論文

Detection of protein by the use of photoluminescence in rare earth-protein-SDBS system

希土類元素—タンパク質—SDBS系の蛍光特性を利用したタンパク質の検出

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概要：希土類元素—タンパク質—ドデシルベンゼンスルホン酸ナトリウム（SDBS）系における蛍光増感効果を調べた。Tb^{3+}-SDBS-タンパク質系の蛍光は大変弱い。しかしながら、この系にGd^{3+}やエタノールを加えると、蛍光が著しく増加した。コラーゲンの場合には、この効果が最大であった。この結果をもとに、タンパク質をナノグラム/mlレベルで感度良く検出する方法を確立した。

Abstract : The fluorescence enhancement effect in rare earth-protein-sodium dodecyl benzenesulfonate (SDBS) system has been studied. The photoluminescence of Tb^{3+}-SDBS-protein system was very weak. However, when Gd^{3+}or ethanol was added into this system, the fluorescence was significantly enhanced. In the case of collagen, this effect was greatest. Based on this, a method was established to determine proteins sensitively at nanogram/ml level.

キーワード：蛍光、希土類元素イオン、タンパク質の検出
Keywords : photoluminescence, rare earth ion, detection of protein

1. Introduction

The fluorescence probe technique using rare earth ions was applied for characterization of many inorganic compounds, the availability in the molecular recognition and chirality sensing of biological substances[1, 2]. Recently, there are many works reported focusing on the detection of proteins[3], because of its important role in the life science. As the fluorescence emitted from the native protein is weak, the new fluorescence methods are necessary to analyze the protein. Thus, a fluorescence enhancement effect in the rare earth-protein system has been studied by Sun et al.[4]. In this paper, we present a new co-luminescence system to detect proteins at nanogram level.

2. Experimental

Protein (BSA, elastin and collagen), $LnCl_3$ $6H_2O$ (Ln= Tb^{3+}, Gd^{3+}, Ce^{3+}, Eu^{3+}, Ho^{3+}) and sodium dodecyl benzene sulfonate (SDBS) were purchased from Wako Pure Chem. Ind., Ltd. The synthetic peptides was synthesized by the reference to the amino acid sequence of hemoglobin. The sequence of amino acid of the peptides shows as follows; Leu-Met-Val-Ser-Thr-Leu-Trp-Arg-Ser-Ile-Arg-Arg. A 5.00 mol/L, Tris-HCl buffer is prepared by dissolving 0.606g of Tris in 100ml deionized water and adjusting the pH to 7.0 with HCl, Tris-HCl, SDBS, Ln^{3+}, protein solutions and additive are added in a 10ml test tube. The mixture is diluted to 7.74ml with water and shaken. The emission spectra were measured with a HITACHI recording absolute spectrofluorophotometer (F-4500) at room temperature. The ultraviolet absorption spectra were recorded on a JASCO V-550 UV spectrophotometer. the circular dichroism(CD) spectra were measured with a JASCO J720 WL spectrophotometer.

3. Results and discussion

3.1 Fluorescence spectroscopy

The emission spectra under excitation at 290 nm of Tb-SDBS (1), Tb-collagen (2), Tb-SDBS-collagen (3), and Tb-Gd-SDBS-collagen(4) systems have shown in Fig. 1. The emission peak at 545 nm is attributed to the $^5D_4$-$^7F_5$ transition of Tb^{3+}. Although the intensity of Tb^{3+}-SDBS-collagen is stronger than that of Tb-collagen system and Tb^{3+}-SDBS system, its intensity is weak. However, the fluorescence intensity of the system is greatly enhanced by Gd^{3+}. The optimum conditions for the determination of protein are as follows; 1.0 x 10^{-6} g/ml collagen, 5.0 x 10^{-3} M Tris-HCl, 1.0 x 10^{-4}M SDBS, 1.0 x 10^{-5} M Tb^{3+}, 1.5 x 10^{-4} M

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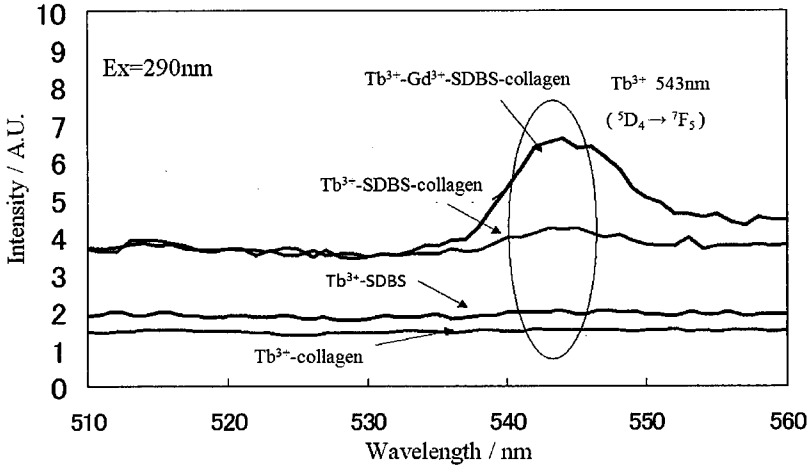

Fig. 1 Fluorescence spectra. Conditions; Tb^{3+}: 1.0 x 10^{-5} mol/l; Gd^{3+}: 1.5 x 10^{-4} mol/l; SDBS: 1.0 x 10^{-4} mol/l, collagen: 1.0 x 10^{-6} g/ml.

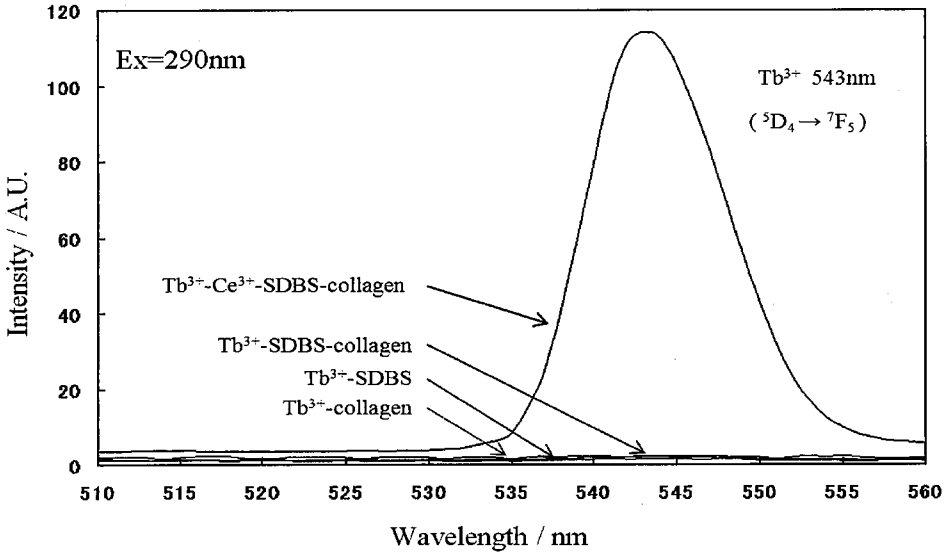

Fig. 2 Fluorescence spectra. Conditions; Tb^{3+}:1.0 x 10^{-5} mol/l; Ce^{3+}: 1.5 x 10^{-4} mol/l; SDBS: 1.0 x 10^{-4} mol/l, collagen:1.0 x 10^{-6} g/ml.

Gd^{3+}. The effect of other rare earth ions was tested. The fluorescence intensity of Tb^{3+}-Eu^{3+} and Tb^{3+}-Ho^{3+} -SDBS-collagen is the same as that of Tb^{3+}-Gd^{3+}-SDBS-collagen. However , when Gd^{3+} ions changed to Ce^{3+} ions, the fluorescence enhancement effect extremely increased as shown in Fig.2. Although the optimum excitation wavelength in the Ce^{3+}-SDBS-collagen system is 255nm, the fluorescence of Ce^{3+} ions(peak at 350nm) under 290nm excitation was slightly observed. Since the excitation wavelength of the Tb^{3+} ions(peak at ca.350nm) was consistent with the fluorescence of Ce^{3+} ions. Moreover, the interference of some ions was tested and shown in Table 1.

Table 1 Interference from forein substance

Foreign substance	concentration(mol/l)	change of Ir*
NH_4^+, SO_4^{2-}	1.0 x 10^{-4}	+0.25
Mg^{2+}, OH^-(p H=8.0)	1.0 x 10^{-4}	-0.5
Acetic acid	1.0 x 10^{-4}	-1.0
Citric acid	1.0 x 10^{-4}	-1.0
Ethanol	1.0 x 10^{-5}	-0.12
	1.0 x 10^{-4}	+0.6
	1.0 x 10^{-3}	-0.45

* The emission intensity of Tb^{3+}-SDBS-collagen system is 1

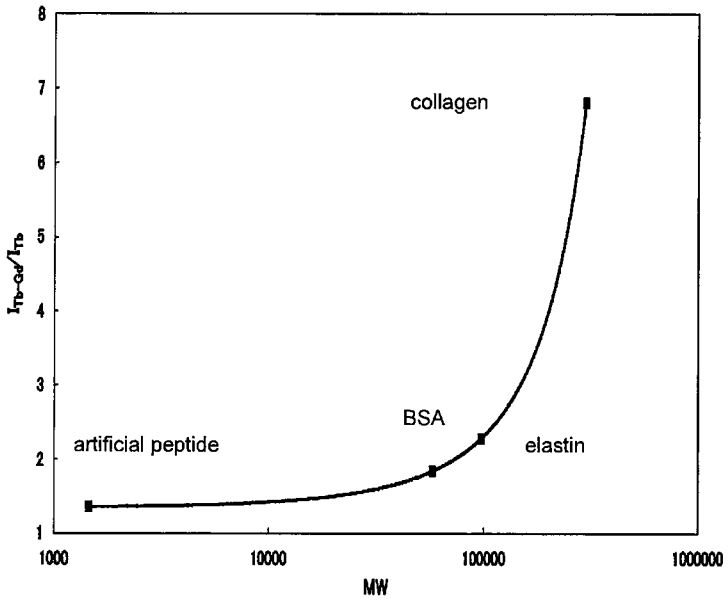

Fig. 3 Dependence of the relative emission intensity of Tb^{3+} on the molecular weight of protein. The emission intensity for Tb^{3+}-SDBS-Gd^{3+}-protein system is 1.

Sun et al.[4] was reported that the addition of some amino acid(L-Ala, L-His, L-Arg., etc) resulted in the enhancement effect. But, when the acid was introduced the system, the fluorescence of Tb^{3+} ions almost disappeared. In the case of ethanol which is one of the denaturant of protein, the enhancement effect was observed at optimum concentration (ca. 1.0 x 10^{-4} mol/l). There is the difference in the enhancement effect between four proteins, as shown in Fig.3. This effect increased with the molecular weight of protein and was greatest for collagen.There is linear relationship between the fluorescence intensity of these system and the concentration of proteins. The limit of detection for collagen was shown in Table2.

3.2 The interaction between protein, SDBS and rare earth ions

From the measurement of UV spectra, it was observed that the peak of BSA at 200 nm decreased and moved to longer wavelength (ca.215nm) when SDBS was added in succession. Since the peak at 200 nm can reflect the framework conformation of the protein[5], the change of UV spectra after addition of SDBS indicates that the binding between SDBS and protein molecules would lead to substantial changes of protein conformation. Although both BSA and SDBS are negatively charged in the solution of pH 7.0, there will be a variety of physicochemical interaction. Previous studies[6] shows that the one of interaction is to be the results of aromatic ring stacking between SDBS and residues of tryptophan, tyrosine or phenylalanine residues in BSA. Tb^{3+} could bind with the negatively charged amino acid residue in protein and form the BSA-Tb^{3+} associate. That is, it is clear that the number of amino acid residue is dependent on the kind of the proteins.

Under the experimental conditions, Tb^{3+}-BSA-SDBS solution remained transparent. However, when Gd^{3+} is added into the system, it was found that the solution became cloudy like diluted milk. This means that the larger complex particles are formed. Tb^{3+}or Gd^{3+} could bind with the

Table 2 The limit of detection for collagen

System	linear range(g/ml)	Limit of detection
Tb^{3+}-Gd^{3+}-SDBS	1.0 x 10^{-6} — 5.0 x 10^{-5}	140 ng/ml
Tb^{3+}-Ce^{3+}-SDBS	5.0 x 10^{-7} — 8.0 x 10^{-5}	25 ng/ml
Tb^{3+}-Gd^{3+}-SDBS-ethanol	1.0 x 10^{-6} — 5.0 x 10^{-5}	6.6 μ g/ml

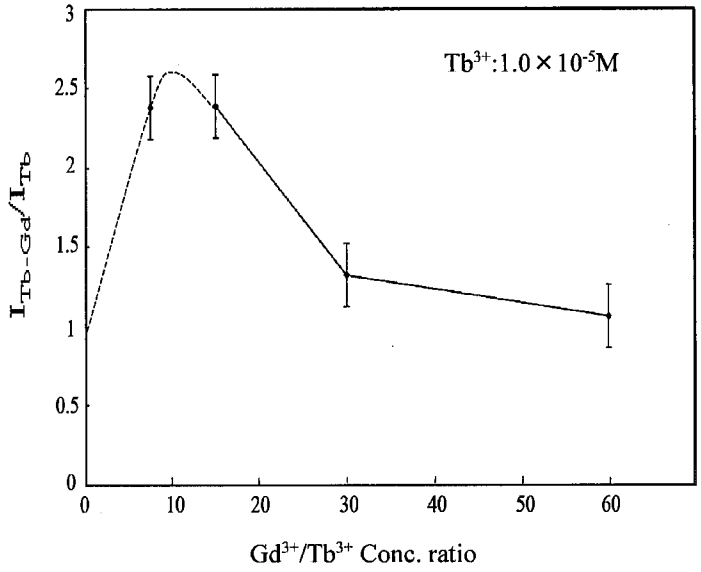

Fig. 4 Dependence of the relative intensity of Tb^{3+} for Tb^{3+}-SDBS-Gd^{3+}-BSA system on the concentration of Tb^{3+} is 1.0 x 10^{-5} mol/l.

negatively charged amino acid residues in protein and form the complex with BSA-SDBS as described in elsewhere[4]. Figure 4 shows the Gd^{3+} concentration dependence of the fluorescence enhancement effect. The Gd^{3+} concentrations are given by these relative values to a definite Tb^{3+} concentartion (1.0 x 10^{-5} M). The enhancement effect was maximum at ca. Gd^{3+}/ Tb^{3+}=10 and decreased with the increase of Gd^{3+} concentration. As the concentration of Gd^{3+} was higher than that of $Tb^{3+}$and the self-aggregation of SDBS shortened the distance between these two ternary complexes, we hypothesize that there are large numbers of Gd^{3+} complexes around Tb^{3+}complexes. However, the fluorescence intensity of Tb^{3+} decreased with the concentration of Gd^{3+} beyond Gd^{3+}/ Tb^{3+}=10. Since the characteristic emission of in the GD-SDBS-BSA system was not observed under the experimental condition, the excess Gd-SDBS-BSA complex could inhibit the efficiency energy transfer of Gd-Tb-SDBS-BSA complex, as described above.

When BSA or SDBS was excited at 290 nm, the emission peak of BSA and SDBS was observed at ca. 340 nm. Next, when Tb^{3+} was added into BSA or SDBS, the fluorescence of 340 nm decreased. On the other hand, when Tb^{3+} was added to the binary system of BSA-TBS, the fluorescence peak of Tb^{3+} was observed, as shown in Fig.1. In the binary complexes of BSA-Tb^{3+} and SDBS-Tb^{3+}, the energy transfer efficiency is low and, in ternary complex of Tb^{3+}-SDBS-BSA, the energy transfer between any two substances is more efficient than that in the relevant binary complex. So, Tb^{3+} would combine with SDBS and then Tb^{3+} would be bound to BSA through SDBS.

Many proteins are labile and readily modified by alternation in pH, by heating in aqueous solution, etc. The fluorescence was first observed with Tb-SDBS-protein system, as shown in Fig.1. This would be the result of denatured conformation of protein that makes more tryptophan residues expose on the surface of increase the binding ratio Tb^{3+} and proteins. That is, the polarity of the microenvironment around the protein is changed and the addition of SDBS could stabilize the unfolded structure of protein. The CD spectra was obtained, in order to clarify the denaturation of proteins by adding SDBS and ethanol. As shown in Fig.5, the BSA-SDBS and BSA- ethanol; system show a weakening CD band in the range of 210-240 nm. CD spectroscopy is remarkably sensitive to the secondary structure (α-helix, β-sheet, etc.) of protein[7]. However, it is difficult to decide the structure of protein after denaturation because analyses of these CD spectra for the secondary structure of protein has been unreliable. That is, it is considered that the optimum energy transfer in Tb^{3+}-Ln^{3+}-SDBS-protein system is occurred with the denaturation of protein. As the fluorescence emitted from the native protein is very weak, the fluorescence methods for the detection of proteins, it is necessary to enhance the fluorescence intensity in the system including proteins.

It can be seen that the addition of rare earth ions, optimum

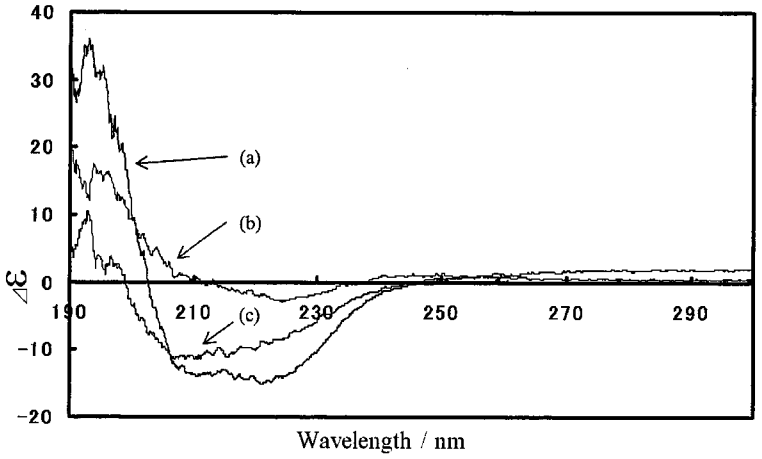

Fig. 5 CD spectra for (a) BSA(1.0 x 10-5 mol/l), (b) BSA-SDBS(1.0 x 10-4 mol/l), (c) BSA-ethanol(1.0 x10-4 mol/l).

surface-active agents and denaturants is one of good method.

Conclusions

In this work, it was found that Tb^{3+} could combine with protein and SDBS, and emitted the characteristic fluorescence of Tb^{3+}. When Gd^{3+} or ethanol was added into the above system, the fluorescence is significantly enhanced. These phenomena would be correlated with the result of denatured conformation protein and be applied to the detection of protein at nanogram level.

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