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Development of a Fluorescent Protein Based FRET Biosensor for Determination of Protease Activity

İbrahim İNCİR[1], Özlem KAPLAN[2], Sema BİLGİN[3], İsa GÖKÇE*[4]

Abstract

Proteases are closely associated with many pathological conditions. Efficient detection of protease activity may be useful for diagnosis, prognosis, and the development of new therapeutic biomolecules. Fluorescent Resonance Energy Transfer (FRET) is defined as the non-radioactive energy transfer that occurs between two fluorophores. Fluorescent proteins are widely used in FRET biosensors because they can be genetically encoded and compatible with cells. Fluorescent Protein based FRET (FP-FRET) biosensors are used to monitor biological processes such as enzyme activity, intracellular ion concentration, conformational changes, protein-protein interactions. In this study, it was aimed to detect protease activity using an FP-FRET biosensor and TEV protease was chosen as a model enzyme. The plasmid encoding the mNeonGreen-TEV-mRuby3 fluorescent protein-based FRET biosensor was constructed. The gene of the designed FP-FRET biosensor was expressed in *Escherichia coli* DH5α cells using recombinant DNA techniques and purified using Ni-NTA affinity chromatography. As a result, the activity of the TEV protease enzyme was determined by emission measurements performed in the spectrofluorometer using the produced FP-FRET biosensor. The usability of the designed FP-FRET biosensor in the determination of protease enzyme activity was demonstrated.

Keywords: Recombinant DNA Technology, Fluorescent Proteins, Fluorescent Resonance Energy Transfer (FRET), Biosensor, *E. coli*

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1. INTRODUCTION

Proteases have an important role in various biological processes such as cell proliferation, digestion and differentiation. Protease activity may be abnormal in pathological conditions such as cancer, neurodegenerative disorders, arthritis, allergies and infections [1]. New and improved protease detection systems may have a fundamental role in the diagnosis of these diseases and drug development.

Förster or fluorescent resonance energy transfer (FRET), first described by Theodor Förster in 1946; It is a mechanism by which a donor fluorophore in its excited state transfers its energy to an adjacent acceptor fluorophore, causing the acceptor to emit characteristic fluorescent light [2]. For energy transfer between fluorophores, the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule must overlap [3]. Three main types of fluorophores are used in FRET biosensors: small organic dyes, fluorescent proteins, and quantum dots. Unlike organic dyes and quantum dots, fluorescent proteins are widely used because they can be genetically encoded and compatible with cells. Biosensor systems created using the FRET technique have occupied an important place in biotechnology and biological studies [4, 5]. These genetically encoded biosensors can be transferred to cells, tissues or the whole organism by transfection of plasmid DNA. The biggest advantage of such a biosensor approach is that it is a non-invasive method and does not damage cells. Fluorescent proteins are in most cases non-toxic to host cells and can remain stable in cells for a long time thanks to their high intracellular stability [2, 6]. In this context, fluorescent proteins and FRET-based biosensors have become powerful tools for *in vitro* and *in vivo* applications [6–8]. In FP-FRET sensors, the selection of the FRET pair (donor and acceptor fluorophores) is the most important parameter for the performance of the biosensors to be designed [9]. The diversity of FPs with improved optical properties has made it possible to select FRET pairs with high FRET efficiency and to develop high-sensitivity FRET biosensors [2]. The color variety of monomeric FPs covers almost the entire visible spectrum, from violet to far red [10].

Shaner et al. obtained a tetrameric yellow fluorescent protein from *Branchiostoma lanceolatum* and later developed a monomeric protein called mNeonGreen, which is more photostable and has significant advantages in terms of rapid maturation. [11]. The mNeonGreen protein (26.6 kDa) has excitation wavelengths at 506 nm and emission wavelengths at 517 nm [11]. mNeonGreen is widely used to investigate biological processes in various organisms including bacterial and eukaryotic cells [12].

The brighter mRuby protein was developed as a result of continuous and intense optimization of the wild-type red fluorescent protein called EqFP611 (derived from *Entacmaea quadricolor*) [13]. Good performance in most fusion structures and resistance to acidic environments makes mRuby useful for many cell biology applications. mRuby has evolved into an effective fluorescent protein for imaging of mammalian cells, flow cytometry analyzers and FACS [14]. Studies in mammalian cells have shown that mRuby3 is the brightest and most photostable monomeric red fluorescent protein known and functions as an efficient FRET acceptor. The mRuby3 protein (26.6 kDa) has excitation and emission at 558 and 592 nm, respectively [15]. Bajar et al. showed that the mNeonGreen-mRuby3 pair is one of the best choices in terms of photostability, brightness and FRET dynamic range when used in cultured cells [2].

In this study, mNeonGreen-TEV-mRuby3 plasmid was designed as a model to determine TEV protease activity. These fluorescent proteins were linked by a 17 amino acids sequence (GGSLEENLYFQGEFSGG) that also contained the TEV protease recognition site. Expression of the designed FP-FRET biosensor was carried out in *E. coli* DH5α cells. Using the produced FP-FRET biosensor, the activity of TEV protease enzyme was determined spectrofluorometrically. With this study, the usability of the produced recombinant mNeonGreen-mRuby3 FRET pair in the determination of protease activity has been clearly demonstrated.

2. MATERIALS AND METHODS

2.1. Plasmid design

The DNA sequence for the mNeonGreen protein was obtained from NCBI (KC295282.1). Then, the DNA sequence was codon optimization for *E. coli* K12 organism using JCat codon optimization program (http://www.jcat.de/). The DNA sequence of the mRuby3 protein was obtained from the Addgene database (plasmid catalog number: 74234). The DNA sequence of the biosensor was designed as 5'-mNeonGreen-TEV protease recognition sequence-mRuby3-3' (mNG-TEV-mRuby3). The histidine tag (6x-His) and the stop codon were added to the 3' end of the mRuby3 DNA sequence, respectively. In addition, codons of serine and glycine amino acids were added to both sides of the TEV cleavage site for optimization of linker length, its flexibility and stabilization (Figure 1). The designed DNA sequence of FRET pair was purchased from Biomatik as cloned into the pET-30a vector.

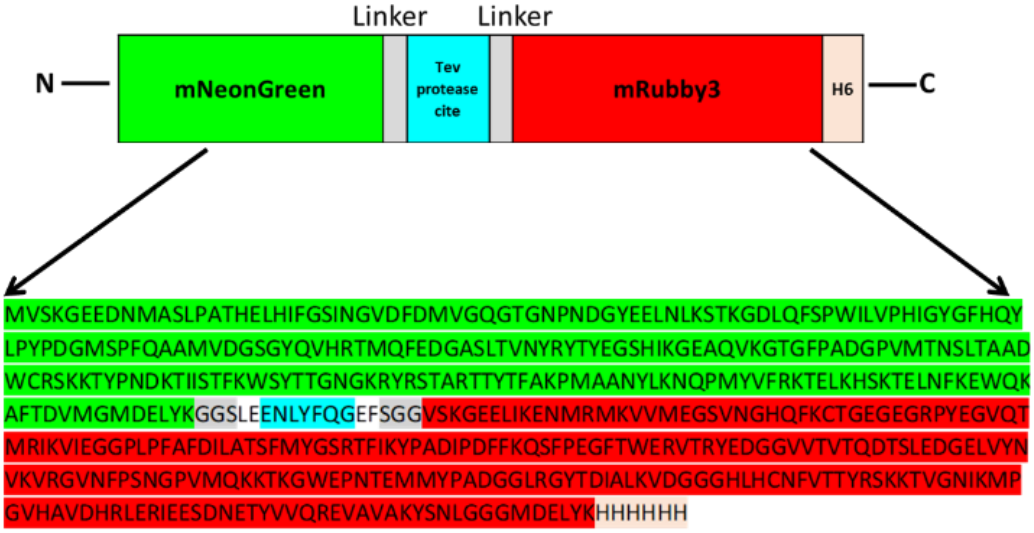

Figure 1 Modular organization and amino acid sequence of the mNG-TEV-mRuby3 protein

2.2. Expression and partial purification of recombinant proteins

The mNG-TEV-mRuby3 protein pair was expressed in *E. coli* DH5α cells. The plasmid mNG-TEV-mRuby3 was used for transformation of *E. coli* DH5α cells by heat shock. The cells were spread on LB agar medium containing 50 µg/ml kanamycin. A single colony was taken and 4 ml of inoculated LB broth (containing 50 µg/ml kanamycin) was grown overnight. The overnight culture was transferred into 600 ml of LB medium containing 50 µg/ml kanamycin and was then incubated at 37 °C at 240 rpm for 20 hours. The cells were collected by centrifugation at 8 000 rpm for 10 minutes and suspended by adding lysis buffer (25 mM Tris-HCl and 300 mM NaCl and pH: 7.8). After the addition of lysozyme enzyme (1 mg/ml), PMSF (0.1 mM), and benzamidine (0.1 mM), the cells were lysed with the help of a sonicator. The cell lysate was centrifuged at 30 000 rpm for 1 hour. The mNG-TEV-mRuby3 fusion protein was purified using Ni-NTA column. 12.5 mg of the mNG-TEV-mRuby3 fusion protein was obtained from 1 L of bacterial culture.

Wash buffer (10 mM imidazole, 25 mM Tris-HCl and 300 mM NaCl and pH: 7.8) and elution buffer (300 mM imidazole, 25 mM Tris-HCl and 300 mM NaCl and pH: 7.8) were used in the purification step. Samples taken at certain steps and purified protein were analyzed in 10% SDS-PAGE.

TEV protease protein was expressed in *E. coli* BL21 pLysE cells. The production and purification processes of TEV protease enzyme were carried out similar to the protocol detailed in

our previous studies [16, 17]. In summary, plasmid encoding TEV protease protein (pRK793) was used for transformation of *E. coli* BL21 pLysE cells by heat shock. These cells were grown in LB medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The cells were lysed with a sonicator and protein partial purification from lysate was performed by nickel affinity chromatography.

2.3. Analysis of TEV protease enzyme activity

Purified the mNG-TEV-mRuby3 fusion protein and TEV protease were dialyzed against 1x TEV protease buffer (25 mM Tris-HCl and 150 mM NaCl and pH:8). Then, mNG-TEV-mRuby3 fusion protein (6 mg/ml) was incubated with TEV protease enzyme (3.5 mg/ml) at 30 °C for 3 hours as indicated in Table 1.

Table 1 Cleavage of the mNG-TEV-mRuby3 with TEV protease enzyme (A: 1x TEV protease buffer without mercaptoethanol, B: 1x TEV protease buffer containing β-mercaptoethanol)

	1	**2**	**3**	**4**	**5**	**6**
TEV protease enzyme	-	50 µl	65 µl	-	25 µl	25 µl
1x TEV buffer	165 µl A	150 µl A	100 µl A	140 µl A	140 µl A	140 µl B
mNG-TEV-mRuby3 protein	35 µl	-	35 µl	35 µl + 25 µl B	35 µl	35 µl

After the protease cleavage process was completed, the color change in the microtubes was observed. Samples of TEV protease cleavage products were analyzed in SDS-PAGE. The emission wavelengths of the reaction mixtures obtained after incubation of the mNG-TEV-mRuby3 FRET sensor with the TEV enzyme were recorded in a spectrofluorometer (Jasco FP-8300) at 37 °C. TEV protease cleavage products were excited at 506 nm (5 nm bandwidth) and emission wavelengths between 510-700 nm were shown.

The experimental steps of this study are summarized in Figure 2.

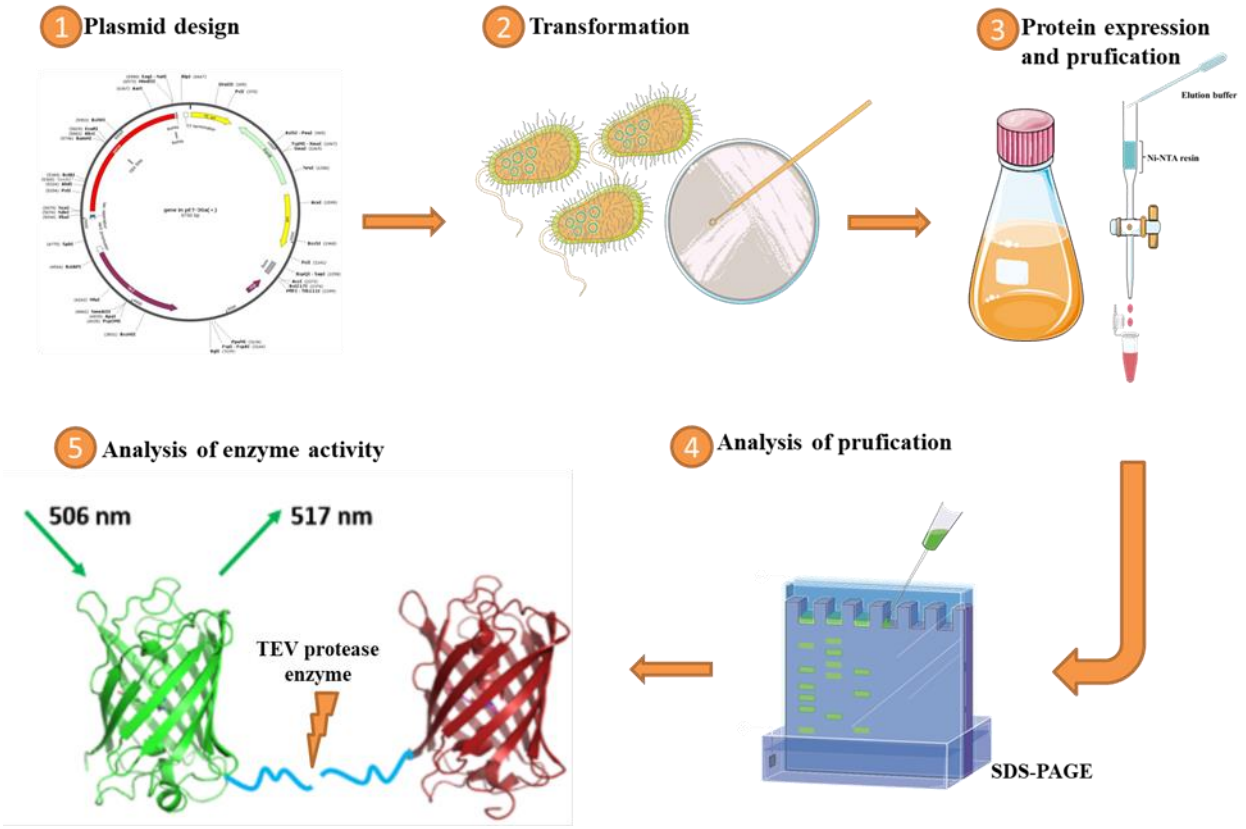

Figure 2 A summary of the experimental steps used in the development of a fluorescent protein-based FRET biosensor for determination of protease activity

3. RESULTS AND DISCUSSION

3.1. Expression and partial purification of the mNG-TEV-mRuby3 FRET sensor

The mNG-TEV-mRuby3 fusion protein was produced in *E. coli* DH5α cells and purified by nickel affinity chromatography. Samples taken during production and partial purification were analyzed in SDS-PAGE. As expected, the 55 kDa mNG-TEV-mRuby3 fusion protein was obtained (Figure 3).

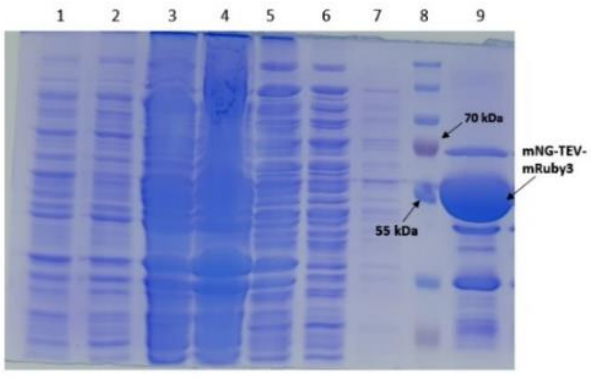

Figure 3 Analysis of the production and partial purification of the mNG-TEV-mRuby3 FRET pair in SDS-PAGE. Lanes 1 and 2 are cell culture samples 2 hours and 4 hours after cell inoculation, respectively. Lane 3, sample of pellet after sonicator. Lanes 4 and 5 sample pellet and supernatant after ultracentrifugation, respectively. Lane 6, flowing filtrate after passing the supernatant through the column. Lane 7, sample obtained by passing wash buffer through the column. Lane 8, protein marker (Thermo Scientific Prestained). Lane 9, sample obtained by passing elution buffer through the column.

3.2. Analysis of TEV protease activity with the mNG-TEV-mRuby3 FRET sensor

The mNG-TEV-mRuby3 FRET sensor was incubated with the TEV protease enzyme in the amounts given in Table 1. When the TEV protease cleavage site in the mNG-TEV-mRuby3 FRET sensor is cleaved by the TEV protease enzyme, it is expected to cause a color and emission change in this FP-FRET biosensor. In addition, a reaction was prepared to examine

whether β-Mercaptoethanol in the TEV protease buffer has an effect on the color change in the mNG-TEV-mRuby3 FRET sensor (example number 4 in Table 1). No direct effect of β-Mercaptoethanol on color change was observed. As expected, the color change in the FP-FRET sensor occurred after the related region was cleaved by the TEV enzyme (Figure 3B). After cleavage with TEV protease, the reaction mixture was analyzed by 10% SDS-PAGE. As indicated in Table 1, where the protease degradation reaction components are shown, there was only the mNG-TEV-mRuby3 FRET pair (approximately 55 kDa) in lanes 1 and 4. In lane 2, only TEV protease enzyme with a molecular weight of approximately 27 kDa was observed. In lanes 3, 5 and 6, as a result of cleavage of the mNG-TEV-mRuby3 FRET pair (55 kDa) with TEV protease added to the microtubes, mNeonGreen (28 kDa) and mRuby3 (27 kDa) fragments were obtained (Figure 4A).

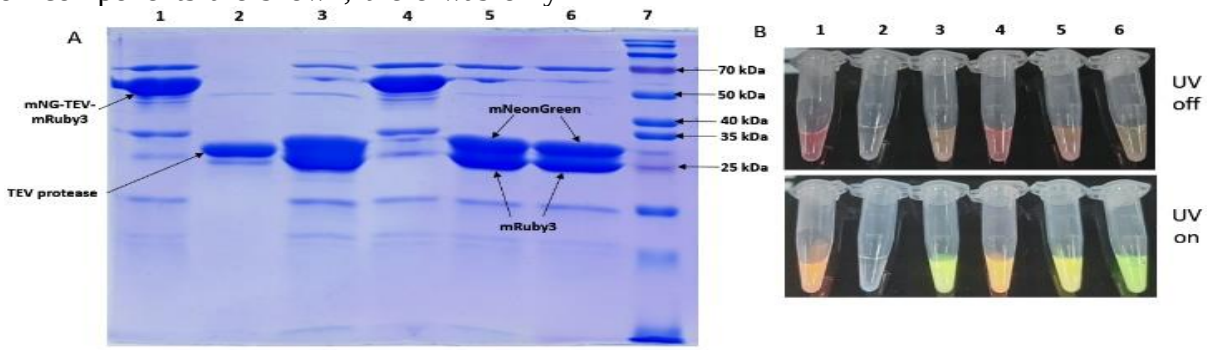

Figure 4 A. Analysis of mNG-TEV-mRuby3 FRET sensor cleavage by TEV protease enzyme in SDS-PAGE. Lanes 1 and 4 contain only FP-FRET biosensor, lane 2 contains only TEV protease enzyme. Lanes 3, 5 and 6 are the products of the FP-FRET biosensor after cleavage with TEV protease. Lane 7, Protein marker (Bio Basic Prestained). B. Image under UV light after mNG-TEV-mRuby3 FRET sensor cleavage by TEV protease enzyme after applying the protocols given in Table 1.

After the mNG-TEV-mRuby3 FRET sensor was cleaved with the TEV enzyme, measurements of emission wavelengths were carried out in the spectrofluorometer. In reactions without TEV protease enzyme, the TEV protease binding site between the mNG-TEV-mRuby3 FRET pair was preserved. As shown in Figure 5. the mNG-TEV-mRuby3 FRET sensor excited at 506 nm wavelength peaks at two wavelengths, 517 nm and 572 nm. The peak at 517 nm is the emission wavelength of the mNeonGreen fluorescent protein. Then, the excited mNeonGreen uses some of its energy as emission energy and transfers the rest to the mRuby3 fluorescent protein, causing the mRuby3 protein to be excited. In this way, the induced mRuby3 fluorescent protein emits at a wavelength of 572 nm (Figure 5A).

In reactions involving the TEV protease enzyme, the connection between the mNG-TEV-mRuby3 FRET pair was cleaved, resulting in two separate fluorescent proteins, mNeonGreen and mRuby3. This indicates that direct resonance energy transfer between the two fluorescent proteins will not occur. In other words, it was observed that resonance energy transfer did not occur by emitting at 517 nm wavelength as a result of excitation of the mNG-TEV-mRuby3 FRET sensor at 506 nm wavelength. In summary, if resonance energy transfer had occurred, a significant peak at 572 nm would be expected. In addition, a weak peak at 572 nm is seen in the graph. This situation can be evaluated as some mNG-TEV-mRuby3 FRET sensors in the reaction tube not cutting with the TEV enzyme or the cutting process is not completed (Figure 5B).

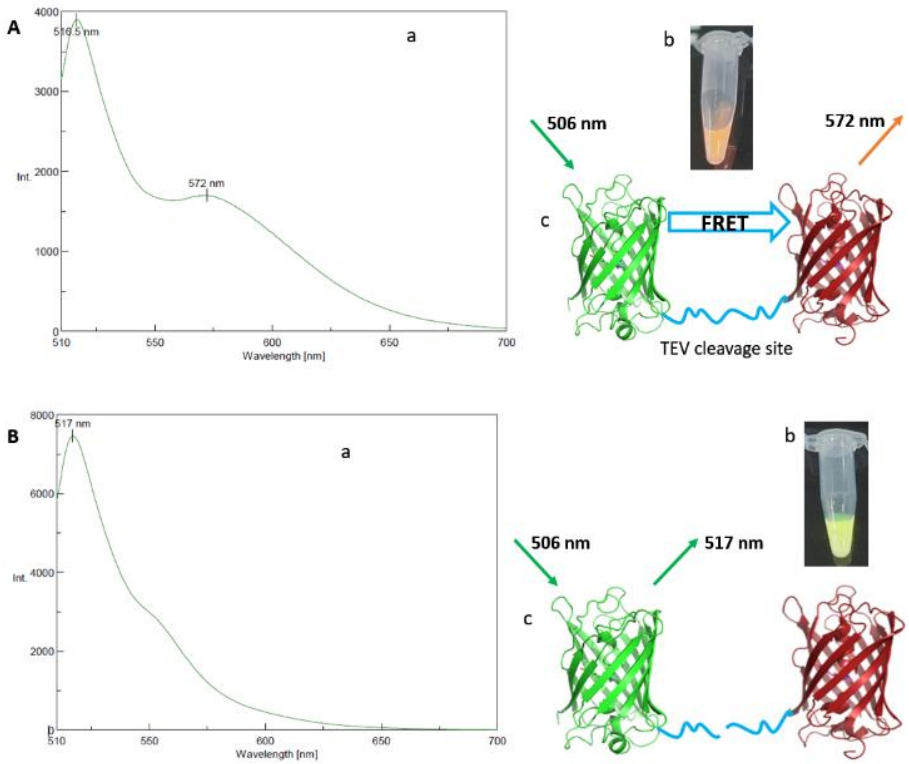

Figure 5 A. Images of the produced mNG-TEV-mRuby3 FRET sensor. Emission wavelength graph of the produced FP-FRET sensor (a), image of the FP-FRET sensor under UV light (b), schematized version of biosensor (c). B. Images of the mNG-TEV-mRuby3 FRET sensor after the TEV protease activity. Emission wavelength graph of the FP-FRET sensor after protease activity (a), image of the biosensor under UV light after the TEV protease activity (b), schematized version after the TEV protease activity (c).

In all FRET biosensors, the selection of the optimum FRET pairs is the most important parameter for the performance of the biosensors to be designed [9]. Bajar et al. (2016) demonstrated that the mClover3-mRuby3 or mNeonGreen-mRuby3 pair is one of the best choices in terms of photostability, brightness, and FRET dynamic range when used in cultured cells [2]. In addition, linker length is one of the important parameters in FP-FRET biosensors. In most cases, steric hindrance or folding interaction between the proteins of interest can occur when the linker joining the fluorescent pairs is not long and flexible enough. Therefore, the linker should be flexible, soluble and resistant to proteolysis and should not have a secondary structure or aggregate [18].

The design of genetically encoded FP-FRET biosensors shows some differences according to the intended use. In general, a large number of different biosensors have been developed for various cellular targets, such as changes in ions, molecules, enzymatic activity, oxidation-reduction events, membrane potential and channel conformation, or stages of the cell cycle [19]. Therefore, there is no ideal linker length used in FP-FRET biosensors. Different linker lengths are used according to the purpose of the study. For example, 20 amino acids for Factor Xa protease activity [20], 25 amino acids for trypsin enzyme activity [21], 20 amino acids for Caspase-3 [22] and 12 amino acids length linker for MMP-2 [23] were used. In a study using the mNeonGreen and mRuby3 proteins, a linker consisting of 8 amino acids was used to examine the FRET activity [24]. Also, when it comes to linker flexibility, the amino acid that gives the greatest flexibility to a peptide chain is glycine with the smallest side chain. Therefore, the linker region between the fluorescent protein pairs was designed to contain the glycine sequence [18]. Flexible polypeptide linkers composed of glycine and serine amino acids are important components of engineered fusion proteins. Some studies have been done on linkers containing glycine and

serine repeats. It was observed that FRET activity decreased with increasing linker length, and FRET activity decreased in linkers containing less glycine. In addition, flexibility was reduced in linkers with low glycine content [25].

In this study, a linker containing 17 amino acids (GGSLEENLYFQGEFSGG) was used in the designed mNG-TEV-mRuby3 biosensor. Within this linker length there were the sequences of the TEV protease cleavage site and restriction enzymes. In addition, the GGS sequence was added to both ends of the linker symmetrically in order to obtain linker stabilization, flexibility and effective linker length. It has been clearly demonstrated that the designed structure is effective in generating FRET pairs. By genetically modifying the protease recognition sequence, a different FP-FRET biosensor that can be used to determine the activity of other protease enzymes can be simply designed.

4. CONCLUSION

There are standard approaches to looking at enzymes, such as analysis of transcripts (i.e. mRNAs) or protein levels, but these cannot reflect the specific activity of the enzyme under certain conditions. Activity-based probes have been designed for the detection of enzyme activity. However, activity-based probes negatively affect enzyme activity due to covalent binding. This situation limits the visibility of dynamic processes [26]. Dynamic and continuous detection of enzyme activity in living cells is possible with FRET sensors. FRET can visualize relevant molecular events in living or fixed cells that are difficult to image for other molecular techniques, thanks to its unique functional distance range. Therefore, FRET has become a key technique for studying the activity of enzymes in cells. Continuous detection of enzyme activities (proteases, phosphatases, polymerase, kinases, telomerase, etc.) is an important parameter in medical diagnosis [27].

Genetically encoded FP-FRET sensors allow the monitoring of certain biochemical or biological recognition processes in the cell by preserving the data flow of time and space [28]. This makes fluorescent proteins and FP-FRET sensors powerful tools for *in vivo* and *in vitro* applications [6].

In this study, the mNG-TEV-mRuby3 FRET sensor designed to monitor TEV protease activity was expressed in *E. coli* DH5α cells. The TEV protease enzyme used in the study was also expressed in *E. coli* BL21 pLysE cells using recombinant DNA techniques. TEV protease activity was analyzed in SDS-PAGE and spectrofluorometer using the mNG-TEV-mRuby3 FRET sensor as a target. With this study, the utility of the mNeonGreen-mRubby3 FRET pair in the determination of protease activity was demonstrated. These findings provide strong evidence that mNG-mRuby3 FRET sensors can be developed that can be used in new protease activity assays. If needed, protease recognition sites can be changed by genetically modifying, and versatile new mNG-mRuby3 FRET sensors can be easily developed.

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The Declaration of Conflict of Interest/ Common Interest

The authors confirm that this article content has no conflicts of interest

Authors' Contribution

İbrahim İncir, Özlem Kaplan, Sema Bilgin and İsa Gökçe formed the research idea and designed the experiments. İbrahim İncir performed the experiments. İbrahim İncir, Özlem Kaplan, Sema Bilgin and İsa Gökçe analyzed the data. İbrahim İncir created the first draft of the manuscript which was edited by all the authors.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

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