NOTE

Potential use of Cypridina luciferin for quantifying alpha 1-acid glycoprotein in human serum

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Received: 9 April 2022 / Accepted: 16 September 2022 / Published online: 7 October 2022
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Abstract

Alpha 1-acid glycoprotein (AGP) is an acute phase protein in mammals, including humans. The amount of AGP in human serum varies in response to certain diseases; thus, many efforts have been made to develop methods for quantifying human AGP. We recently discovered that luminescence occurs merely by mixing Cypridina luciferin with human AGP under human serum-free neutral or basic buffer conditions. In this study, we tested an application of Cypridina luciferin for quantifying AGP contained in human serum. Our luminescence spectrum measurements of Cypridina luciferin with human serum samples showed that the maximum emission wavelength with human serum (480 nm) differed from that with human AGP (464 nm) due to the abundant presence of endogenous human serum albumin (HSA). Furthermore, the luminescence intensities of Cypridina luciferin with human AGP in HSA-depleted human serum were consistent with those in a human serum-free basic buffer, but those in human serum were not. These results indicated that depletion of HSA in human serum was required to use Cypridina luciferin for quantifying AGP in human serum. Additionally, we found that the luminescence intensity of Cypridina luciferin with bovine AGP was approximately tenfold lower than that with human AGP.

Keywords Alpha 1-acid glycoprotein · Orosomucoid · Cypridina luciferin · Luminescence · Human serum · Plasma protein

Introduction

Alpha 1-acid glycoprotein (AGP), also called orosomucoid, is a plasma glycoprotein isolated from various mammals [1–8]. Biochemical and pharmacological studies have characterized AGPs in different species, such as humans, cattle, and dogs, and have revealed their multiple biological functions, including their ability to bind drugs [9–13]. AGP is also known as an acute phase protein, and previous quantitative analyses of AGP contained in the plasma or sera of healthy and diseased mammals have revealed that the amount of AGP varies in response to certain diseases [13–15]. Therefore, AGP has attracted attention as a marker to track disease progression and evaluate recovery in humans, companion animals, and farm animals. Many efforts have also been made to develop methods for quantifying human AGP [16–24].

An ideal method for quantifying AGP in clinical examinations would require no special skills, expend minimal time, use no harmful reagents, and exhibit high cost-efficiency. Two promising techniques for clinical examination are chemiluminescence and bioluminescence, which are light-producing phenomena resulting from chemical reactions of unique compounds (e.g., luminol, AMPPD, and luciferin) with chemical substances or enzymes [25]. Chemiluminescence and bioluminescence can be detected with high sensitivity using an instrument that anyone can operate easily, and diverse applications of these phenomena have been reported [26–30]. Common enzyme-linked immunoassay systems using chemiluminescence or bioluminescence can be used to quantify AGP, but these systems are not ideal for clinical examinations, especially because of their time-consuming assay process, which requires the use of multiple reagents.

Recently, we discovered that human AGP causes the luminescence of Cypridina luciferin, a marine natural product that emits light through its reaction with molecular oxygen in the presence of a specific enzyme, Cypridina luciferase (Scheme 1) [31–33]. The luminescence of Cypridina luciferin occurs immediately merely by mixing it with human AGP under human serum-free neutral or basic conditions.
buffer conditions. Moreover, we observed that the luminescence intensity of Cypridina luciferin was proportional to the amount of human AGP under a human serum-free basic buffer condition, at least at 0.4–10 μg mL⁻¹, which seemed to be a reliable quantitative range for detecting the AGP level in a disease state [13]. However, the luminescence of Cypridina luciferin with AGP contained in human serum is still not examined.

In this study, to test an application of Cypridina luciferin for quantifying AGP in human serum, we analyzed the luminescence of mixtures of Cypridina luciferin and human serum samples deliberately spiked with various amounts of human AGP. Additionally, we tested the ability of bovine AGP to cause the luminescence of Cypridina luciferin to examine whether Cypridina luciferin can be used to quantify another mammalian AGP that shows amino acid sequence similarity to human AGP.

**Experimental**

**Materials**

The commercially available materials used in this study were obtained from the following suppliers. Human serum (pooled human serum collected from 5 healthy males and 5 healthy females; lot number BJ16063A) was from Cosmo Bio (Tokyo, Japan). Human AGP (alpha 1-acid glycoprotein from human plasma; lot number SLBJ6840V) and bovine AGP (alpha 1-acid glycoprotein from bovine; lot number 111M7401V) were from Sigma-Aldrich (St. Louis, MO, USA). Human serum albumin (HSA) (albumin, human serum, F-V; lot number M8H7013) was from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA) (albumin from bovine serum, fatty acid/IgG/protease-free; lot number CAQ4454), chlorpromazine hydrochloride, Tris–HCl buffers, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate decahydrate, sodium chloride, glycine, and sodium hydroxide were from FUJIFILM Wako Pure Chemical (Osaka, Japan). Cypridina luciferin (Cypridina luciferin hydrobromide) was from ATTO (Tokyo, Japan).

All materials were used without further purification. The aqueous solution of Cypridina luciferin used in the following experiments was prepared by diluting the concentrated solution with distilled water. The concentration of Cypridina luciferin in the original concentrated solution was determined spectrophotometrically using the reported molar absorption coefficient [34]. Concentrations of human AGP, HSA, bovine AGP, and BSA solutions were determined spectrophotometrically using the corresponding molar extinction coefficient at 280 nm, calculated by the peptide property calculators available at [http://biotools.nubic.northwestern.edu/proteincalc.html](http://biotools.nubic.northwestern.edu/proteincalc.html) or [https://www.biosyn.com/peptidepropertycalculator/peptidepropertycalculator.aspx](https://www.biosyn.com/peptidepropertycalculator/peptidepropertycalculator.aspx).

**Depletion of human serum albumin in human serum**

Ten milliliters of human serum was centrifuged at 12,000×g for 3 min at 4 °C, followed by filtration through a polyvinylidene fluoride (PVDF) filter equipped with a glass fiber filter (0.45 μm; AS ONE, Osaka, Japan) to give approximately 9 mL of filtrate. One milliliter of the filtrate was diluted with 9 mL of 20 mM sodium phosphate (pH 7.0) to give 10 mL of “diluted human serum”. Two milliliters of this diluted serum was applied to an HSA-depletion column (1 mL of HiTrap Blue HP column; Cytiva, Uppsala, Sweden) after equilibration of the column with 20 mM sodium phosphate (pH 7.0) in accordance with the manufacturer’s protocol, using a 10 mL polypropylene syringe (TERUMO, Tokyo, Japan). Approximately 2 mL of the unbound fraction was collected as “HSA-depleted human serum”. Components of human serum that were bounded to the column were eluted with 10 mL of 20 mM sodium phosphate (pH 7.0) containing 2 M NaCl, and all of the elution (approximately 10 mL) was collected as “endogenous HSA”. This procedure is summarized in Supplementary Scheme S1 (Online Resource 1). To exchange solutions of a portion of these obtained human serum samples, 500 μL of each sample was concentrated using an Amicon Ultra-0.5 centrifugal filter device with an NMWL of 10 kDa (Millipore, Billerica, MA, USA), and the resultant concentrates were reconstituted to the original sample volume with 20 mM sodium phosphate conditions.
and 50 μL of a 4000 μg mL⁻¹ solution of HSA in 200 mM of a mixture of 25 μL of 20 mM sodium phosphate (pH 7.0) in a luciferin or distilled water was manually added to 75 μL HSA, or both, 25 μL of a 20 μM aqueous solution of Cypridina luciferin or distilled water was manually added to 50 μL of the “diluted human serum”, the “HSA-depleted human serum”, the “endogenous HSA”, or 20 mM sodium phosphate (pH 7.0) that was twofold diluted with 400 mM Tris–HCl (pH 9.0), in a 0.2 mL micro-tube (0.2 mL thin-walled tube; Thermo Fisher Scientific, Waltham, MA, USA), followed by immediate measurement of the luminescence emission spectrum at room temperature. To measure human serum samples without an exchange of solutions (see the Experimental section “Depletion of human serum albumin in human serum” and Supplementary Scheme S1 in Online Resource 1), 50 μL of a 10 μM aqueous solution of Cypridina luciferin or distilled water was manually added to 50 μL of the “diluted human serum”, the “HSA-depleted human serum”, or 20 mM sodium phosphate (pH 7.0). The concentration and reconstitution were repeated three times, and the resultant human serum samples were used in the following experiments.

Measurement of luminescence emission spectra

The luminescence emission spectra were measured using a LumifLspectrocapture high-sensitivity charge-coupled device (CCD) spectrophotometer (AB-1850C; ATTO) with the following settings: measurement mode, single; measurement time, 5 min; slit width, 0.5 mm; camera gain, high; diffraction grating, 150 lines mm⁻¹; and shutter for measurement, automatic. To measure human serum samples without an exchange of solutions (see the Experimental section “Depletion of human serum albumin in human serum” and Supplementary Scheme S1 in Online Resource 1), 50 μL of a 0.16–50 μg mL⁻¹ solution of HSA in 200 mM Tris–HCl (pH 9.0). The concentration and reconstitution were repeated three times, and the resultant human serum samples were used in the following experiments.

Linear regression analysis of the relation between luminescence intensity of Cypridina luciferin and the amount of human AGP or bovine AGP

In the luminescence intensity measurements for the linear regression analysis of human AGP, 50 μL of a 10 μM aqueous solution of Cypridina luciferin was automatically added to 50 μL of a 0–40 μg mL⁻¹ solution of human AGP in 200 mM Tris–HCl (pH 9.0) in a white 96-well plate using the injector equipped with the multimode microplate reader at 5 s after the start of the measurement. In the luminescence intensity measurements for the linear regression analysis of bovine AGP, 50 μL of a 1.6 μM aqueous solution of Cypridina luciferin was automatically added to 50 μL of a 0.16–50 μg mL⁻¹ solution of human AGP in 100 mM Gly-NaOH (pH 9.5) in a Nunc white 96-well plate using the injector equipped with a luminometer (Phelios AB-2350; ATTO), followed by immediate measurement of luminescence intensity over 60 s at room temperature using the luminometer. The obtained data were subjected to linear regression analysis using Excel (Microsoft 365; Microsoft, Redmond, WA, USA).

Measurement of luminescence intensity from the mixtures of Cypridina luciferin and human serum samples spiked with human AGP

Two-hundred microliters of “diluted human serum” or “HSA-depleted human serum” without an exchange of solution (see the Experimental section “Depletion of human serum albumin in human serum” and Supplementary Scheme S1 in Online Resource 1) was diluted with 800 μL of 200 mM Tris–HCl (pH 9.0). One-hundred microliters of these fivefold diluted samples were mixed with 100 μL of 200 mM Tris–HCl (pH 9.0) containing 0–20 μg mL⁻¹ human AGP, and the luminescence intensity of each prepared sample was measured. In the measurements, 50 μL of a 10 μM aqueous solution of Cypridina luciferin was automatically added to 50 μL of each prepared sample in a white 96-well plate using the injector equipped with the multimode microplate reader at 5 s after the start of the measurement. The obtained data were subjected to linear regression analysis using Microsoft Excel (Microsoft 365).

Measurement of luminescence intensity from the mixtures of Cypridina luciferin and bovine AGP under various pH conditions

Fifty microliters of a 0.2 μM aqueous solution of Cypridina luciferin was automatically added to 50 μL of a 50 μg mL⁻¹
solution of bovine AGP in 100 mM Tris–HCl (pH 7.5, 8.0, 8.5, and 9.0) or Gly-NaOH (pH 9.0, 9.5, 10.0, and 10.5) in Nunc white 96-well plates (Thermo Fisher Scientific) using the injector equipped with a luminometer (Phelios AB-2350; ATTO), followed by immediate measurement of luminescence intensity over 60 s at room temperature using the luminometer.

Results and discussion

Luminescence spectrum of Cypridina luciferin with human AGP contained in human serum

To determine whether human AGP contained in human serum causes the luminescence of Cypridina luciferin, we first measured the luminescence spectrum of the mixture of Cypridina luciferin and human serum under a Tris–HCl (pH 9.0) buffer condition, which is an optimal condition for the induction of Cypridina luciferin luminescence by human AGP [33]. The luminescence spectrum was successfully obtained, but the wavelength at maximum relative intensity (480 nm) differed from that (464 nm) in the luminescence spectrum of the mixture of Cypridina luciferin and human AGP under the human serum-free buffer condition (Fig. 1a, b). Shimomura previously noted that BSA caused significant luminescence of Cypridina luciferin [35], and Campbell’s research group reported that HSA caused luminescence of coelenterazine which, like Cypridina luciferin, is an imidazopyrazinone-type luciferin [36]. Additionally, other research groups reported the effects of albumins on the luminescence intensity of a Cypridina luciferin analog and the signal-to-noise ratio of bioluminescence imaging using coelenterazine [37, 38]. Although the ability of HSA to cause luminescence of Cypridina luciferin was inferior to that of human AGP as shown in our previous study [33], HSA is more abundant (35–50 mg mL⁻¹ [39, 40]) than human AGP (0.4–1.0 mg mL⁻¹ [10, 13]) in human serum. Therefore, we considered the possibility that the luminescence of Cypridina luciferin with endogenous HSA in human serum caused the difference in wavelength at the maximum relative intensity of luminescence spectra (Fig. 1a, b). When we measured the luminescence spectrum of the mixture of Cypridina luciferin and HSA, the wavelength at maximum relative intensity was 474 nm (Supplementary Fig. S1a in Online Resource 1). Furthermore, when Cypridina luciferin was mixed with both human AGP and HSA at a concentration comparable to that in healthy human serum, the luminescence spectrum showed a maximum relative intensity at 473 nm (Fig. 1c). These results suggested that the luminescence of Cypridina luciferin is caused by both endogenous AGP and abundant endogenous HSA in human serum.

To further determine whether the luminescence of Cypridina luciferin is caused by both endogenous HSA and endogenous AGP in human serum, we measured the luminescence spectrum of the mixture of Cypridina luciferin and HSA-depleted human serum (see Supplementary Scheme S1 and Fig. S2 in Online Resource 1) under a Tris–HCl (pH 9.0) buffer condition. We found that the wavelength at maximum relative intensity (457 nm) was similar to that (464 nm) in the luminescence spectrum of the mixture of Cypridina luciferin and human AGP under a human serum-free buffer.

Fig. 1 Luminescence emission spectra of Cypridina luciferin with human serum samples in 100 mM Tris–HCl (pH 9.0). a “diluted human serum,” b authentic human AGP (2 μg, ~48 pmol), c a mixture of human AGP (1 μg, ~24 pmol) and authentic HSA (100 μg, ~15 nmol), d “HSA-depleted human serum.” Black lines in panels (a) to (d), the addition of Cypridina luciferin (0.28 μg, 500 pmol); grey lines, without Cypridina luciferin. Human serum samples are shown in Supplementary Scheme S1 (Online Resource 1)
condition (Fig. 1b, d). Moreover, the luminescence of the mixture of Cypridina luciferin and HSA-depleted human serum was reduced by the addition of chlorpromazine (CPZ) (Supplementary Fig. S3 in Online Resource 1), which binds to human AGP and shows an inhibitory effect on the luminescence of Cypridina luciferin with human AGP [33, 41]. When we measured the luminescence spectrum of the mixture of Cypridina luciferin and the endogenous HSA separated by the HSA-depletion of human serum, we found that the wavelength at maximum relative intensity (473 nm) was similar to that (474 nm) in the luminescence spectrum of the mixture of Cypridina luciferin and HSA (Supplementary Fig. S1a and 1b in Online Resource 1).

Collectively, these results indicated that abundant endogenous HSA in human serum also caused the luminescence of Cypridina luciferin. Therefore, we considered that the luminescence of Cypridina luciferin caused by endogenous HSA overlapped with that caused by endogenous AGP in human serum.

**Luminescence intensity of Cypridina luciferin with human AGP contained in human serum**

To test the application of Cypridina luciferin for quantifying AGP contained in human serum, we measured the luminescence intensities of mixtures of Cypridina luciferin and either human serum or HSA-depleted human serum deliberately spiked with various amounts of human AGP. As the amount of spiked human AGP increased, the total values of luminescence intensity over 60 s increased proportionally under both the condition with human serum and the condition with HSA-depleted human serum (Fig. 2a). However, under the former condition, the values of luminescence intensity were higher and the slope of the calibration curve was more gentle. Additionally, the luminescence intensities of Cypridina luciferin with human AGP spiked to HSA-depleted human serum were consistent with those with human AGP under a human serum-free basic buffer condition (Fig. 2b), whereas those with human AGP spiked to human serum were lower (Fig. 2c). Therefore, the amount of human AGP spiked to human serum was underestimated against a calibration curve of human AGP determined by luminescence measurement using Cypridina luciferin (Fig. 2a and Supplementary Table S1 in Online Resource 1). When we estimated the HSA concentrations in human serum and HSA-depleted human serum as shown in Supplementary Table S2, the estimated concentrations of HSA reasonably explained why the luminescence intensities of Cypridina luciferin under the condition with human serum were higher than those under the condition with HSA-depleted human serum (Fig. 2a and Supplementary Fig. S5). Additionally, based on the result that the estimated concentration of HSA exceeded the concentration of Cypridina luciferin under the condition with human serum, the underestimation of human AGP spiked to human serum may be explained by the competition between human AGP and the abundant endogenous...
HSA for Cypridina luciferin (Supplementary Table S1 in Online Resource 1).

Next, we evaluated the quantitative accuracy of the method using Cypridina luciferin under a condition with human serum and a condition with HSA-depleted human serum, by comparing it with the quantification of human AGP using a commercially available ELISA kit. Under the condition with human serum and the condition with HSA-depleted human serum, the amount of human AGP was over-estimated compared to the result obtained by the ELISA kit (Supplementary Tables S3 and S4 in Online Resource 1). Furthermore, we noticed that the luminescence intensity of Cypridina luciferin with human AGP at a concentration of a few micrograms per milliliter was not considerably different from that with HSA at around a concentration of 10 μg per milliliter. Therefore, when the concentration of human AGP is a few micrograms per milliliter or less, the signal-to-noise ratio of luminescence of Cypridina luciferin with human AGP decreases, accompanied by a decrease in the quantitative accuracy. Possibly for the same reason, as the concentration of spiked human AGP decreased, the quantitative accuracy of the method using Cypridina luciferin decreased (Supplementary Table S1 in Online Resource 1).

Collectively, these results showed that the presence of endogenous HSA in human serum considerably disrupted the process of using Cypridina luciferin to quantify AGP in the serum, and HSA-depletion of human serum was required to improve the quantitative accuracy of the method using Cypridina luciferin. It takes time to deplete HSA from human serum using a conventional prepacked column, as we did in the current study (see Supplementary Scheme S1 in Online Resource 1), but commercially available albumin-depletion reagents and centrifugal HSA-depletion columns can readily and rapidly remove HSA from human serum. As a result, the method using Cypridina luciferin to quantify AGP contained in human serum depleted of endogenous HSA seems promising. Another possible way to avoid the disruption by endogenous HSA is to further dilute human serum more than 200-fold (see the Experimental sections “Depletion of human serum albumin in human serum” and “Measurement of luminescence intensity from the mixtures of Cypridina luciferin and human serum samples spiked with human AGP”). Further dilution could prevent the disruption because the luminescence of Cypridina luciferin with HSA decreases as the concentration of HSA decreases (Supplementary Fig. S4 in Online Resource 1). However, due to the detection limit of human AGP in the method using Cypridina luciferin, such further dilution of human serum will make it impossible to quantify the AGP in the serum (Fig. 2a and Supplementary Fig. S5 in Online Resource 1). Notably, Nishihara et al. reported the successful development of HuLumino1, a coelenterazine analog, which produces light specifically in the presence of HSA [42]. Therefore, a promising alternative way to avoid the disruption by endogenous HSA is the approach that focuses on decreasing the affinity of HSA to Cypridina luciferin and invalidating the ability of HSA to cause luminescence of Cypridina luciferin. In future studies, we expect to develop Cypridina luciferin analogs that specifically react with human AGP to emit light more brightly. The use of such Cypridina luciferin analogs for quantifying AGP contained in human serum would improve the quantitative accuracy and detection limit of AGP compared with the method using Cypridina luciferin shown in the present study. The improvement of the detection limit of AGP would also enable us to quantify urinary AGP, which has attracted attention as a biomarker in human urine and is present in a 1,000-fold lower concentration than serum AGP [43–45].

**Luminescence intensity of Cypridina luciferin with a homologous AGP to human AGP**

Although amino acid sequences of mammalian AGPs differ depending on species, human AGP shows an amino acid sequence similarity to other mammalian AGPs. Therefore, we further examined the potential of Cypridina luciferin to quantify bovine AGP, which shows amino acid sequence identity of 57% to human AGP in a BlastP analysis, as another mammalian AGP. Luminescence measurement of the mixture of Cypridina luciferin and bovine AGP showed that bovine AGP caused luminescence of Cypridina luciferin as human AGP does, and that the luminescence intensity was maximized under basic buffer conditions (Supplementary Fig. S6 in Online Resource 1). However, the total value of the luminescence intensity of Cypridina luciferin with bovine AGP over 60 s was approximately tenfold lower than that with human AGP (Fig. 3). This result suggested that the molecular interaction with Cypridina luciferin differed between bovine AGP and human AGP despite their amino acid sequence similarity.

When we measured the luminescence intensities of different concentrations of Cypridina luciferin with bovine AGP or human AGP, the obtained data tended to follow the Michaelis–Menten equation. Therefore, we calculated the $K_m$ values of bovine AGP and human AGP based on the assumption that both AGPs act as *Cypridina* luciferase for Cypridina luciferin. The calculation showed that the $K_m$ value of bovine AGP was tenfold or higher than that of human AGP (Supplementary Table S5 in Online Resource 1). Therefore, we estimated that human AGP had a higher affinity for Cypridina luciferin than bovine AGP. Supporting this estimation, a previous pharmacological study revealed a difference in the microviscosity of the drug-binding sites between human AGP and bovine AGP [46]. In the additional calculation of the $K_m$ value of HSA, whose ability to cause the luminescence...
Potential use of *Cypridina* luciferin for quantifying alpha 1-acid glycoprotein in human serum

of *Cypridina* luciferin is inferior to that of human AGP, the calculated $K_m$ value of HSA for *Cypridina* luciferin was also higher than that of human AGP (Supplementary Table S5 in Online Resource 1). Based on these results, further investigation of the affinity of bovine AGP to *Cypridina* luciferin is likely required to understand the weaker luminescence of *Cypridina* luciferin with bovine AGP than with human AGP. As shown in Supplementary Fig. S7 (Online Resource 1), the luminescence intensity of *Cypridina* luciferin seemed to be proportional to the bovine AGP concentration, but the weaker luminescence of *Cypridina* luciferin with bovine AGP is a drawback in quantifying bovine AGP in terms of sensitivity. Further biochemical and crystallographic characterizations of bovine AGP and other mammalian AGPs with *Cypridina* luciferin may pave the way for developing *Cypridina* luciferin analogs that can react with the AGPs to produce sufficient light for high-sensitive quantification of the AGPs. Moreover, although AGPs and HSA have no significant amino acid sequence similarity to *Cypridina* luciferase, such investigation could provide clues to solve the longstanding question of how *Cypridina* luciferin causes the luminescence of *Cypridina* luciferin with excellent quantum yield ($\phi_{BL} = \sim 0.30$) [35, 47, 48].

**Conclusions**

In this study, we analyzed the luminescence of *Cypridina* luciferin with human serum samples to test the application of *Cypridina* luciferin for quantifying AGP in human serum. In luminescence measurements of mixtures of *Cypridina* luciferin and either human serum or HSA-depleted human serum deliberately spiked with various amounts of human AGP, the luminescence intensities under the condition with HSA-depleted human serum—but not those under the condition with human serum—were consistent with the intensities determined by luminescence measurement of *Cypridina* luciferin with human AGP under a human serum-free buffer condition. This result indicates that *Cypridina* luciferin is a promising compound for quantifying AGP in HSA-depleted human serum. Additionally, the luminescence measurement of the mixture of *Cypridina* luciferin and bovine AGP showed that bovine AGP caused the luminescence of *Cypridina* luciferin. Although the luminescence intensity of *Cypridina* luciferin with bovine AGP was lower than that with human AGP, the observed luminescence phenomenon suggests the potential of *Cypridina* luciferin to quantify AGPs, which show amino acid sequence similarity to human AGP. Future development of *Cypridina* luciferin analogs could result in methods for quantifying AGP in various mammalian sera or urines via luminescence measurement.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s44211-022-00191-7.

**Acknowledgements** We thank Ms. Mami Komatsu (AIST, Japan) for assistance of experiments. This work was supported by JSPS KAKENHI Grant Number JP19K20205 (to SK), JP18KK0199 (to YM), and a strategic grant for contribution to Hokkaido from AIST (Japan). The authors would like to thank to KN International, Inc. (https://www.kninter.co.jp) and Enago (www.enago.jp) for the English language review.

**Author contributions** SK and YM conceived and designed the study. SK performed the experiments. SK and YM analyzed the data. SK and YM wrote and reviewed the manuscript.

**Data availability** The datasets generated and/or analyzed in the present study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors have no competing interests to declare that are relevant to the content of this article.

**Ethics approval** Human sera used in the present study were prepared in accordance with the appropriate US Food Drug Administration (FDA) regulations and with the ethical standards of Cosmo Bio Corporation.
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