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Use of hGluc/tdTomato pair for sensitive BRET sensing of protease with high solution media tolerance

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ABSTRACT

Due to the complicated media, monitoring proteases in real physiological environments is still a big challenge. Bioluminescence resonance energy transfer (BRET) is one of the promising techniques but its application is limited by the susceptibility to buffer composition, which might cause serious errors for the assay. Herein we report a novel combination of BRET pair with humanized *Gaussia* luciferase (hGluc) and highly bright red fluorescence protein tdTomato for sensitive and robust protease activity determination. As a result, the hGluc/tdTomato BRET pair showed much better tolerance to buffer composition, pH and sample matrices, and wide spectral separation ($\Delta\lambda$: ~110 nm). With the protease sensor built with this pair, the detection limit for enterokinase reached 2.1 pM in pure buffer and 3.3 pM in 3% serum. The proposed pair would find broad use in both *in vitro* and *in vivo* assays, especially for samples with complicated matrix.

1. Introduction

Proteases, which are widely present in organisms, represent important regulatory nodes via participating in various physiological processes [1–3] and those from microorganisms may also pose threats to human health as bacterial toxins [4] and are usually the main targets of viral disease prevention and therapy [5]. Monitoring proteases' activities and determining their recognition sites have been widely studied [6,7]. The complicated environment of naturally existing proteases presents a huge challenge for protease detection in real physiological samples, such as cytoplasm and body fluids. This is usually solved by separation of proteases from the matrices, while the more economical approach is to establish methods with very low interference from sample matrices and thus avoid the separation process. Several technologies such as Bioluminescence resonance energy transfer (BRET) [8–10], protein fragment complementation assays [11] and mass spectrum analysis [12] all adopt this principle, while bioluminescence based techniques are always preferred due to their high sensitivity and cheaper instruments.

The BRET assay based on the ratiometric technique which depends on the distance between a donor and an acceptor strictly has been widely used to detect proteases [8–10] and other inter- or intra-molecular interactions [13–15] because of its high sensitivity, excellent reproducibility and low background. For detection of proteases, the donor and the acceptor are linked by a protease recognizable fragment, the donor catalyzes its substrates and light emits during this process; at the same time, energy transfers non-radiatively to the acceptor due to its proximity to the donor and thus the acceptor emits light, that is, the BRET occurs. When the linker is broken by the protease and thus separates the donor and the acceptor, the BRET diminishes. The proteases will be detected by monitoring the change of BRET. BRET is sensitive but may suffer from serious influence of the sensing environment [16,17]. The classical BRET system with *Renilla* luciferase (Rluc)/green fluorescent proteins (GFPs) as the donor–acceptor pair may exhibit obvious signal variation when changing the reaction buffer [16]. This means the samples with different matrix or prepared in different solutions would have different signal response for the same protease concentration, which may be a huge source of errors for this type of assay. To solve this problem, it is necessary to explore more efficient donor–acceptor pairs.

Humanized *Gaussia* luciferase (hGluc) is smaller but much brighter than Rluc [18,19]. When it coupled with a kind of GFPs, enhanced yellow fluorescent protein (EYFP), it has been proved to be an appropriate combination for sensitive assay based on BRET [17]. However, similar to Rluc/GFPs, BRET with hGluc/EYFP pair suffers influence from buffer changes too. As discussed in Ref [17], the dependence of BRET signal on buffers is most probably arisen from the acceptor GFPs that are susceptible to the environment. The red fluorescent protein tdTomato is a rather attractive acceptor owing to its high quantum yield (0.69), high molar extinction

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coefficient (138,000 M^{-1} cm^{-1}) and high photostability [20,21]. It has been widely used as a labeling protein [22,23]. These properties may make it a promising acceptor for a stable and sensitive BRET system. tdTomato has been used as a BRET acceptor coupled with click beetle green luciferase (CBG) for protease detection [8], but this pair displayed small spectral separation ($\Delta\lambda \sim 40$ nm) and thus might limit the assay sensitivity.

In the present work we try to surmount the limitations mentioned above by combining the highly bright hGluc and highly photostable red fluorescent protein tdTomato together to develop a new BRET pair. hGluc is more suitable as a donor because its emission (em=470 nm) is far away from that of tdTomato (em=580 nm), and overlaps well with the excitation of tdTomato (ex=554 nm). The pair gave rather consistent signals for different buffers and selected pHs, and is insensitive to complicated sample matrix such as serum. The pair has a large spectral separation ($\Delta\lambda \sim 110$ nm) and high assay sensitivity was attained. Using enterokinase as a model, the detection limit was as low as 2.1 pM after 4 h incubation, 35 times better than that acquired with hGluc/EYFP pair.

2. Materials and methods

2.1. Materials

The plasmids containing gene of hGluc: pcDNA3.1–zipper–hGluc (1) and pcDNA3.1-zipper-hGluc (2) were generous gifts of Prof. Stephen W Michnick (Université de Montréal, Canada) [24]. tdTomato was amplified from pLVX-IRES-tdTomato (Clontech, CA, US). A *Gaussia* luciferase (Gluc) assay reagent, which contains the substrate coelenterazine, and enterokinase were purchased from New England Biolabs (UK). The rabbit serum was from a healthy rabbit provided by the animal laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences. The restriction endonucleases were purchased from TaKaRa (Japan). Oligonucleotides were synthesized by Sangon (Shanghai, China). All other chemicals used in the experiments were of analytical grade. Millipore water was used for all experiments.

2.2. Expression and purification of proteins

The fused plasmids, pET28a-hGluc–tdTomato, pET28a-hGluc-EK-tdTomato and pET28a-tdTomato-EK-hGluc (EK symbolized the linker GSDDDDKEF containing sequence which could be cleaved by enterokinase), were constructed as described in Ref. [17], substituting the EYFP gene by tdTomato from the corresponding clones. For the first and second clones, the gene of tdTomato was amplified by the primers F: 5′ AAGGAT GAATTC GTGAGCAAGGGCGAGG 3′, R: 5′ ATTTAC CTCGAG CTTGTACAGCTCGTCCAT; and for the last clone, the gene was amplified by the primers F: 5′ CAAACA CATATG GTGAGCAAGGGCGAGGAGGTCAT 3′, R: 5′ AAGCAA GAATTC CTTATCGTCATCGTCGGATCCCTTGTACAGCTCGTCCATGCCG 3′.

The plasmids pET28a-hGluc was constructed with hGluc gene amplified with primers F: 5′ ACACAT CATATG AAGCCCACCGAGAACAA 3′, R: 5′ ATTAAC CTCGAG GTCACCACCGGCCCCCTT 3′containing Nde I and Xho I cleavable sites, respectively, and then digested and inserted into pET28a digested by the same restriction endonucleases.

All the constructing sequences of the proper clones were finally reconfirmed by DNA sequencing.

2.3. Protein expression and purification

The detailed procedures were the same as those in Ref. [17]. All the fusion targets and hGluc were expressed in *Escherichia coli* BL21 cells and purified by affinity chromatography with HisTrap FF crude column (GE Healthcare) on the AKTA purifier (GE Healthcare). Finally, all the purified proteins were ultra-filtrated in Amicon Ultra-15 centrifugal filter device (Millipore) to remove excess imidazole and concentrate the fusion proteins in mg mL^{-1} range. The sizes of the proteins were analyzed on SDS-PAGE. Their concentrations were assessed by bicinchoninic acid (BCA) assay (Pierce Rockford, IL).

2.4. Solutions preparation

Three different buffer components: tris(hydroxymethyl)aminomethane (Tris), N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and imidazole were dissolved in Millipore water, separately, to prepare buffers with concentration 20 mM and pH 7.30–7.40.

HCl (36% v/v) was added in the imidazole buffer (20 mM) to adjust the pH to obtain imidazole solutions with various pH values.

The enterokinase reaction buffer was prepared by dissolving Tris, NaCl and $CaCl_2$ in Millipore water to the final concentration of 20, 50 and 2 mM, respectively, and adjusted to pH 7.4.

2.5. The orientation assessment

The two fusion proteins hGluc-EK-tdTomato, tdTomato-EK-hGluc and hGluc alone were added in Tris buffer to a final concentration of 0.3 μM in a total volume of 60 μL. Then the BRET signals were recorded on a LS55 Luminescence Spectrometer (PerkinElmer, UK) in bioluminescence mode (Delay: 1.00 ms; Gate: 1.00 ms; Cycle: 20 ms; Flash count: 1) upon addition of 40 μL of the Gluc assay reagent. The spectra were recorded in a range from 420 to 700 nm. The spectrum of the Tris buffer plus 40 μL of the Gluc assay reagent was recorded as background, which was deducted correspondingly from the spectra of BRET and hGluc by the software FL WinLab (PerkinElmer). From the background deducted spectra, BRET ratios were calculated by emissions at 580 nm/470 nm minus emissions at 580 nm/470 nm of hGluc. The commercial statistics software DPS v7.55 was used for analyzing the significance of the differences in the BRET ratios whenever applicable.

2.6. Measurements in different solutions

The fusion protein hGluc-EK-tdTomato and hGluc alone were diluted in the solutions prepared above (20 mM Tris, 20 mM HEPES, 20 mM imidazole and 20 mM imidazole with different pH adjusted by HCl mentioned in Section 2.4) to record the signals. The proteins were prepared at about 0.3 μM in a total volume of 60 μL. The spectrum of each buffer was measured as background to be subtracted from the bioluminescent signals. Measurements and calculations were performed following the same procedures as described in Section 2.5.

2.7. The specificity assessment

Enterokinase (final concentration of 1900.0 pM) was mixed with hGluc-tdTomato (final concentrations: 1.0 μM) or hGluc-EK-tdTomato (final concentration: 1.0 μM) in the enterokinase reaction buffer to a total volume of 20 μL. Controls were also prepared, in which enterokinase was replaced by an equal volume of the enterokinase reaction buffer. The mixtures were incubated at room temperature (around 25 °C) for 4 h. When the reactions between enterokinase and hGluc-tdTomato or hGluc-EK-tdTomato have finished, 40 μL of the enterokinase reaction buffer was added in the finished reaction system to a final volume of 60 μL, respectively. And then, 40 μL of Gluc assay reagent was added in the 60 μL of system containing the finished reaction,

respectively, and immediately the signals were measured. Measurements and calculations were performed following the same procedures as described in Section 2.5.

2.8. BRET assays for protease activities

For enterokinase assay, various concentrations of enterokinase (1900.0 pM, 380.0 pM, 76.0 pM, 38.0 pM, 15.0 pM, 7.5 pM, 3.0 pM, 1.5 pM, 0.8 pM, 0.3 pM and 0.0 pM, serially diluted by 1 mg mL^{-1} BSA) were mixed with hGluc-EK-tdTomato (final concentration: 1.0 μM) in the enterokinase reaction buffer to a total volume of 20 μL. The mixtures were incubated at room temperature for 4 h. After the reaction, 40 μL of the enterokinase reaction buffer was supplemented to the reaction solution to a final volume of 60 μL. BRET spectra and ratios were measured and calculated after mixing the enzymatic reaction mixtures with 40 μL of the Gluc assay reagent. The change in BRET ratio was calculated by subtracting BRET $ratio_{blank}$ from BRET $ratio_{sample}$, where BRET $ratio_{blank}$ represents the BRET ratio obtained when no enterokinase added, and BRET $ratio_{sample}$ is the BRET ratio obtained after enterokinase with an indicated concentration reacted with the BRET sensor. Measurements and calculations were performed following the same procedures as described in Section 2.5.

2.9. Protease activity assay in serum

Various concentrations of enterokinase (serially diluted in rabbit serum to final concentrations of 38.0 pM, 15.0 pM, 7.5 pM, 3.0 pM and 0.0 pM) were mixed with hGluc-EK-tdTomato (final concentration: 1.0 μM) in the enterokinase reaction buffer to a total volume of 20 μL with a final serum concentration of 3% (v/v). Measurements were taken following the same procedures as described in Section 2.8.

3. Results and discussion

3.1. Sensor construction

Fig. 1 shows the spectra of hGluc, tdTomato and the fusion protein hGluc-EK-tdTomato expressed and purified in this study. The emission of the expressed hGluc overlapped well with the excitation of the expressed tdTomato, and BRET occurred for the fusion hGluc-EK-tdTomato. These results demonstrated that correct fusion has been successfully obtained. The SDS-PAGE result also showed that the full length fusion of hGluc-EK-tdTomato was expressed (data not shown).

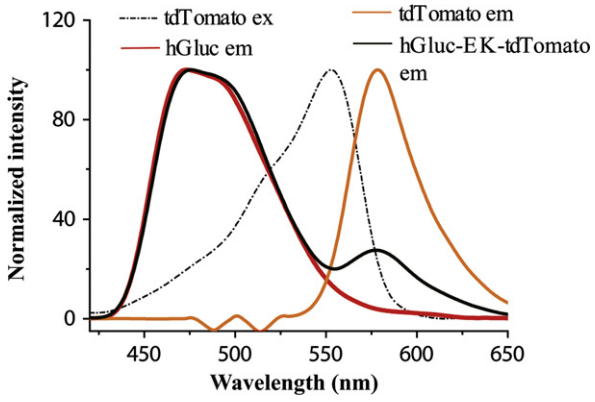

Fig. 1. Excitation spectrum of tdTomato (tdTomato ex, the dashed black line) and emission spectra of hGluc (hGluc em, the red line), tdTomato (tdTomato em, the orange line) and hGluc-EK-tdTomato (hGluc-EK-tdTomato em, the black line). The signals were normalized to their maximum emissions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As mentioned above, tdTomato is prominent among numerous red fluorescent proteins with high brightness and good photostability [20,21]. These two properties are usually required by BRET and thus render tdTomato particularly promising as a promising acceptor. When tdTomato was combined with hGluc genetically through a linker sequence (4 aa or 9 aa), BRET occurred in the resulted pair hGluc/tdTomato and displayed larger spectral separation (~110 nm) (Fig. 1, the black line) compared to other BRET systems, such as the hGluc/EYFP pair [17].

3.2. Effect of orientation

The BRET efficiency is greatly affected by the relative orientation of donors and acceptors [16,25]. To investigate the proper orientation of the hGluc/tdTomato pair, two fusions with hGluc inserted in either N-terminus (hGluc-EK-tdTomato) or C-terminus (tdTomato-EK-hGluc) with respect to tdTomato were assessed in Tris buffer. hGluc at N-terminus yielded higher BRET ratio (0.232 ± 0.003, mean ± SD) than that at C-terminus (0.172 ± 0.014, mean ± SD). This result differed from those obtained in previous works. When Rluc is used as the donor and GFPs as the acceptors, the preferable orientation is always that the donor is at C-terminus [16,25]. However, our previous work utilizing hGluc/EYFP pair showed that the orientation hardly has any effect [17]. The discrepancy might be a result of the structural difference of these proteins. In addition, the orientation with hGluc at N-terminus led to higher yield of target fusion due to the different expression levels of the two moieties. Therefore, the fusion with hGluc at N-terminus (hGluc-EK-tdTomato) was used in the following study.

3.3. The effects of solutions

tdTomato is derived from DsRed, which demonstrated high photostability and resistance to pH [20]. It can be speculated that tdTomato might inherit the properties of pH-stability and high photostability from DsRed. hGluc activity is relatively stable within the pH range 7–9 [26], and however, is expected to be negatively affected by serum, which is the common matrix for many kinds of proteases. Normally, serum severely weakens the output of Rluc [27] and other luciferase with maximum emission around 480 nm [28]. To assess whether these factors affected the hGluc/tdTomato pair and to evaluate its sensitivity to solution media, the BRET ratios were recorded for protease solutions with different media.

Three commonly used buffers, Tris, HEPES and imidazole, were utilized for this assessment. They were used for comparison because it was proved that they influenced the BRET ratio of hGluc/EYFP pair in different levels [17]. As shown in Fig. 2A, the discrepancy of the ratios in HEPES and in imidazole was not significant ($P > 0.05$), while they were significant in Tris and in the other two buffers ($P < 0.05$). However, the ratios did not increase significantly, only 8% higher in Tris than that in HEPES or imidazole. Therefore, the hGluc/tdTomato system was rather robust against the change of buffer components.

The effect of pH was also assessed in the range from 7 to 10, which almost covered the pH conditions for most biological reactions. Solutions of 20 mM imidazole were used to do the assessment. The BRET ratios were similar at pH 7.35 and pH 8.12 without any significant discrepancies ($P > 0.05$), while lower at pH 8.78 and 9.50 with significant discrepancy compared to the former two values ($P < 0.05$) (Fig. 2B). At pH 9.50, the BRET ratio was the lowest, but it decreased only 9% compared with the

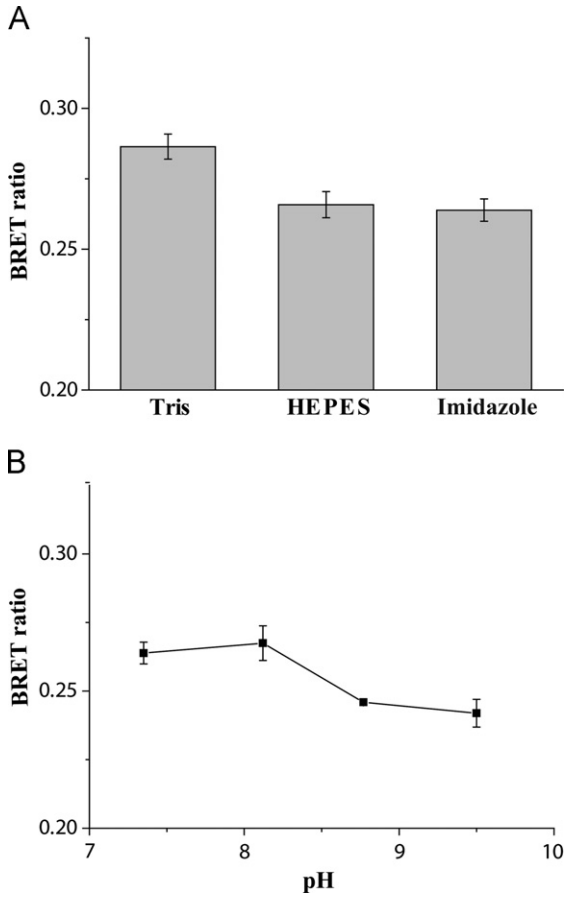

Fig. 2. Effects of the buffers on BRET. (A) The effect of buffer components on BRET ratios of the fusion probe hGluc-EK-tdTomato. The probe was at 0.3 μM in 60 μL of buffers. All the buffers were composed of 20 mM corresponding chemicals, pH 7.30–7.40. (B) The effect of pH on BRET ratios of the fusion probe in imidazole buffer (20 mM, pH adjusted with HCl). The protein concentration was 0.3 μM in 60 μL of buffers. Error bars represent SD, $n=3$.

highest value at pH 8.12 ($P<0.05$). This suggested the effect of pH on the BRET ratio of hGluc/tdTomato was not significant and thus the pair showed good stability in the studied pH range.

The effects of solution components and pH on hGluc/tdTomato were different from those on hGluc/EYFP. Since the difference of the two pairs just lies in their acceptors, it was their acceptors that caused this difference.

Subsequently, serum containing solution was also assessed. Because the components of a proper cleavable buffer for enterokinase (20 mM Tris, 50 mM NaCl, 2 mM $CaCl_2$, and pH 7.4) were commonly used in biological related buffers, this buffer was chosen in the assessment and rabbit serum was added in this buffer. With the increased concentration of serum, the signal output sharply decreased compared to that in pure buffer as seen by naked eyes (data not shown). Because 3% of serum could guarantee a reasonably strong signal from hGluc/tdTomato, the BRET ratio was measured at this serum concentration. The average BRET ratio in solution in the absence of serum was 0.232 and slightly increased to 0.256 in the presence of serum, about 10% increase in serum. This suggested that low concentration of serum could slightly increase BRET ratio possibly due to its protective role on protein conformation, and it did not affect the performance of hGluc/tdTomato pair significantly even though it did negatively affect the luminescence of hGluc. This result also implied that the activity of hGluc itself would not influence its BRET ratio significantly; hence the acceptor might be the main cause of the environment sensitivity of BRET systems. Therefore, it can be reasonably speculated that the hGluc/tdTomato pair would have high tolerance to a wide range of matrix containing different components and pHs as long as those conditions did not impair the activity of tdTomato severely. This tolerance might make hGluc/tdTomato pair a promising indicator for biological analysis with BRET, especially for the cases under complicated conditions. A good stability would be very useful for biological related applications because biological matrixes are always considerably intricate with complicated components and diverse pHs. The system that is stable to the environment would be much easier to operate and obtain reliable results.

3.4. Detection of enterokinase activity

Enterokinase participates in the pro-enzyme activation during the digestion process. It is cheap and easily available and thus is usually utilized as a model for protease research. The assay of enterokinase activity was based on the hGluc/tdTomato pair by inserting enterokinase recognized sequence DDDDK between hGluc and tdTomato (hGluc-EK-tdTomato). Before detection, the specificity of the proposed sensor was assessed by evaluating its response to a similar BRET probe without DDDDK, hGluc-tdTomato (linker: GSEF). The BRET ratios of hGluc-tdTomato were 0.259 ± 0.019 (mean $\pm$ SD) and 0.248 ± 0.007 (mean $\pm$ SD)

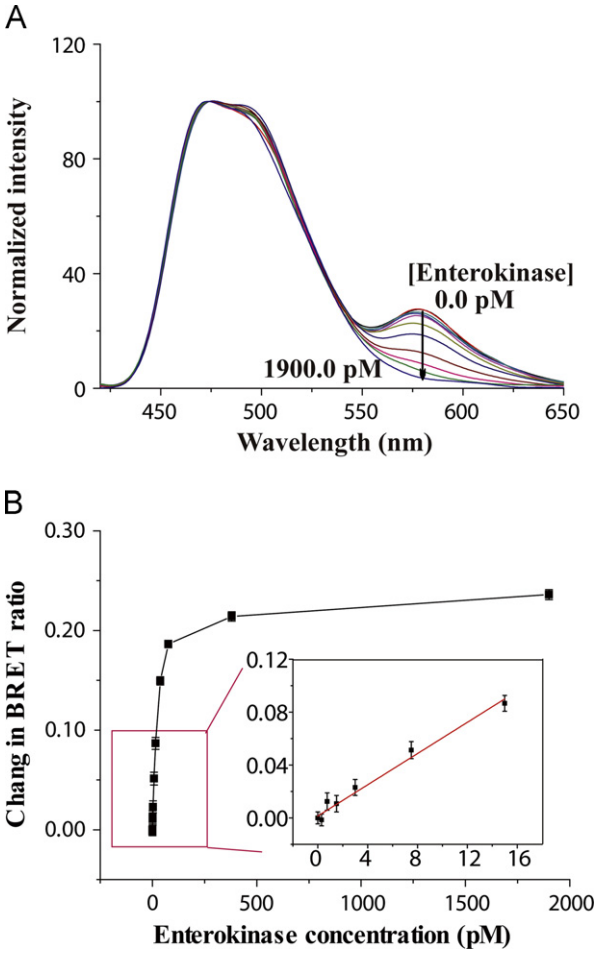

Fig. 3. Detection of enterokinase with hGluc-EK-tdTomato sensor. (A) Representative emission spectra in the presence of the indicated concentration of enterokinase. All spectra were normalized to the emission at λ_{470} nm. (B) The changes in BRET ratio were plotted versus enterokinase concentrations (means $\pm$ SD, $n=3$). Inset: linear calibration curve fitted by OriginPro 8.0. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

without and with enterokinase, respectively. The difference between these two BRET ratios was not statistically significant with $P > 0.05$. However, for its target probe hGluc-EK-tdTomato, the BRET ratio changed significantly from 0.232 ± 0.003 (mean $\pm$ SD) in the absence of enterokinase to -0.007 ± 0.004 (mean $\pm$ SD) in the presence of enterokinase. It decreased about 102% in BRET ratio due to the enterokinase proteolytic reaction. The significant decrease of BRET ratios demonstrated that enterokinase was specific to its target probe hGluc-EK-tdTomato.

Using the fusion probe hGluc-EK-tdTomato, the activity of different concentration enterokinase was further analyzed. The BRET spectra normalized at $\lambda_{470\ nm}$ showed that the emission at $\lambda_{580\ nm}$ declined gradually along with the increasing concentration of enterokinase from 0.0 to 1900.0 pM (Fig. 3A, showed with the black arrow). Up to 1900.0 pM, the emission of tdTomato disappeared from the spectrum (Fig. 3A, the lowest blue line), revealing that the fusion probe was cleaved completely. Oppositely, the change in BRET ratio augmented with the increased concentration of enterokinase (Fig. 3B), linearly in the range from 0.0 pM to 15.0 pM with R^2 of 0.98 (the linear relation: $y = 5.9E-3x + 1.4E-3$) (Fig. 3B, the inset). The detection limit of the sensor calculated from the calibration curve based on 3 times of the standard deviation of the blank BRET ratios reached 2.1 pM.

Enterokinase spiked in serum samples was also measured. The emission at λ_{580} nm decreased along with the increased concentration of enterokinase in the range of 0.0–38.0 pM (Fig. 4A).

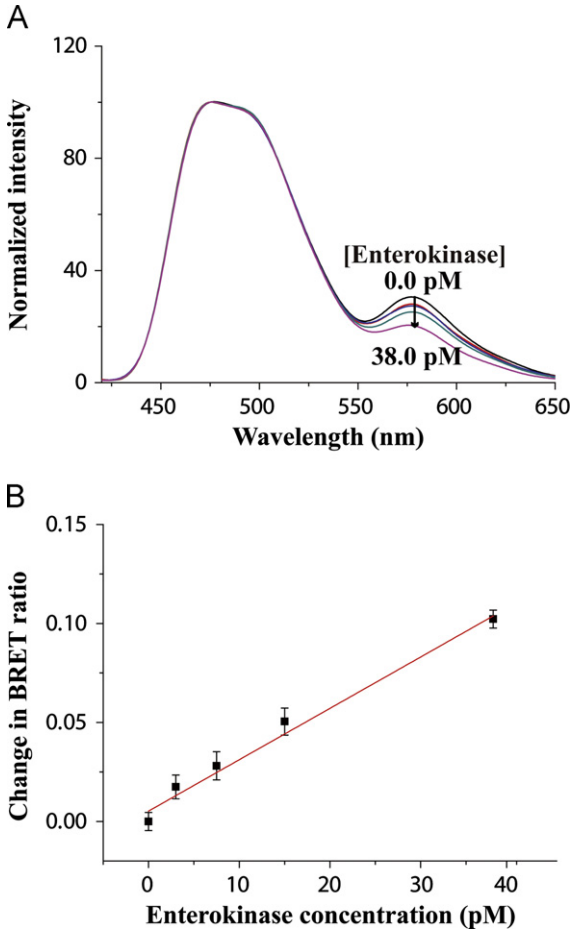

Fig. 4. Detection of enterokinase with hGluc-EK-tdTomato sensor in rabbit serum. (A) Representative emission spectra in the presence of the indicated concentration of enterokinase. All spectra were normalized to the emission at λ_{470} nm. (B) Linear calibration curve of changes in BRET ratio versus enterokinase concentrations fitted by OriginPro 8.0 (means $\pm$ SD, $n = 3$).

Accordingly, the change in BRET ratio increased linearly along with the increased concentration of enterokinase with R^2 of 0.99 (the linear relation: $y = 2.6E-3x + 5.2E-3$) (Fig. 4B). The calculated detection limit in serum was 3.3 pM, close to that in the pure buffer. This suggested that the robust hGluc/tdTomato system would reduce the serum interference effectively, and thus, achieve the similar detection performance for complicated samples as that in the pure buffer.

Compared with the similar sensors based on hGluc/EYFP pair under the same conditions, the enterokinase sensor based on hGluc/tdTomato pair showed a detection limit of 35-fold lower in buffers and 30-fold lower in serum [17]. Obviously, the proposed BRET pair could be used to construct better protease assays than that reported previously. Although only enterokinase was used as a model, a similar performance of the hGluc/tdTomato sensor for other proteases could be expected as long as the proteases did not cleave hGluc and tdTomato non-specifically and the media of target proteases would not suppress the activities of both moieties severely [8,25,29,30].

4. Conclusions

Highly bright tdTomato and hGluc were used as the acceptor and the donor to construct a novel BRET pair. Both hGluc and tdTomato were relatively robust to the environment and the resulting BRET pair showed high tolerance toward the solution components and pH. This tolerance would enhance its applications to complicated biological matrix. Moreover, the pair showed a good spectral resolution, which might contribute greatly to the high sensitivity of the assay. We expect that the pair would find more applications for in vitro or in vivo BRET assays in the future.

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