Food Chemistry 262 (2018) 67–71

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A novel reaction-based fluorescent probe for the detection of cysteine in milk and water samples

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ARTICLE INFO

Keywords:
Fluorescent probe
Cysteine
Sulfonate esters
Visual sensor

ABSTRACT

A novel fluorescent probe 3′-hydroxy-3-oxo-3H-spiro [isobenzofuran-1,9′-xanthene]-6′-yl-2,4-dinitrobenzenesulfonate (probe **1**) was designed and synthesized as a visual sensor for the detection of cysteine levels in milk and water samples. The addition of cysteine to the solution of probe **1** resulted in an increase in fluorescence intensity and color change, from light yellow to yellow-green. The distinct color response indicated that probe **1** could be used as a visual sensor for cysteine. Cysteine can be detected quantitatively at concentrations between 0 and 400 μM and the detection limit of the fluorescence response to the probe was 6.5 μM. This suggests that probe **1** could be used as a signaling tool to determine the cysteine levels in samples, such as milk and water.

1. Introduction

L-cysteine is one of 20 essential amino acids and an important sulfur-containing compound (Zivkovic et al., 2017). L-cysteine is a permitted food additive and thought to be of nutritional benefit when included in processed cereal-based food, food for infants and young children, infant formula and nutritional supplements (Gilsenan, Lambe, & Gibney, 2002; Hopkins et al., 2015; Le Donne, Piccinelli, Sette, & Leclercq, 2011). The Joint FAO expert committee (JECFA) has classed L-cysteine as a flavoring agent. The European Food Safety Authority (EFSA) has also approved its use in food products for infants, and in animal feedstock (EFSA FEEDAP Panel, 2013). In industrial application, L-cysteine is used in pharmaceuticals, cosmetics, baking and as a flavoring additive (Oldiges, Eikmanns, & Blombach, 2014; Wada, & Takagi, 2006). One example is cysteines effect on the functionality of wheat starch, particularly after starch gelatinization, resulting in it being an effective reducing agent in the production of French-bread, crackers and cookies (Majzoobi, Farahnaky, Jamalian, & Radi, 2011; Majzoobi, Raissjalali, Jamalian, & Farahnaky, 2015; Li et al., 2015). Furthermore, in flavor chemistry, cysteine is a compound in foods as well as being a source of sulfur in the production of natural and processed flavors (Roland, Schneider, Razungles, & Cavelier, 2011; Starkenmann, & Niclass, 2011; Starkenmann, Troccaz, & Howell, 2008; Yaghmur, Aserin, & Garti, 2002).

Cysteine plays an important role across a broad range of industries, including food, cosmetic and medical applications. Therefore, the ability to detect and determine cysteine levels in a diverse array of products would be beneficial. To date, several methods have been developed to determine the cysteine content in a substance. Firstly, mercaptide-forming reactions using metal ions have been used (Nishiuchi, Kohmura, & Wakabayashi, 2011). More recently, fluorescent probes have emerged as an attractive tool for the selective detection of cysteine, due to its simplicity, high selectivity and sensitivity (Lin, Chen, Xian, & Chang, 2015; Wang et al., 2017). In particular, a large number of fluorescent probes for biological thiols have been developed based on the cleavage of disulfide bonds (Jung et al., 2012; Jung, Chen, Kim, & Yoon, 2013; Liu et al., 2014), sulfonate esters and sulfonamide (Ji et al., 2010; Niu et al., 2015), native chemical ligation (Lee, Kim, Yin, & Yoon, 2015; Yang et al., 2014; Zhang, Zhang, Liu, Yi, & Sun, 2015), aromatic substitution–rearrangement (Busschaert, Caltagirone, Van Rossom, & Gale, 2015; Kowada, Maeda, & Kikuchi, 2015; Niu et al., 2013), cyclization with aldehydes (Liu, et al., 2013; Rusin et al., 2004; Sudeep, Joseph, & Thomas, 2005), conjugate addition–cyclization with acrylates (Guo, Nam, Park, & Yoon, 2012; Yang, Guo, & Strongin, 2011; Zhang, et al., 2015) and Michael reactions (Chen, Zhou, Peng, & Yoon, 2010; Lin, et al., 2015; Ros-Lis, et al., 2004). Although many small molecular fluorescent probes have been used to detect cysteine, most were used to image living cells. Further study is required to develop a detection method for cysteine in food. The aim of this work is to design and synthesize an efficient fluorescent probe for cysteine, investigate its specificity for cysteine and explore its use as a visual sensor for the detection of cysteine in milk and water

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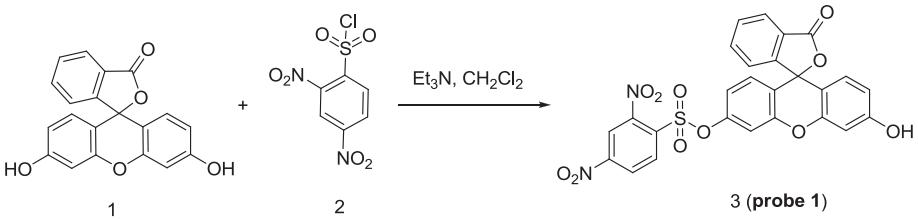
https://doi.org/10.1016/j.foodchem.2018.04.084
Received 2 May 2017; Received in revised form 5 April 2018; Accepted 21 April 2018
Available online 23 April 2018
0308-8146/



Scheme 1. Synthesis of probe **1**; Fluorescein (1), 2,4-dinitrobenzene sulfonyl chloride (2).

samples.

2. Materials and methods

2.1. Chemicals

Fluorescein (98%), 2,4-dinitrobenzene sulfonyl chloride (98%), trimethylamine (99%), L-asparagine (Asp), L-alanine (Ala), L-valine (Val), L-phenylalanine (Phe), L-histidine (His), L-leucine (Leu), L-serine (Ser), L-isoleucine (Ile), L-tryptophan (Trp), L-lysine (Lys), L-arginine (Arg), L-proline (Pro), L-glycine (Gly), L-methionine (Met), L-tyrosine (Tyr), L-glutamic acid (Glu), L-threonine (Thr), cysteine (Cys), glucose, phenol (C_6H_5OH), potassium iodide (KI), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), calcium chloride ($CaCl_2$), manganese(II) chloride ($MnCl_2$), Zinc chloride ($ZnCl_2$), ferric chloride ($FeCl_3$), aluminum chloride ($AlCl_3$) and chromium chloride ($CrCl_3$) were obtained from J&K Scientific Ltd (Beijing, P.R. China). HPLC grade chloroform ($CHCl_3$) and dimethyl sulfoxide (DMSO) were purchased from Beijing Huaxue Shiji Company (Beijing, P.R. China).

2.2. Instruments

NMR spectra were performed on a Bruker AV 300 MHz NMR spectrometer (^{1}H NMR at 300 MHz, ^{13}C NMR at 75 MHz) using tetramethyl silane (TMS) as an internal standard. High resolution mass spectroscopy (HRMS) was performed using a Bruker Apex IV FTMS spectrometer. Fluorescence spectra were recorded using a Hitachi F-4600 fluorescence spectrometer with a temperature controller.

2.3. Preparation of probe *1*

Fluorescein (1 g, 3 mmol) and 2,4-dinitrobenzene sulfonyl chloride (0.8 g, 3 mmol) were dissolved in $CHCl_3$ (10 mL). The mixture was stirred for 15 min and trimethylamine (one drop) in $CHCl_3$ (10 mL) was slowly added. The reaction mixture was heated to reflux in an oil bath for 5 h and the reaction progress monitored using thin layer chromatography (TLC). The precipitate was collected by evaporation, and the crude residue was subjected to silica gel chromatography eluted with petroleum/ethyl acetate, (v/v, 10:1). The product was determined using TLC (a new point, which differed from the material points) and petroleum/ethyl acetate solvent was removed, resulting in probe 1 being a yellow solid (105 mg). The structure of probe **1** was confirmed using ^{1}H NMR, ^{13}C NMR and HRMS.

2.4. Preparation of solutions of probe *1* and analytes

Probe **1** stock solutions (1 mM) were prepared using HPLC grade DMSO. The analytes L-asparagine (Asp), L-alanine (Ala), L-valine (Val), L-phenylalanine (Phe), L-histidine (His), L-leucine (Leu), L-serine (Ser), L-isoleucine (Ile), L-tryptophan (Trp), L-lysine (Lys), L-arginine (Arg), L-proline (Pro), L-glycine (Gly), L-methionine (Met), L-tyrosine (Tyr), L-glutamic acid (Glu), L-threonine (Thr), cysteine (Cys), glucose, KI, $MgCl_2$, NaCl, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $FeCl_3$, $AlCl_3$, $CrCl_3$ were dissolved in distilled water (10 mM). 2-Hydroxy-1-ethanethiol was prepared in DMSO (10 mM). The stock solutions were diluted with distilled water to the desired concentration and used immediately.

2.5. Preparation of milk and water samples

Six commercial samples which included one brand of mineral water (bottle, 500 mL), one brand of purified water (bottle, 500 mL), and four brands of milk (cardboard, 250 mL; pasteurized and full fat milk) were purchased from a local supermarket (Beijing, P.R. China). Tap water was collected (in glass container, 500 mL) from the running water supply network system in Beijing. Prior to the cysteine analysis, the milk samples were diluted 60 times using deionized water. A 20 μL sample solution of each sample was used for the determination of cysteine content of the solution. Different amounts (0.03 mM, 0.05 mM) of Cys were added to the samples and the fluorescence signal at 520 nm for each sample was recorded. Each test was repeated 3 times.

2.6. The determination of Cys and sample analysis

The preparation of the test system: DMSO (0.48 mL) was added to a solution of probe **1** (0.02 mL), the tested sample was added, and the cuvette was filled to a total volume of 2 mL using phosphate buffer solution (PBS, pH = 11). After mixing, the spectrum was recorded. The parameters used for the fluorescence spectrophotometer were: λ_{ex} = 337 nm, λ_{em} = 347 nm, slit width: 5 nm, 5 nm, voltage: 700 v, sensitivity: 2 and temperature: 37 °C.

3. Results and discussion

3.1. Probe *1* synthesis

Probe **1** was produced using an esterification reaction, shown in Scheme 1. Fluorescein (1) was reacted with 2,4-dinitrobenzene sulfonyl chloride (2) in the presence of trimethylamine as a catalyst, to produce probe **1**. The identification and structure of probe **1** was confirmed using ^{1}H NMR, ^{13}C NMR and HRMS Supplementary Fig. A1-3.

^{1}H NMR (300 MHz, DMSO), δ (ppm): 10.25 (s, 1H), 8.91 (d, J = 2.7 Hz, 1H), 8.48 (dd, J = 9.3, 2.7 Hz, 1H), 8.00 (d, J = 7.2 Hz, 1H), 7.72–7.85 (m, 2H), 7.42 (d, J = 9.3 Hz, 1H), 7.26–7.36 (m, 2H), 7.00 (dd, J = 8.7, 2.4 Hz, 1H), 6.91 (d, J = 8.7 Hz, 1H), 6.70 (d, J = 0.9 Hz, 1H), 6.61 (s, 2H).

^{13}C NMR (300 MHz, DMSO): δ (ppm): 169.1, 160.2, 156.1, 154.2, 152.8, 152.4, 151.9, 142.8, 140.5,136.4, 136.4, 130.9, 130.3, 129.7, 126.3, 125.4, 124.6, 122.5, 121.5, 117.0, 116.2, 113.7, 109.6, 108.5, 102.8, 82.4.

HRMS (ESI): calcd for $[M + H]^+$ 563.089030, found 563.089107.

3.2. The Cys sensing properties of probe *1*

The fluorescence response of probe **1** (10 μM) to Cys was studied in 10 mM PBS (pH 7.4) with DMSO (v/v, 3:1) at 37 °C. As shown in Fig. 1a, the fluorescence signal of probe **1** (520 nm) significantly increased (excited at 337 nm) within 27 mins of the introduction of Cys, indicating that at these conditions the reaction was complete after 27 min. The effect of pH was investigated in the range of 6–12 (Fig. 1b). Overall, the fluorescence intensity of probe **1** was not affected between pH 6 and 9. However, between pH 9–12 the fluorescent intensity increased and pH 11 was chosen for subsequent studies. The fluorescence responses of probe **1** (10 μM) in the absence and presence of Cys