Selective Detection of Mercury Ion by a Fluorescent Sensor Based on Dipeptide Containing Cysteine

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Two fluorescent peptidyl chemosensors containing cysteine and a dansyl group (DNS-γGlu-Cys, DNS-Cys-Glu) were prepared via Boc solution phase peptide synthesis for monitoring heavy metal ions on the basis of phytochelatin peptide sequence. DNS-Cys-Glu displayed a selective fluorescence turn-off response to Hg^{2+} over other heavy metal ions. In contrast, the addition of various metal ions to DNS-γGlu-Cys resulted in no significant fluorescent changes. For DNS-Cys-Glu, because the thiol group of Cys is adjacent to the sulfonamide group, they can bind to Hg^{2+} by cooperative electrostatic interactions. As a result, the quenching of the fluorescence intensity can be explained in terms of effective electron transfer from the excited dansyl fluorophore to the bound Hg^{2+}. DNS-Cys-Glu could be applicable to homogeneous Hg^{2+} detection.

Keywords : mercury ion, dipeptide, fluorescence, phytochelatin, cysteine

1. Introduction

In recent years, the developments of selective and sensitive fluorescent chemosensors for heavy and transition metal ions represent a challenge that continues to attract considerable attention because of their potential use in medicinal, environmental, and biological fields [1, 2]. In particular, mercury ion (Hg^{2+}), is one of the most dangerous and widespread global metal pollution ions, which represents a major toxicity to the environment and human body even in low concentrations [3, 4]. Since mercury has recognized accumulative and persistent characters and can easily penetrate into the skin and cell membranes, it can be accumulated in the body and cause human health problems such as irreversible damages to DNA and the central nervous system, and mitosis impairment [5, 6]. Therefore, several techniques have been developed for Hg^{2+} detection and quantification based on spectrophotometry [7], electrochemical [8], atomic absorption spectrometry [9], and fluorescence [10, 11] methods. Among them, fluorescence analysis has great importance due to its high sensitivity, selectivity, versatility, rapid response time and simple handling.

Phytochelatins are small cysteine-rich peptides, synthesized by higher plants, algae and some fungi, have capability to detoxify heavy metal ions by forming ion-phytochelatin complexes [12]. Phytochelatins have a primary structure which consists of repeating sequences of the γ-glutamyl-cysteinyl dipeptide and a carboxy terminal glycine (PCn, γGlu-Cys)n-Gly). The repeating value n can range from 2 to 11, but it is generally in the range from 2 to 6 [12, 14]. It has been demonstrated in vitro that phytochelatins were bound with heavy metal ions such as Hg^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} [15-17]. It is inferred that the thiol group of Cys plays an important role in the complexation of phytochelatin and heavy metal ions. It is expected that phytochelatin analogue peptides have capability to be a candidate for detection of heavy metals.

Fluorescent peptide sensors have also been designed successfully for the detection of various metal ions [18-20]. Because peptides do not usually have fluorescent properties to be essential for sensing, a fluorophore group needs to be
conjugated to them for developing fluorescent peptide sensors. Peptide sensors have the following advantages. Peptides consisting of natural amino acid can be readily designed and synthesized in conventional solid or solution phase synthesis. The sensitivity and selectivity of peptide probes can be optimized by further tuning of the amino acid sequences. Hydrophilic amino acids have been introduced into the structural design in order to improve the water-solubility. Peptide sensors are generally working well in aqueous solution or mixed organic-aqueous solution due to their good solubility.

In the present study, we have attempted to synthesize the two short peptides with a dansyl group (DNS) as a fluorescent probe which has similar structure to phytochelatin as shown in Scheme 1 and to evaluate their recognition properties to various metal ions by fluorescence spectrometry. DNS was chosen as a fluorophore because it is sensitive to the microenvironment and its sulfonamide group could interact with metal ions [21]. DNS-γGlu-Cys was designed as a PC analogue, in contrast, DNS-Cys-Glu was designed in order to construct an effective fluorescent heavy metal sensor by shortening the distance between DNS and Cys.

2. Experimental
2.1 Materials
All chemicals were obtained from commercial suppliers and used without further purification. N-α-Boc-L-glutamic acid α-benzyl ester (Boc-Glu-OBzl, where Boc and Bzl are tert-butoxycarbonyl and benzyl, respectively) and N-α-Boc-S-p-methoxybenzyl-L-cysteine (Boc-Cys(4-MeOBzl)-OH, where 4-MeOBzl is 4-methoxybenzyl) were purchased from Bachem AG and Peptide Institute, Inc., respectively. L-Glutamic acid α, γ-dibenzyl ester tosylate (Tos·Glu(OBzl)-OBzl) was obtained from Novabiochem. Trifluoroacetic acid (TFA), dicyclohexylcarbodiimide (DCC), dancyll chloride (DNS-Cl), methanesulfonic acid (MSA), N-hydroxybenzotriazole (HOBT), and N-hydroxysuccinimide (HOSu) were purchased Tokyo Chemical Industries Co., Ltd.

2.2 Measurements
NMR spectra were obtained at 400 MHz on a JEOL JNM-EX400 with TMS as internal standard at room temperature. Fluorescence spectra were recorded on a JASCO FP-750 spectrofluorometer. All fluorescence measurements were carried out in DMSO:water (1:99, v:v) containing 5 mM TrisHCl buffer (pH 7.0). Fluorescence spectra of the peptide sensors in the presence of various metal cations (Ag+, Cd2+, Co2+, Fe2+, Hg2+, Mg2+, Ni2+, Pb2+, and Zn2+ as chloride anion) were measured by excitation with 340 nm.

2.3 Synthesis
2.3.1 DNS-γGlu-Cys
To Boc-Cys(4-MeOBzl)-OH (1.71 g, 5.00 mmol) was added TFA (5 mL) and the mixture was stirred at room temperature for 1 h and evaporated in vacuo to give an oily product. To the oily product and NaHCO3 (0.84 g, 10.0 mmol) dissolved in 50 mL of water was added HOSu ester of Boc-Glu-OBzl (2.25 g, 5.00 mmol) in 100 mL of THF [22-24]. After 2 h of stirring at room temperature, the solution was concentrated, acidified with 0.5 N HCl, and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered, concentrated, and washed with hexane to give a white precipitate. To the precipitate deprotected with TFA (5 mL) was added DNS-Cl (2.95 g, 10.8 mmol) and triethylamine (2.29 mL, 15.0 mmol) in 300 mL of acetone. After 20 h of stirring at 5°C, the solvent was removed reduced pressure. The crude product was purified by column chromatography on silica gel column with CHCl3:acetone (1:1, v:v) as eluent. The purified product was treated with MSA (15 mL) containing anisole (1.5 mL) at room temperature for 1 h and the mixture was triturated with diethyl ether to give 1.70 g (75.7%) of DNS-γGlu-Cys. 1H NMR (DMSO-d6, ppm): δ1.70-1.89 (m, 2H), 2.08-2.17 (t, 2H), 2.68-2.72 (m, 2H), 3.01-3.19 (s, 6H), 3.73-3.80 (t, 1H), 4.29-4.35 (t, 1H), 7.60-7.78 (m, 3H), 8.21-8.59 (m, 3H).
2.3.2 DNS-Cys-Glu

To Boc-Cys(4-MeOBzl)-OH (3.42 g, 10.0 mmol), Tos·Glu(OBzl)-OBzl (4.98 g, 10.0 mmol), triethylamine (1.53 mL, 10.0 mmol), and HOBt (1.61 g, 12.0 mmol) dissolved in 100 mL of dichloromethane, was added DCC (2.47 g, 12.0 mmol) and the mixture was stirred at room temperature for 48 h. The white precipitate was filtered off and the filtrate was washed with 0.1 N NaHCO₃, 0.1 N HCl, and water. The organic layer was dried over anhydrous sodium sulfate, filtered, concentrated, and dried in vacuo to give a white precipitate. The precipitate was treated with TFA (10 mL) at room temperature for 1 h in order to deprotect the Boc group and the reaction mixture was evaporated in vacuo to give an oily product. To the oily product was added DNS-Cl (3.85 g, 13.9 mmol) and triethylamine (2.58 mL, 18.5 mmol) in 500 mL of acetone. After 20 h of stirring at 5°C, the solvent was removed reduced pressure. The crude product was purified by column chromatography on silica gel column with ethyl acetate : hexane (1:2, v:v) as eluent. The purified product was treated with MSA (6 mL) containing anisole (0.6 mL) at room temperature for 1 h and the mixture was triturated with diethyl ether to give 0.66 g (14.6%) of DNS-Cys-Glu. 1H NMR (D₂O, ppm): δ 1.73-1.83 (t, 2H), 2.31-2.50 (t, 2H), 2.52-2.60 (m, 2H), 3.32-3.48 (s, 6H), 3.78-3.88 (t, 1H), 3.90-3.95 (t, 1H), 7.72-7.97 (m, 3H), 8.24-8.67 (m, 3H).

3. Results and discussion

Two dipeptides were prepared by the conventional active ester method in solution phase peptide synthesis, adding Boc amino acid N-hydroxysuccinimide ester to an amino acid with a free amino group and a protected side chain. DCC has been used to synthesize N-hydroxysuccinimide active esters, which are colorless derivatives with good stability. After Boc group was selectively removed by TFA, the dancyl group was coupled with a free amino group of the dipeptides with protected side chains. The protecting groups of the dipeptides bearing a dansyl group were eliminated with MSA in the presence of anisole in order to synthesize the fluorescent peptide sensors (DNS-γGlu-Cys, DNS-Cys-Glu).

Because the peptide sensors have poor solubility in water, their stock solutions for fluorescence measurements were prepared in a small amount of DMSO. The all fluorescence measurements were carried out in DMSO-H₂O (1:99, v/v) containing 5 mM Tris-HCl pH 7.0 buffer.

The fluorescence response of DNS-Cys-Glu in the presence of each metal ion (Ag⁺, Cd²⁺, Co²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Ni²⁺, Pb²⁺, Zn²⁺) was observed in order to investigate its selectivity for metal ions. Figure 1 shows fluorescent spectra of DNS-Cys-Glu upon the addition of 10 folds equivalent of each metal ion relative to DNS-Cys-Glu. Apparently, a significant fluorescence quenching of DNS-Cys-Glu was observed only when Hg²⁺ was added. On the other hand, the fluorescence response of DNS-Cys-Glu promoted only a small change in the presence of other metal ions, even at high concentrations as 60 μM. Only Ag⁺ showed slight fluorescence enhancement. These results indicated a high selectivity of DNS-Cys-Glu toward to Hg²⁺ over other metal ions, in particular, other soft acid metal ions such as Cu²⁺, Pb²⁺, Ag⁺, which are potential competitors and provided some limitations in many papers [25-29].

In order to elucidate the sensitivity for DNS-Cys-Glu toward Hg²⁺, the changes fluorescence spectra of DNS-Cys-Glu were measured when titrated with Hg²⁺. As shown in Figure 2(a), the fluorescence intensity decreased gradually with increasing the concentration of Hg²⁺. This result demonstrated that DNS-Cys-Glu exhibited a turn-off fluorescent response for Hg²⁺. The maximum fluorescence wavelength was slightly shifted from 553 to 525 nm. This blue shift could be interpreted as an indication of moving the dansyl fluorophore to a less polar environment upon metal ion binding.
Figure 2. (a) Fluorescent titration spectra of DNS-Cys-Glu (6 µM) in DMSO-H₂O (1:99, v/v) containing 5 mM Tris-HCl pH 7.0 buffer in the presence of Hg²⁺ at different concentrations, [Hg²⁺] = 0, 6, 60, 600, 3000, and 6000 µM upon excitation at 340 nm.

(b) Normalized fluorescence intensity of DNS-Cys-Glu (6 µM) as a function of Hg²⁺ concentration. I₀ and I denote the fluorescence intensity at 553 nm in the absence and in the presence of Hg²⁺, respectively.

Figure 2(b) shows the plot of the fluorescence intensity as a function of the concentration of Hg²⁺ in order to determine the detection limit of DNS-Cys-Glu. Fluorescence response is linearly proportional to the concentration of Hg²⁺ in the range of 6 to 6000 µM. The value of linearly dependent coefficient (R²) is found to be 0.987. It was found that the minimum detection limit of DNS-Cys-Glu was approximately 1 µM for Hg²⁺ from Figure 2(b).

Figure 3 shows the fluorescence intensity of DNS-γ-Glu-Cys and DNS-Cys-Glu at 553 nm upon addition of various metal ions in order to compare the binding affinity of two peptide sensors. DNS-Cys-Glu showed high selectivity for Hg²⁺. In contrast, Ag⁺, Cd²⁺, Co²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Ni²⁺, Pb²⁺, and Zn²⁺ did not almost give rise to significant changes in the fluorescent intensity of DNS-γ-Glu-Cys even at high concentration as much as 60 µM.

It is well known that Hg²⁺ can offer a strong and favorable electrostatic interaction with sulfur and nitrogen atoms which can act as soft binding sites for metal cations [30, 31]. For DNS-Cys-Glu, the thiol group of Cys and the sulfonamide group adjacent to dansyl group can bind to Hg²⁺ by electrostatic interactions. The quenching of the fluorescence intensity can be explained in terms of effective electron transfer from the excited dansyl fluorophore to the bound Hg²⁺ with DNS-Cys-Glu [32, 33]. On the other hand, DNS-γ-Glu-Cys may be incapable of binding these metal ions because of longer distance between the thiol group of Cys and the sulfonamide group. These results indicated that the peptide sensors need to have suitable distance and conformation between the thiol group of Cys and the sulfonamide group for binding and recognizing Hg²⁺.

4. Conclusion

In conclusion, two short fluorescent peptide sensors containing cysteine and DNS were synthesized by Boc solution phase peptide synthesis on the basis of the amino acid sequence of phytochelatin. DNS-Cys-Glu successfully exhibited a fluorescent turn-off response for Hg²⁺ in aqueous solution with a low detection limit. The peptide sensor could be a potentially useful for detecting Hg²⁺ in aqueous solution.
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