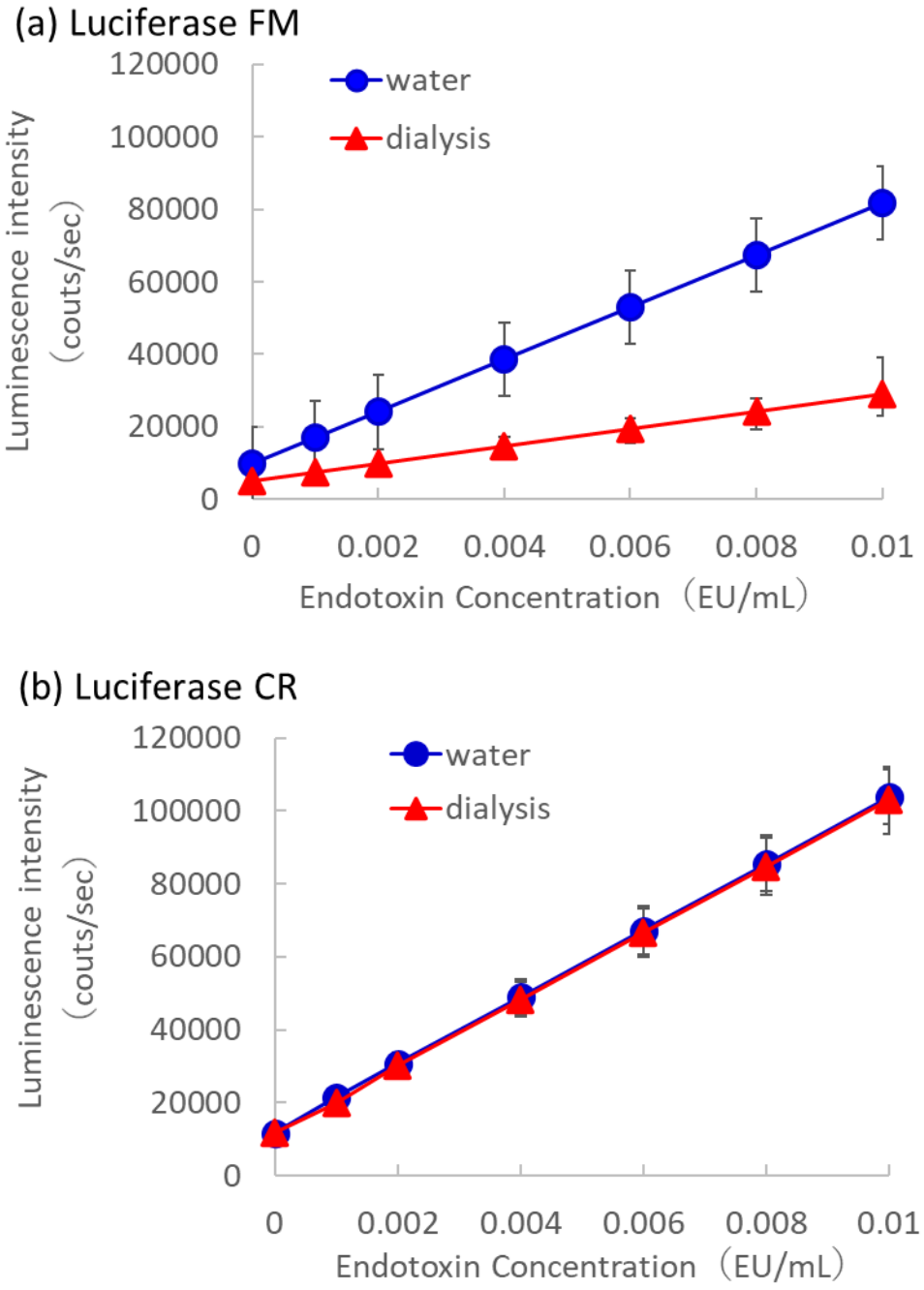

Fig. 3-2 Relationship of luminescence intensity versus endotoxin concentrations. Luminescence intensities of the luciferase reaction were measured at various concentrations of endotoxin using luciferase FM (a) and luciferase CR (b). These experiments were performed in the absence and presence of dialysis. Values shown are the means ± SD (n = 3).

correlation coefficient was 0.99 or more. The new bioluminescence-based LAL test meets the validation guidelines and enables rapid and sensitive detection of endotoxin. Measurement of endotoxin by the bioluminescence-based LAL test using salt-resistant luciferase CR achieved the criteria required for the validation of endotoxin measurement in medical dialysis. This improved test will be useful for endotoxin and microbiological contamination control during dialysis. The salt-resistant luciferase CR enabled a broader usage of firefly luciferase in more conditions, including ones with high concentrations of salt ions.

3.5. References

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Chapter 4: Conclusions

Endotoxin is a cell wall component of gram-negative bacteria that causes fever and shock symptoms upon entering the bloodstream. Therefore, it is crucial to measure endotoxin in pharmaceutical products and prevent contamination in order to avoid medical accidents. The allowable endotoxin levels set by the ISO (23500) and Japanese Society of Dialysis Medicine are 0.03 and 0.001 EU/mL, respectively. Bioluminescence-based endotoxin measurement method can be used to rapidly measure endotoxin in water with high sensitivity. However, the luminescence reaction of luciferase is inhibited by sodium chloride, which resulted in low values. Therefore, the bioluminescence-based endotoxin measurement method is not suitable for the dialysis sample containing sodium chloride. On the other hand, mutations in the *luc* gene have been known to produce mutant luciferase enzymes with properties significantly different from those of the wild-type gene. For example, some mutant luciferase enzymes have different bioluminescence spectra, increased enzyme stability, or different catalytic activity. However, mutant luciferase that alleviates inhibition by salt has not been reported. In this study, I generated a mutant luciferase enzyme that retains its activity in the presence of various concentrations of sodium chloride. I developed the bioluminescence-based endotoxin measurement method using the salt-resistant luciferase.

In this study, it was found that the luminescence of luciferase is inhibited by chloride ion. I first obtained two mutant luciferase enzymes whose inhibition were alleviated (Chapter 2). Two random mutations per 1000 bp were introduced into the entire firefly luciferase gene. Mutations were randomly introduced into *P. pyralis luc* and then mutant luciferase enzymes that retained more than 60% of their activities under dialysis condition were selected. Hence, two mutant luciferase enzymes were obtained from approximately twenty thousand transformants (Val288Ile, Glu488Val). I determined the mutations to be Val288Ile and Glu488Val. Furthermore, site-directed mutagenesis was conducted to investigate the effect of substitution with other amino acids at positions 288 and 488 of luciferase. The mutation that showed the highest salt resistant were Val288Ile and Glu488Val. In addition, the double mutant (luciferase CR), which is a combination of the two mutations, has improved salt resistant compared to the single mutation. The luciferase activities of luciferase CR under dialysis condition were 95% or more activity of those in the absence of sodium chloride. It also maintained higher activity than wild-type luciferase at various sodium chloride concentrations. The emission wavelength of luciferase CR was not different from that of wild-type luciferase, and the maximum wavelength was 556 nm. When ATP was measured in the presence of sodium chloride, it showed higher luminescence intensity for various concentrations of ATP than for mutant luciferase (luciferase FM). As a result, it was concluded that luciferase CR can be used as a salt-resistant luciferase.

Next, I developed the bioluminescence-based endotoxin method using luciferase CR (Chapter 3). According to the guidelines on validation of the LAL test, the measured values of endotoxin in medical dialysis must be 75–125% of those tested in water. The luminescence intensities of luciferase FM were decreased by 67% in dialysis, whereas those of luciferase CR were retained more than 95% of that in water. Chromogenic and turbidimetric methods are commonly used to measure endotoxin. However, chromogenic methods show low sensitivities (detection limit of approximately 0.01–0.1 EU/mL) and turbidimetric methods are time-consuming (over 60 min). The detection limit and measurement time for endotoxin in the new bioluminescence-based measurement method were 0.00025 EU/mL and 20 min, respectively. The bioluminescence-based LAL test with luciferase CR meets the validation guidelines and enables rapid and sensitive detection of endotoxin.

In the recent years, the usefulness of on-line hemodiafiltration (HDF) treatment, in which dialysis is directly replenished in bloodstream for treatment, has been reported, and the number of patients undergoing treatment is increasing [1]. In on-line HDF treatment, if endotoxin is mixed in the dialysis, it will enter the bloodstream directly. Therefore, it is very important to make sure that endotoxin is not contaminated. When performing online HDF treatment, for safety reasons, endotoxin is measured immediately before treatment to confirm that it is not contaminated. Therefore, as on-line HDF treatment becomes widespread, a highly sensitive and rapid

measurement method for endotoxin will become indispensable. In the future, it is expected that the bioluminescence-based endotoxin method in dialysis developed in this study will be used.

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