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Note

Monitoring of enzymatic cleavage reaction of GST-fusion protein on biolayer interferometry sensor

Sena Tarumoto, Sei Inoue, Rina Yanagimoto, Takashi Saitoh

Faculty of Pharmaceutical Sciences, Hokkaido University of Science, Sapporo, Hokkaido 006-8585, Japan

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Biolayer interferometry (BLI) is an optical sensor-based analytical method primarily used for analyzing interactions between biomolecules. In this study, we explored the application of BLI to observe the cleavage reaction of glutathione S-transferase (GST)-tagged fusion protein by human rhinovirus (HRV) 3C protease on a BLI sensor as a new application of the BLI method. The soluble domain of the Tic22 protein from *Plasmodium falciparum* was expressed and purified as a GST-tagged fusion protein, GST-Tic22, in *Escherichia coli*. A cleavage sequence for HRV 3C protease was inserted between the GST tag and the soluble domain of Tic22. First, we confirmed that GST-Tic22 was specifically cleaved at the inserted sequence by HRV 3C protease using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following this, GST-Tic22 was immobilized on a BLI sensor, and enzymatic cleavage by the HRV 3C protease was monitored. We observed that the soluble domain of Tic22 was cleaved and released into the buffer over time, and this reaction was dependent on the enzyme concentration. This result demonstrates that the BLI method can be used to evaluate the cleavage of the GST tag by the HRV 3C protease in real time under different conditions. This method enables a more efficient search for the optimal conditions for the tag cleavage reaction in fusion proteins, a process that has historically required a substantial amount of time and effort.

Key words: cleavage enzyme, HRV 3C protease, tagged protein, real-time monitoring

Significance

BLI is a method primarily used for analyzing interactions between biomolecules. In this study, a recombinant protein with a GST tag and an HRV 3C cleavage site was prepared for the Tic22 protein of *Plasmodium falciparum*. The site-specific enzymatic cleavage reaction of this GST-Tic22 was confirmed using SDS-PAGE. Subsequently, cleavage was monitored on a BLI sensor. The results of this study suggest that the BLI method can be effectively used to investigate the optimal conditions for the cleavage of tags in recombinant proteins.

Introduction

Biolayer interferometry (BLI) is an analytical technique that uses optical sensors to study interactions between biological molecules [1]. In this method, molecules (ligands) are immobilized on the tip of an optical fiber (referred to as a biosensor). Light reflects off the surface where the ligand is immobilized. White light passing through the optical

Corresponding author: Takashi Saitoh, Faculty of Pharmaceutical Sciences, Hokkaido University of Science, 15-4-1, Maeda, 7-jo, Teine-ku, Sapporo, Hokkaido 006-8585, Japan. ORCID iD: https://orcid.org/0000-0001-6533-4667, e-mail: saito-t@hus.ac.jp

fiber is reflected at two interfaces: the boundary between the fiber tip and the ligand, and the boundary between the ligand and the buffer. The light reflected from these two interfaces, which have different refractive indices, interferes with each other, creating an interference wave. This interference wave is influenced by the ligand, causing its phase too change according to the length of the ligand and its interaction with the target molecule (analyte). Therefore, it is possible to observe changes in ligand molecules in real time. In typical BLI measurements, a protein or other ligand is first immobilized on the sensor of an optical fiber using strong and selective molecular interactions, such as an antigen–antibody reaction. Next, the sensor is soaked in a solution containing an analyte that interacts with the ligand, such as proteins, nucleic acids, or potential drug compounds. The phase change over time is then recorded. By measuring different concentrations of the analyte, information on the association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium dissociation constant (K_D) of molecular interactions can be obtained [2-4]. Although there are few examples, the BLI method has been used to monitor enzymatic reactions [5,6].

The apicoplast of *Plasmodium falciparum* is a unique organelle, surrounded by four membranes, that is considered a plastid derived from secondary endosymbiosis with algae, despite not performing photosynthesis [7-9]. This organelle is essential for the survival of *Plasmodium falciparum,* and being absent in animals, serves as an attractive target for new antimalarial drugs. [10-11]. Similar to other symbiotic organelles, such as mitochondria, the apicoplast possesses its own DNA. However, many of the proteins utilized by the apicoplast (apicoplast proteins) are synthesized in the cytoplasm and transported into the apicoplast through translocators in each of the four membranes. Among these, the translocator in the innermost membrane (the fourth membrane), derived from algae, is known as the translocator of the inner envelope membrane of the chloroplast (Tic) complex [12,13]. This complex is also present in chloroplasts, and the homology between chloroplasts and apicoplasts is relatively high. The Tic22 protein is responsible for recognizing apicoplast proteins that pass through the third membrane to the Tic complex [14,15].

Protein expression and purification using fusion proteins have become essential in the field of protein engineering. Among the many tags, the glutathione S-transferase (GST) tag is one of the most widely used due to its ability to improve the solubility of the expressed protein and enable simple purification methods [16]. After obtaining the target recombinant protein as a tagged fusion protein, it is often necessary to cleave the tag. Various proteases, such as thrombin, are used for tag cleavage; however human rhinovirus (HRV) 3C protease is frequently preferred due to its very high sequence specificity at the cleavage site [17]. Consequently, GST fusion proteins with HRV 3C protease cleavage sites are commonly used in recombinant protein production. In the present study, we purified the soluble domain of *Plasmodium falciparum* Tic22 as a GST fusion protein (GST-Tic22) using a high-expression system in *E. coli.* An HRV 3C protease cleavage site was inserted between GST and Tic22 to observe the enzymatic cleavage reaction of GST-Tic22 on the BLI sensor. Additionally, the dependence of the cleavage reaction on enzyme concentration was confirmed, demonstrating the viability and utility of monitoring the enzymatic cleavage reaction of GST-fusion proteins using the BLI method.

Materials and Methods

Preparation of GST-tagged Tic22 Protein

The cDNA of *Plasmodium falciparum* contains more than 80% adenine and thymine, making cloning and other common genetic manipulations difficult [18-20]. To address this, the cDNA of the soluble domain of *Plasmodium falciparum* Tic22 (amino acids 67–270) was synthesized with codon optimization for *E. coli.* (Takara Bio). This gene was cloned into the pGEX-6P1 vector (Cytiva) using the BamHI and EcoRI restriction sites. The plasmid was transformed into *E. coli* BL21(DE3) pLysS using the heat shock method. *E. coli* was cultured in Luria-Bertani medium containing 100 μg/L ampicillin at 37°C until the optical density reached 0.6. Isopropyl β-D-thiogalactopyranoside was then added at a final concentration of 0.5 mmol/L, and the cultures were incubated overnight at 20°C. *E. coli* cells were harvested by centrifugation (5000 × g, 20 min, 4°C). The collected cells were resuspended in 50 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl buffer, disrupted by sonication, and the supernatant was collected by centrifugation (12000 × g, 30 min, 4°C). For affinity purification, Glutathione Sepharose 4 B resin (Cytiva) was added to the supernatant. After washing the resin with 50 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl buffer, GST-Tic22 was eluted with a buffer containing 25 mmol/L reduced glutathione. The sample was concentrated using an ultrafiltration membrane and further purified using size-exclusion chromatography (SEC) (Superdex 200 increase 10/300 GL, Cytiva). Detection was performed by measuring absorbance at 280 nm. The molecular weight corresponding to the elution volume was calculated using a calibration curve generated from the elution volumes of standard proteins: bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

Enzymatic Cleavage Reaction by HRV 3C Protease

HRV 3C protease was purchased from Takara Bio. First, 100 μL of 0.6 mg/mL GST-Tic22 in 10 mmol/L HEPES- HCl (pH 7.4) and 150 mmol/L NaCl was incubated with 5.0×10^{-9} kat HRV 3C protease for 16 h at 4°C to cleave the GST tag from the fusion protein. The cleavage reaction by HRV 3C protease was confirmed by subjecting both treated and

untreated samples to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie brilliant blue.

BLI

BLI was performed using OCTET K2 (ForteBio) at 30°C, and anti-GST biosensors with immobilized anti-GST antibodies were used (ForteBio). The buffer used for the measurement was 10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 0.01% bovine serum albumin, and 0.05% Tween 20. The sensor was first soaked in the buffer for 300 s to establish the baseline, then soaked in the buffer containing 0.1 mg/mL GST-Tic22 for 300 s to immobilize GST-Tic22 on the sensor. This was followed by another 300-second soak in the buffer again to wash off any unbound GST-Tic22. Subsequently, the sensor was soaked in a buffer containing different concentrations of HRV 3C protease (0 kat/L, 1.7×10^{-4} kat/L, and 1.7×10^{-4} kat/L) to monitor the enzymatic cleavage of the GST tag from Tic22.

Results and Discussion

Purification of GST-Tic22

The composition of GST-Tic22 used in the present study, along with the SDS-PAGE results before and after affinity column purification, are shown in Supplementary Figure S1. In the extraction fraction obtained with a buffer containing 25 mmol/L reduced glutathione, GST-Tic22 appeared almost as a single band corresponding to its expected molecular weight of approximately 50 kDa. The results of SEC performed on this sample, as well as the SDS-PAGE gel image of several fractions, are presented in Figure 1. GST-Tic22 eluted as a peak at an elution volume of approximately 12.8 mL on SEC, with the molecular weight at this elution volume estimated to be approximately 142 kDa based on the calibration curve. This suggested that GST-Tic22 avoided aggregation and likely formed a dimer or trimer. Given that GST is known to dimerize [21], it is likely that GST-Tic22 also forms a dimer, with an approximate molecular weight of 100 kDa. The high elution volume relative to the expected size is likely due to the shape of the GST-Tic22 molecule. The SEC fractions marked with asterisks (*) on the SDS-PAGE gel (Figure 1) were selected for subsequent cleavage experiment with HRV 3C protease.

Cleavage Reaction by HRV 3C Protease

The cleavage of GST-Tic22 by the HRV 3C protease was confirmed by SDS-PAGE (Figure 2). Post-cleavage, the band corresponding to GST-Tic22 completely disappeared, replaced by two bands of comparable intensity between 20 and 25 kDa, as well as a weakly stained band. Given that the GST tag has an approximate molecular weight of 24 kDa and the soluble domain of Tic22 is approximately 26 kDa, the two prominent bands correspond to the GST tag and the

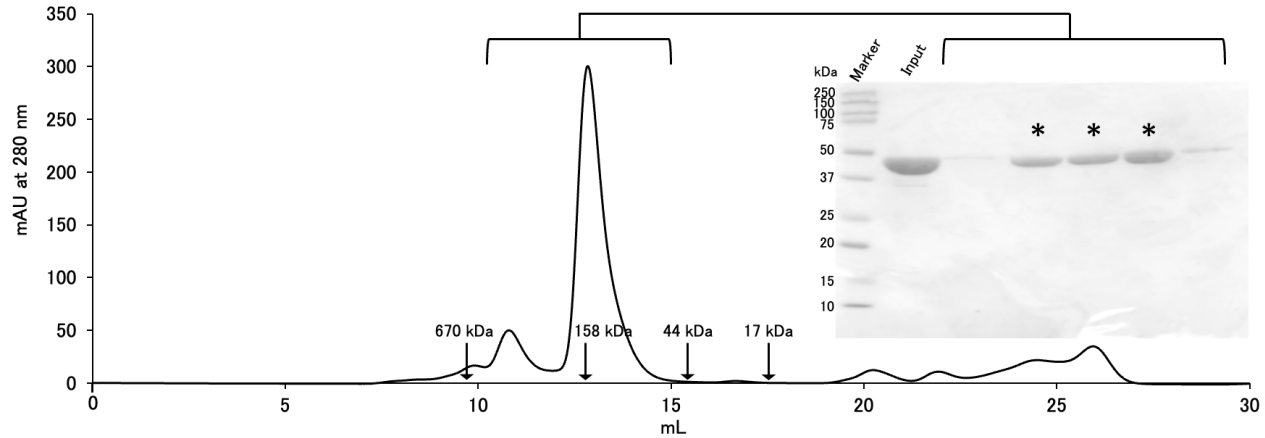

Figure 1 Size-exclusion chromatography of glutathione S-transferase (GST)-Tic22 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Following affinity chromatography purification, GST-Tic22 was subjected to SEC using a Superdex 200 increase 10/300 GL column (Cytiva) equilibrated with 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl. The elution volumes of the standard proteins using for estimating molecular weight were indicated by arrows. The fractions collected were analyzed by SDS-PAGE analysis on 12% polyacrylamide gel, stained with Coomassie brilliant blue). The fractions marked with asterisk (*) on SDS-PAGE gel were selected for subsequent cleavage experiment with HRV 3C protease.

soluble domain of Tic22. Additionally, the weakly stained band was presumed to be HRV 3C protease, which has a molecular weight of 22 kDa. These results confirm that HRV 3C protease specifically cleaved GST-Tic22 at the intended cleavage site.

Observation of Cleavage Reaction using BLI

In the BLI assay, GST-Tic22 was immobilized onto two anti-GST biosensors using an anti-GST antibody (Figure 3, 300 s to 800 s). The observed phase shift confirmed that GST-Tic22 was successfully captured by both sensors. Subsequently, one of these sensors was soaked in a buffer containing 1.7×10^{-4} kat/L of HRV 3C protease, while the other was soaked in a buffer without HRV 3C protease to monitor relative changes in the sensorgram (Figure 3, from 1200 s). The sensor soaked in the buffer without HRV 3C protease showed no change, whereas the sensor soaked in the buffer with HRV 3C protease exhibited a decrease in the phase shift over time, indicating that the molecules immobilized on the sensor were being cleaved. This result indicates that the cleavage reaction catalyzed by HRV 3C protease gradually progressed over time, with the soluble domain of Tic22 gradually dissociating from the sensor. By 1300 s, the changes in the sensor readings had plateaued, suggesting that nearly all GST-Tic22 had been cleaved into the GST tag and the soluble domain of Tic22. Since the enzymatic reaction by the HRV 3C protease dose not dependent on the type or state of the tag region, but on the amino acid sequence of the cleavage region, the cleavage reaction observed on the BLI sensor was presumed to be the same as that in solution. Based on the results of the BLI

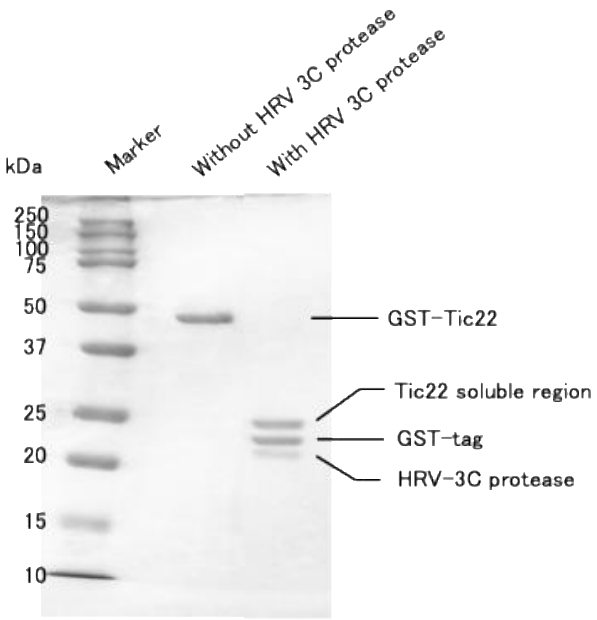

Figure 2 Cleavage of GST-Tic22 by HRV 3C protease. SDS-PAGE analysis of the cleavage reaction of GST-Tic22 by HRV 3C protease (12.5% polyacrylamide gel stained with Coomassie brilliant blue). A 100 µL solution of 0.6 mg/mL GST-Tic22 was incubated overnight at 4°C with 5×10^{-9} kat of HRV 3C protease (Takara Bio) to facilitate cleavage of GST from Tic22.

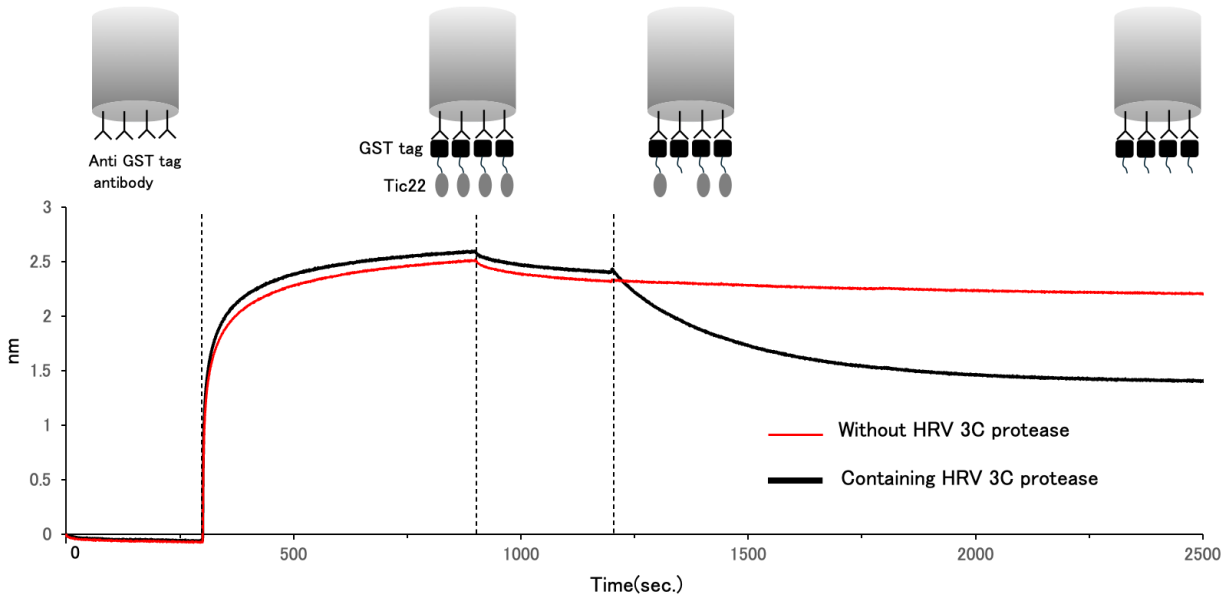

Figure 3 Enzymatic cleavage reaction of GST-Tic22 monitored by BLI. BLI analysis was conducted at 30°C in a buffer containing 10 mmol/L HEPES-NaOH (pH 7.4), 150 mmol/L NaCl, 0.01% bovine serum albumin and 0.05% Tween 20. GST-Tic22 was immobilized on the sensor via an anti-GST tag antibody, starting from 300 s and continuing until 800 s. At 1,200 seconds, the sensor was exposed to a buffer containing 1.7×10^{-4} kat/L HRV 3C protease. The cleavage of GST-Tic22, resulting in the release of the soluble Tic22 domain, was monitored as changes in the sensorgram over time.

experiment, the SDS-PAGE analysis of the GST-Tic22 sample cleaved with HRV 3C protease at 30°C for 1 hour, at the same HRV 3C protease concentration as in the BLI experiment, showed the same results as those obtained after the cleavage reaction at 4°C for 16 hours (Supplementary Figure S2).

GST-Tic22 was then immobilized on two separate sensors and exposed to buffers containing either 1.7×10^{-4} kat/L or 1.7×10^{-5} kat/L of HRV 3C protease, respectively, to monitor sensorgram changes (Figure 4). Sensorgram analysis revealed a slower cleavage reaction at the lower protease concentration than at higher concentration, indicating a concentration-dependent cleavage rate.

These results indicate that the BLI method is effective for real-time monitoring of the GST tag cleavage process by HRV 3C protease under various conditions. Using a BLI system with temperature control, it is possible to measure enzyme reactions at various temperatures. The reaction can also be monitored simultaneously at different enzyme concentrations and under various buffer and pH conditions using 96-well or 384-well plates. In addition, since real-time monitoring is possible, it allows to accurately estimate the time required for the cleavage reaction to complete. This approach facilitates a more efficient search for optimal cleavage conditions, which can streamline the preparation of tagged recombinant proteins for purification and solubilization, a process that traditionally demands considerable time and effort [22,23].

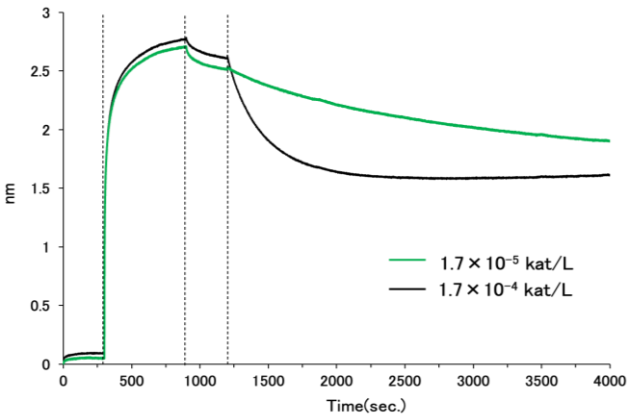

Figure 4 Concentration dependence of HRV 3C protease-mediated cleavage reaction. The enzymatic cleavage reaction of GST-Tic22 at different concentrations of HRV 3C protease (black: 1.7×10^{-4} kat/L and green: 1.7×10^{-5} kat/L) was monitored using BLI. GST-Tic22 was immobilized on the sensor via an anti-GST tag antibody between 300 s to 800 s. At 1,200 s the sensor was then soaked in a buffer containing HRV 3C protease.

Conclusion

The BLI method has been primarily used to observe biomolecular interactions and determine their dissociation and association constants. A comparable technique that provides kinetic parameters is surface plasmon resonance (SPR) [24]. A major difference between these two methods is that while SPR uses a flow cell for measurements, BLI measures interactions by soaking the ligand in the analyte. In this study, we leveraged the advantages of this soaking method and applied it to monitor the enzymatic cleavage reaction of a GST-fusion recombinant protein by HRV C3 protease. These results indicate that BLI is effective for rapidly identifying optimal conditions for tag cleavage during the preparation of new recombinant proteins.

Conflict of Interest

The authors have no competing interests to declare.

Author Contributions

Planned experiments: Takashi Saitoh; expression and purification: Sei Inoue and Rina Yanagimoto; BLI experiments: Sena Tarumoto; writing the paper: Takashi Saitoh.

Data Availability

The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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