Development of Fluorescent Phycocyanin-Cu^{2+} Chemosensor for Detection of Homocysteine

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Abstract: A simple optical chemosensor from phycocyanin (L1), a protein-pigment complex extracted from cyanobacterium *Spirulina platensis*, has been developed. The L1 shows high selectivity toward Cu^{2+} by exhibiting spectral shift and quenching of fluorescence intensity. This L1-Cu^{2+} complex, so called ensemble probe, can be used to detect mercapto biomolecules, and has superior affinity toward homocysteine (Hcy). Upon binding to mercapto biomolecules, the color change from colorless to blue and the fluorescence emission change from colorless to pink were observed. This chemosensor could detect Hcy at concentration as low as 4.71 μM (S/N = 3) with a linear range of 10-100 μM (R^2 = 0.9903). This sensing approach may have found applications in clinical diagnostics.

Keywords: Phycocyanin, copper (II) ion, mercapto biomolecules, homocysteine, turn-on fluorescence.

1. Introduction

Mercapto biomolecules, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play essential roles in physiological processes [1]. For example, Cys is a precursor amino acid of GSH, Coenzyme A and taurine, while Hcy is critical in sulfur metabolism and methionine synthesis [2]. GSH, on the other hand, serves many cellular functions, including maintenance of intracellular redox activities, xenobiotic metabolism, and gene regulation [3]. Elevated levels of mercapto biomolecules have been linked to many diseases such as cancers and cardiovascular disease [4], [5]. However, a deficiency of mercapto biomolecules, can lead to liver damage, muscle and fat loss, and skin lesions [6], [7]. Therefore, the detection of mercapto biomolecules in biological samples is crucially important.

Several methods have been used to detect mercapto biomolecules such as spectroscopy [8], liquid chromatography [9], flow-injection [10], and electrochemical method [11], [12]. Nevertheless, all of these methods are time-consuming and difficult for on-site analyses. Moreover, they require pretreatment procedures, and use costly instruments. Among various strategies, optical methods based on colorimetric and fluorescent probes have attracted much attention due to their simplicity, high selectivity and sensitivity, low cost, and real-time monitoring [13], [14]. As the fluorometric method can reach a very low detection limit, considerable affords have been devoted to develop fluorescent probe for mercapto biomolecules in recent years [15]-[20]. However, owing to the similarity in structure and reactivity of Cys, Hcy, and GSH,

only few fluorescent sensors with ability to discriminate these biomolecules have been reported so far [21]-[25].

Phycocyanin (L1) is a pigment-protein complex that captures light in cyanobacteria, rhodophytes, cryptophytes and glauphytes [26], [27]. L1 is a water-soluble protein that composes of tetrapyrole unit that functions as a chromophore. From the excellent photophysical properties such as long absorption and emission wavelength, high fluorescence quantum yield, large extinction coefficient, and high photostability, this L1 can be used as a fluorescent probe [28]. Herein, we developed a simple chemosensor for mercapto biomolecules detection using an L1-Cu^{2+} ensemble probe. The Cu^{2+} induces a fluorescent turn-off mechanism of L1 upon complexing with L1. However, the turn-on fluorescent response could be restored in the presence of mercapto biomolecules, as shown in Fig. 1. This L1-Cu^{2+} ensemble probe shows high selectivity toward Hcy over other amino acids and mercapto biomolecules. We speculate that the water solubility of L1 and metal binding affinity between Cu^{2+} and Hcy can effectively enhance the selectivity of the probe to Hcy, in comparison with Cys and GSH.

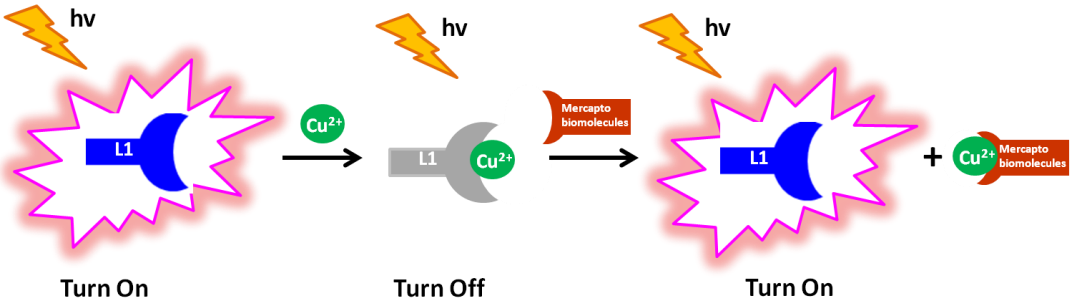

Fig. 1. Schematic drawing of the turn-on chemosensor for mercapto biomolecules detection based on L1-Cu^{2+} ensemble probe.

2. Experimental Section

2.1. Instrumentation

Absorption and fluorescence spectra were recorded on a Tecan microplate reader. The pH of the solution was measured with a Mettler Toledo pH-meter. All measurements were operated at a room temperature (25 °C).

2.2. Materials and Methods

2.2.1. Chemicals

All chemicals were analytical grade and used without further purification. Milli Q water used throughout the experiment was purified through a Millipore system with a resistance of 18 $M\Omega cm^{-1}$. Solution of Cu^{2+} was prepared from its chloride salt. All amino acids and mercapto biomolecules were purchased from Sigma-Aldrich Chemical.

2.2.2. Preparation of L1 from *S. platensis*

L1 was obtained from the cyanobacterium *Spirulina platensis (S. platensis)*, a culture collection maintained at the School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok, Thailand. Extraction, separation and purification of L1 were carried out using procedures reported by Chaiklahan *et al*. [29]. Briefly, extraction was done by using 100 nM phosphate buffers (pH 7.0) at a ratio of 1:100 (w/v) with continuous stirring at 300 rpm at room temperature for 4 h.

The sample was then centrifuged at 4,800 x g for 15 min to remove the cell residue. The crude extract was firstly filtered through a 5 µm membrane at flow rate of 150 $mLmin^{-1}$ and then through a 0.8/0.2 µm membrane at 100 $mLmin^{-1}$. The phycocyanin filtrate was then filterd again through a membrane with a molecular weight cut-off of 50 kDa at 69 kPa and 75 $mLmin^{-1}$. Food-grade phycocyanin powder was obtained after lyophilization . The structure of obtained phycocyanin was shown in Fig. 2.

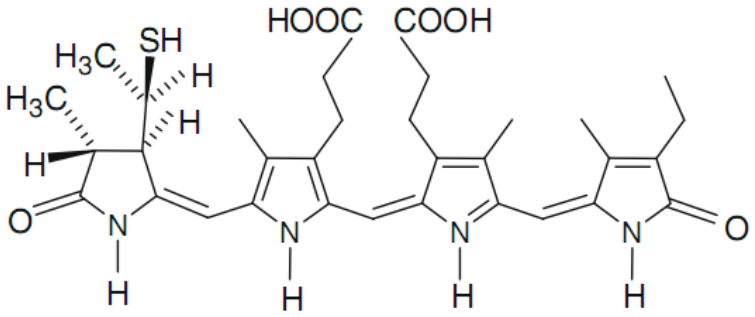

Fig. 2. Structure of phycocyanin.

2.2.3. Preparation of stock solution

Milli Q water was used to prepare metal salt solution of Cu^{2+} (10 mM) and solutions of various amino acids and mercapto biomolecules (10 mM). L1 solution (0.5 gL^{-1}) was prepared in 10 mM HEPES pH 7.0.

2.2.4. Fluorescence measurement of L1-Cu^{2+} with amino acids and mercapto biomolecules

One milliliter of L1 solution (0.5 gL^{-1}) in 10 mM HEPES buffer (pH 7.0) was pipetted into a 2 mL small vial, followed by the addition of 5 µL of 10 mM Cu^{2+} solution in Milli Q water. The L1-Cu^{2+} solution was mixed and left at 25 °C for 15 min. After that, 10 mM of solution containing amino acids or mercapto biomolecules was added into the L1-Cu^{2+} solution for 2 min before the absorption and emission spectra were recorded.

3. Results and Discussion

3.1. Selectivity Properties of L1 toward Cu^{2+}

3.1.1 UV absorption and fluorescence properties of L1 toward Cu^{2+}

The absorption and fluorescence properties of L1 (0.5 gL^{-1}) in the absence and presence of Cu^{2+} in 10 mM HEPES buffer, pH 7.0 were shown in Fig. 3. The L1 was excited at wavelength 510 nm and showed an absorption maximum at 620 nm, which is the characteristic of tetrapyrrole-based chromophore (Fig. 3(a)). Upon addition of Cu^{2+}, the color of L1 changed from blue to colorless (Fig. 3 (a) inset), which is dued to the chelation of tetrapyrole core with Cu^{2+} [30]. This L1 also exhibited the maximum emission at 640 nm when excited at 510 nm and almost 80% of its fluorescence was quenched upon adding Cu^{2+}, as shown in Fig. 3(b) inset.

3.1.2. Response time of L1 toward Cu^{2+}

Once the Cu^{2+} was added into L1, the fluorescence intensity at 640 nm was recorded over time (0-30 min) to investigate the optimum response time of L1 toward Cu^{2+}. From Fig. 4, a sharp decrease in fluorescence intensity was observed within 1 min after adding Cu^{2+} and the intensity continued to decline overtime. There was no significant change in fluorescence intensity after 15 min. Accordingly, the 15-min response time was chosen as the optimal condition that allows maximum quenching of the L1 probe.

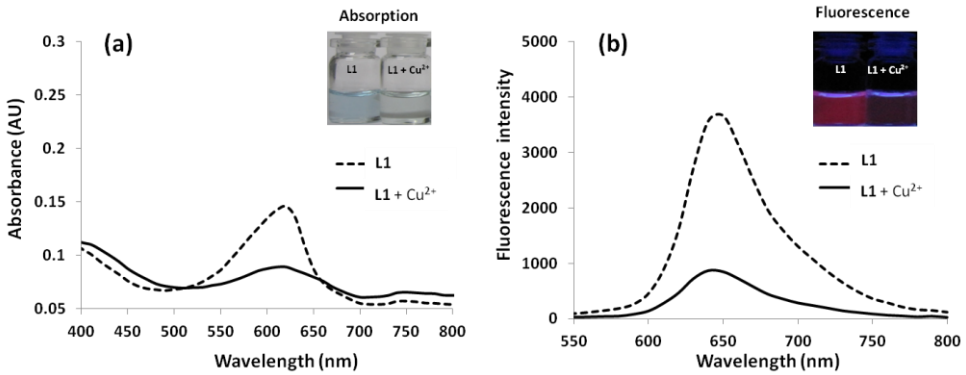

Fig. 3. (a) Absorption and (b) emission spectra of L1 (0.5 gL^{-1}) in 10 mM HEPES (pH 7.0) in the absence and presence of Cu^{2+} (50 uM) (The insets show (a) the color and (b) fluorescence changes of L1 with and without Cu^{2+}).

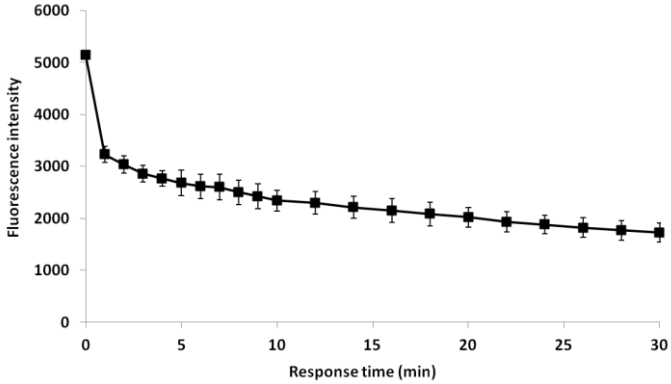

Fig. 4. Response time of L1 (0.5 gL^{-1}) in the presence of Cu^{2+} (50 μM) in 10 mM HEPES, pH 7.0 (Fluorescence intensity was recorded at 640 nm).

3.1.3. Binding constant of L1 toward Cu^{2+}

To examine the binding constant (K_a), fluorescence titration of L1 (0.5 gL^{-1}) was carried out with various concentrations of Cu^{2+} (8 to 100 μM). When the concentration of Cu^{2+} increased, the fluorescence intensity of L1 decreased which accompanied by a slight blue shift of 10 nm, as shown in Fig. 5. We estimated K_a using the emission intensity titration curve according to the equation [31]:

$$\frac{I_F^0}{(I_F - I_F^0)} = (\frac{1}{f})\left[\frac{1}{K_a[M]} + 1\right] \quad (1)$$

where I_F^0 is the fluorescence intensity of L1 at 640 nm, I_F is the emission intensity of L1 at 640 nm upon the addition of different concentrations of Cu^{2+}, f is the fraction of the initial fluorescence which is accessible to the sensor, and [M] is the concentration of Cu^{2+}. In Fig. 5 inset, the data were recorded 2 min after Cu^{2+} being added. The inset shows the fitting of titration curve of L1 in the presence of Cu^{2+} (1:1, v/v). These spectra were measured in 10 mM HEPES (pH 7.0). Excitation wavelength was 510 nm. The K_a value is given by the ratio interception/slope and is determined to be 2.08×10^4 M^{-1}. This higher K_a value indicates a strong binding of Cu^{2+} to tetrapyrrole unit of L1, compared to an iminofluorescein-Cu^{2+} ensemble probe (1.91×10^3 M^{-1}) [32].

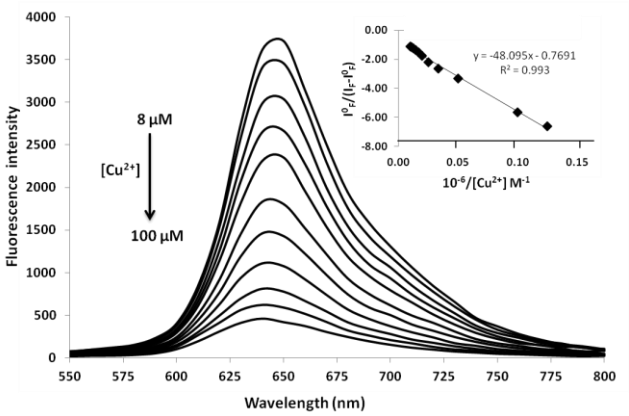

Fig. 5. Fluorescent titration spectra of L1 (0.5 gL^{-1}) in the presence of various concentrations of Cu^{2+} ion (8, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM).

3.2. Selective and Sensitive Properties of L1-Cu^{2+} Ensemble Probe toward Mercapto Biomolecules

3.2.1 Selective recognition of L1-Cu^{2+} ensemble probe

To investigate the ability of L1-Cu^{2+} ensemble probe to detect mercapto biomolecules, the fluorescence emission changes of the probe were examined in terms of selectivity. The selectivity study of L1-Cu^{2+} ensemble probe was carried out with different types of amino acids and mercapto biomolecules in 10 mM HEPES (pH 7.0). The fluorescent intensity at 640 was recorded after applying an excitation energy at 510 nm. Among 15 amino acids and mercapto biomolecules tested, the fluorescent state of Hcy, GSH and Cys were found to restore almost completely, when compared to others (Fig. 6). Upon addition of mercapto biomolecules, we speculated that these mercapto biomolecules can remove Cu^{2+} from the L1-Cu^{2+} complex by interacting with Cu^{2+}. Accordingly, the L1 is free from Cu^{2+} and is able to return to its original state and restore the fluorescence.

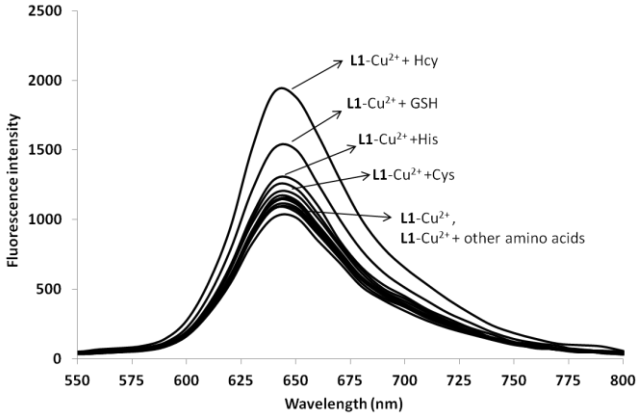

Fig. 6. The fluorescence response of L1-Cu^{2+} ensemble probe (50 μM) in the presence of different types of amino acids and mercapto biomolcules (50 μM of each) in 10 mM HEPES buffer (pH 7.0) (Excitation wavelength was carried out at 510 nm).

3.2.2 Detection limit of L1-Cu^{2+} ensemble probe toward Hcy

To investigate the detection limit of L1-Cu^{2+} ensemble probe for Hcy, various concentrations of Hcy were

added into the probe (50 μM). The fluorescence intensity at 640 nm was plotted against the Hcy concentrations (Fig. 7). We found a linear response of L1-Cu^{2+} ensemble probe toward Hcy within the range of 10 – 100 μM. This Hcy chemosensor gave a detection limit of 4.71 μM (S/N = 3; %RSD = 5.11 - 1.22 %), which is much lower than the free intracellular Hcy concentrations (5 – 15 μM) [33]. Hence, this sensing system may have potential to be used in diagnosis of diseases related with abnormal levels of cellular thiols.

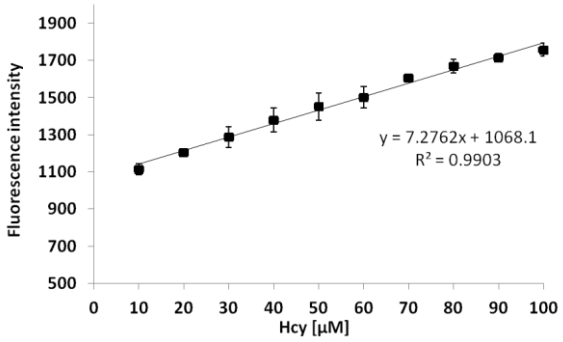

Fig. 7. Fluorescence intensity of L1-Cu^{2+} ensemble probe (50 μM) in the presence of various concentrations of Hcy (10-100 μM) in 10 mM HEPES buffer (pH=7.0).

4. Conclusion

In summary, we have developed L1 based bifunctional fluorescence chemosensor for Cu^{2+} and mercapto biomolecules detection. The L1 showed high selectivity and sensitivity toward Cu^{2+}, based on absorption and emission changes. Upon binding to Cu^{2+}, mercapto biomolecules induced the decomplexation of L1-Cu^{2+} by removing Cu^{2+} from the L1 structure. This Cu^{2+} has high affinity for mercapto biomolecules and therefore leaves the tetrapyrole unit of L1 free from the Cu^{2+}. As a result, the absorbance and fluorescent signal of L1 can be restored in the presence of mercapto biomolecules. Under the optimal conditions, this chemosensor can detect Hcy at concentration as low as 4.71 μM (S/N = 3). We believed that this water soluble L1-Cu^{2+} ensemble probe may have the potential to be used as a Hcy sensor in aqueous biological and medical samples.

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