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Rapid Quantification of Bioactive Lentinan with an Aniline Blue Fluorescent Method

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Abstract

Lentinan is a clinically approved immune modulator and its anticancer and immunomodulatory bioactivity is found to be dependent on its triple helical conformation. Therefore, the development of rapid and convenient method for determination of bioactive lentinan with triple helical conformation holds great promise for the quality control of lentinan healthy products. In this work, an aniline blue fluorescent method was optimized and established to accurately and rapidly detect bioactive lentinan. In the presence of lentinan, the fluorescence intensity of aniline blue with 404 nm excitation and 492 nm emission dramatically enhanced within 15 min in pH 10 glycine-NaOH buffer solution, which allowed the analysis of lentinan in a very simple and fast manner. The method allowed for the sensitive determination of lentinan in the range of 1 to 60 μg/mL with a detection limit of 0.25 μg/mL. Notably, the protocol exhibited excellent selectivity for the determination of triple helical lentinan over other saccharides. The method was successfully applied to the detection of bioactive lentinan in health tonic solution, which demonstrated the method had great potential for quality control of lentinan contained products.

Keywords

Lentinan, Triple Helical Conformation, Aniline Blue, Fluorescence Detection, Health Tonic Solution

1. Introduction

Lentinan is isolated from the fruiting body of *Lentinus edodes* (Shiitake) [1] [2] and is known as a bioactive polysaccharide due to its strong antitumor activity through the activation of human immune system [1] [3] [4] [5] [6]. Lentinan has been approved as an immune modulator for tumor treatment and clinically ap-

plied in combination with chemotherapy [7] [8]. With greater emphasis on the prevention of diseases, consumption of health products containing lentinan has grown in popularity. Recent researches have demonstrated that the immunotherapeutic activity of lentinan is mainly dependent on the polysaccharide's junior and senior structures [9] [10]. Lentinan is a β-glucan with the repeating unit consisting of two β-(l,6)-glucopyranoside branches for every five linear β-(l,3)-glucopyranoside linkages [11] [12] [13] [14]. Owing to the inter and intra molecular hydrogen bonds, lentinan can form the helical structures especially triple helical conformation in aqueous solution [15], which plays critical role in the immunoregulatory function [16] [17] [18]. Therefore, developing a rapid and convenient method for quantification of triple helical lentinan is highly desired to evaluate the quality of lentinan containing healthy products.

At present, there are mainly three kinds of methods to measure β-glucan content, including inorganic chemistry-based method [19] [20], enzymic method [21], and protein specific identification method [22] [23]. The first two kinds of methods utilize acid or exoglucanase to hydrolyze the β-glucan into glucose. The content of β-glucan can be calculated by determination of the generated glucose concentration. Although the detecting sensitivity is high, these two methods suffer unsatisfactory specificity because they can only detect the total glucose content and may obtain higher results than the actual value in the presence of other polysaccharide such as starch [21]. Moreover, these methods are unable to directly measure the content of the triple helical lentinan. Protein specific identification method like enzyme-linked immunosorbent assay [22].and limulus factor G test [24] that uses specific protease and polyclonal antibody for β-glucan detection can meet the requirements of specificity. However, the method is costly and time-consuming. Therefore, there is still a strong need to develop a sensitive, specific, rapid and cost-efficient detection method for helical lentinan.

The fluorimetry method is a powerful tool due to its high sensitivity and simplicity in data collection [24] [25]. In this work, a fluorimetry method using aniline blue as the probe has been established to detect helical lentinan. Aniline blue, with the structure of sodium 4,4'-[carbonylbis-(benzene-4,1-diyl)bis-(imno)] bis(benzene-sulphonate) [26] [27], is a water-soluble weakly fluorescent dye. Upon binding to the triple helical lentinan, the formed complex of aniline blue/lentinan produces strong fluorescence, which is potential and promising for quantitation of bioactive lentinan in health products. Therefore, in this study, we have optimized an aniline blue fluorescent method by investigating a variety of impact factors in fluorescence intensity of the aniline blue/lentinan complex. Furthermore, this fluorimetry method has been employed for direct detection of bioactive lentinan content in oral health tonic liquid.

2. Materials and Methods

2.1. Materials and Reagents

Lentinan with an average molecular weight of 630,000 Da (purity > 98%) was

purchased from Yuanfu Pharmaceutical Science and Technology Co. Ltd. (Shanghai, China). Aniline blue was purchased from Nanjing Wanqing Chemical Glassware Instrument Co. Ltd. (Nanjing, China). Glucose, sucrose, maltose and starch were purchased from Sinopharm Chemical Reagent Co. Ltd. (Nanjing, China). Xylan and xanthan gum were purchased from Aladdin Industrial Co. Ltd. (Shanghai, China). Oral health tonic liquid was supplied by Infinitus Co. Ltd. (Guangzhou, China). All reagents were of at least analytical grade and used without further purification. All solutions were prepared with ultrapure water (18.2 MΩ) purified by a Millipore Simplicity System (Millipore, Bedford, USA).

2.2. Apparatus

Fluorescence intensity and spectra were recorded by Varioskan Flash Multimode Reader (Thermo Scientific). pH meter (Delta 320) was applied to measure the pH values of solutions.

2.3. Reagents Preparation

A series of glycine-NaOH buffer solutions with different pH values were prepared by mixing 0.2 mol/L glycine with NaOH or HCl solutions at different proportions, and the pH values were adjusted by pH meter. 100 μg/mL lentinan stock solution was prepared by dissolving 0.0100 g of lentinan in a 0.5 mol/L NaOH solution and adjusted to pH 10 with 0.2 mol/L glycine, and then diluted with 0.1 mol/L glycine-NaOH buffer solution in a 100 mL volumetric flask. The lentinan stock solution was stored in 4˚C. 0.1% (w/v) of aniline blue stock solution was prepared by dissolving 0.1000 g of aniline blue with 0.1 mol/L glycine-NaOH buffer in a 100 mL volumetric flask and stored in dark to preserve it from light.

2.4. Fluorescence Determination of Lentinan

Different volumes (0 - 6 mL) of lentinan stock solution were mixed with 4 mL aniline blue solution (0.1%), and the mixtures were diluted to 10 mL with pH 10 glycine-NaOH buffer solution in brown volumetric flasks. The resulted solutions were heated at 80˚C for 15 min and then gradually cooled to room temperature. Afterwards 100 μL mixture per well was added into the 96-well black plate, and the fluorescence intensity was measured by Varioskan Flash Multimode Reader. The samples were excited at 404 nm and the emission was collected at 492 nm. The calibration curve of lentinan was constructed by plotting the relative fluorescence intensity against the corresponding lentinan concentration using linear regression model. The limit of detection (LOD) was determined as the concentration of the lentinan in the solution when the signal to noise ratio (S/N) was approximately 3.

2.5. Specificity Assay

0.4 mL lentinan stock solution was mixed with different volumes (0, 0.2, 0.4, 0.6,

0.8, 1, 1.2, 1.4, 1.6, 1.8, and 2.0 mL) of 1 mg/mL other potentially interferential polysaccharides including glucose, sucrose, maltose, starch, cellulose, xanthan gum, and xylan. Then 4 mL 1% aniline blue stock solution was added, and the mixture was diluted to 10 mL with pH 10 glycine-NaOH buffer solution. The resulted solution was heated at 80˚C for 15 min and gradually cooled to room temperature to perform fluorescence assay. In addition, dimethyl sulfoxide (DMSO) was added to investigate the specific binding between aniline blue and the lentinan with triple helical conformation. 1.0 mg lentinan was dissolved in different volumes of DMSO and diluted to 10 mL using pH 10 glycine-NaOH buffer solution with the DMSO concentration of 0, 2%, 10%, and 50%, respectively. Taken 0.4 mL solution into 4 mL 0.1% aniline blue stock solution, and the mixture was diluted to 10 mL with pH 10 glycine-NaOH buffer solution to perform fluorescence assay.

2.6. Fluorescence Detection of Lentinan in Health Tonic Solution

Different volumes (0, 0.25, 0.50 and 0.75 mL) of 0.1 mg/mL lentinan solution were mixed with 0.25 mL health tonic solution, and then 4 mL 0.1% aniline blue stock solution was added. The solution was diluted to 10 mL with pH 10 glycine-NaOH buffer solution. The established fluorescence method was performed to obtain the recovery to evaluate the accuracy of the method.

3. Results and Discussion

3.1. Fluorescence Spectra of Aniline Blue Fluorescent Method

Fluorescence spectra of aniline blue in the absence and presence of lentinan were measured to investigate the feasibility of aniline blue fluorescent method to detect lentinan. As shown in Figure 1, the excitation and emission of aniline blue was very weak in aqueous solution, which could be attributed to the intramolecular C-C rotation of aniline blue. After addition of lentinan, strong fluorescence

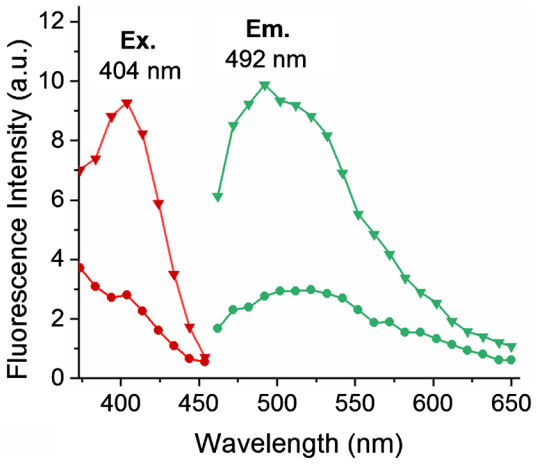

Figure 1. Fluorescence excitation (red line) and emission (green line) spectra of aniline blue in the absence (dotted line) and presence (trigonal line) of 5 μg/mL lentinan in 0.1 M pH 10.0 glycine-NaOH buffer.

enhancement was observed which indicated the formation of aniline blue/lentinan complex. The result suggested that upon binding to the cavity of triple helical structure of lentinan, the intramolecular C-C rotation of aniline blue was inhibited which increases the coplanarity and leaded to an enhanced fluorescence for lentinan detection. For the following experiments, 404 nm and 492 nm were chosen as the excitation and emission wavelength, respectively.

3.2. Optimization of Detection Conditions

Several detection conditions of the aniline blue fluorescent method that may affect the assay of lentinan were investigated, including the concentration of aniline blue stock solution, incubation pH, temperature, and time. When the concentration of aniline blue stock solution was lower than 0.1% (w/v), the fluorescence intensity of aniline blue/lentinan complex increased along with the increasing concentration of aniline blue (**Figure 2(a)**). When the concentration was higher than 0.1% (w/v), the fluorescence intensity decreased sharply, which may due to the self-quenching effect of aniline blue molecules. Thus, a concentration

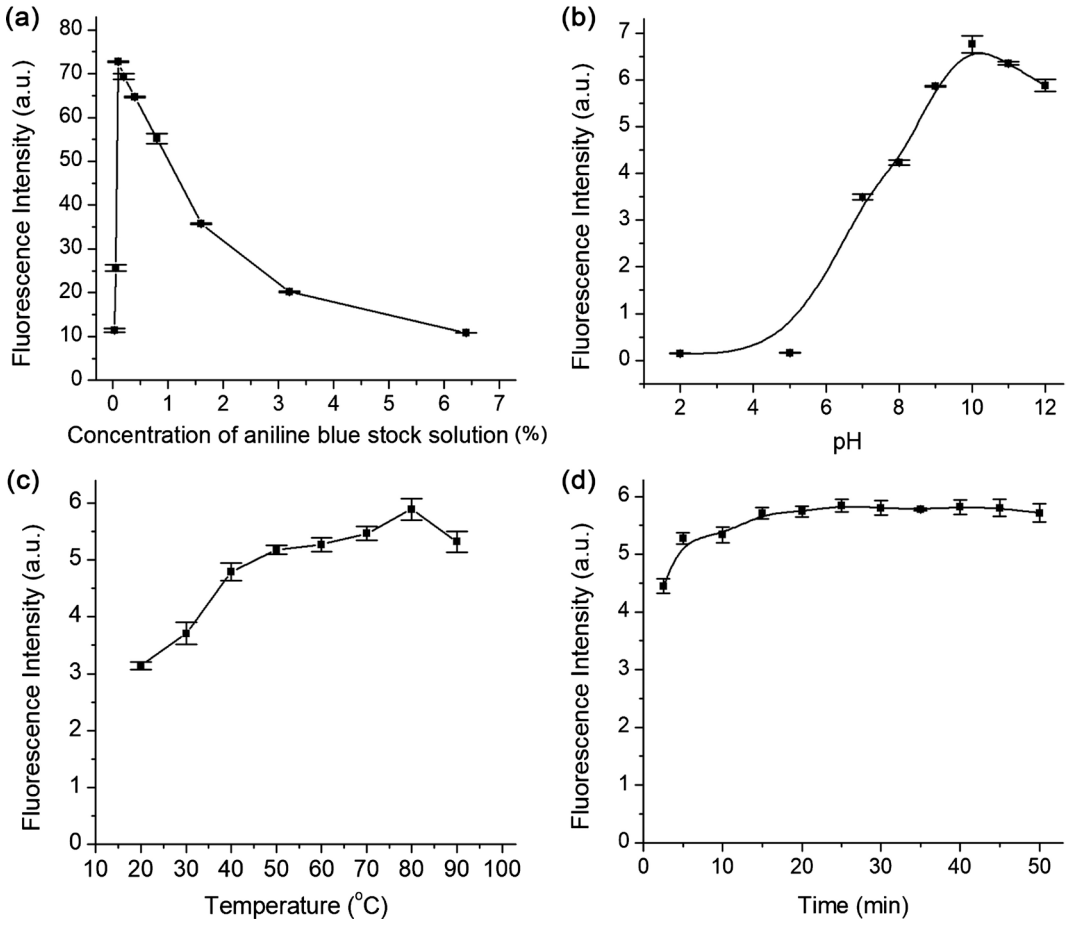

Figure 2. (a) Fluorescence intensity of aniline blue (λ_{ex}= 404 nm, λ_{em}= 492 nm) with various concentrations of aniline blue stock solution from 0.025% to 6.4% (w/v) in the presence of 60 μg/mL lentinan. Effects of (b) pH, (c) temperature, and (d) incubation time on the fluorescence intensity (λ_{ex}= 404 nm, λ_{em}= 492 nm) of aniline blue in the presence of 4 μg/mL lentinan in glycine-NaOH buffer. Data are means ± SD (n = 3).

of 0.1% (w/v) aniline blue stock solution was selected for the following experiments.

The effect of pH on the fluorescence intensity of aniline blue/lentinan complex was also investigated. The fluorescence intensity was observed in glycine-NaOH buffer solution with the pH ranging from 2 to 12 (**Figure 2(b)**). The results showed that the fluorescence intensity was very weak under weakly acidic medium. This observation indicated that the acidic pH could influence the intra- and inter-molecular hydrogen bonds of lentinan, which damaged the triple helical structure of lentinan [28] and inhibited the formation of aniline blue/lentinan complex. However, the fluorescence intensity increased conspicuously with the pH from 6 to 12, and the intensity reached a maximum value at pH 10 which was selected as the optimum incubation pH.

The effect of incubation temperature from 20˚C to 90˚C on the fluorescence intensity was examined (**Figure 2(c)**). The fluorescence intensity increased gradually along with the temperature from 20˚C to 80˚C, suggesting that higher temperature could prompt the molecular motion of aniline blue to bind with lentinan. While the temperature was higher than 80˚C, the fluorescence intensity unexpectedly declined, indicating that excessive temperature may lead to the nonradiative transition probability of aniline blue molecules. For the following experiments, 80˚C was chosen as the optimum reaction temperature.

The influence of time was also investigated. The fluorescence intensity increased rapidly within 10 min (**Figure 2(d)**). After 15 min, it remained nearly constant, indicating a fast binding between aniline blue and lentinan. Therefore, an optional incubation time of 15 min was selected to ensure a rapid detection of lentinan.

3.3. Sensitivity and Selectivity for Lentinan Detection

To evaluate the sensitivity of this aniline blue fluorescent approach for quantitative analysis under the optional conditions, the assay was practiced at different amounts of lentinan. As shown in **Figure 3**, a good linear relationship between relative fluorescence intensity and lentinan concentrations was obtained over the range from 1 to 60 μg/mL (R^2 = 0.9997). The LOD (the concentration when S/N was approximately 3) was determined to be as low as 0.25 μg/mL, demonstrating the high sensitivity of the aniline blue fluorescent method.

Discrimination of lentinan from a large excess of other saccharides is a critical issue for lentinan detection. We examined the selectivity of the aniline blue fluorescence method for lentinan by detecting the response of lentinan in the presence of potentially interfering saccharides, including glucose, sucrose, maltose, cellulose, starch, xanthan gum, and xylan. The results showed that almost no fluorescence change was observed for the addition of those saccharides (**Figure 4**), suggesting the presence of these interferences did not affect the quantitative determination of lentinan. Furthermore, DMSO as a powerful hydrogen bond acceptor was added to break the triple helical structure of lentinan to be coil conformation [9] [29] [30]. With the increasing concentration of DMSO from 0 to 50%, the fluorescence intensity decreased significantly (**Figure 5**), demonstrating

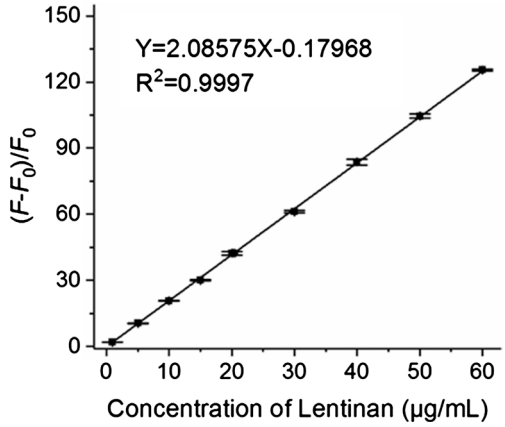

Figure 3. Relative fluorescence intensity (λ_{ex}= 404 nm, λ_{em}= 492 nm) of aniline blue in the presence of increasing concentration of lentinan from 1 to 60 μg/mL. F and F_0 are fluorescence intensities in the presence and absence of lentinan, respectively. Data are means ± SD (n = 3).

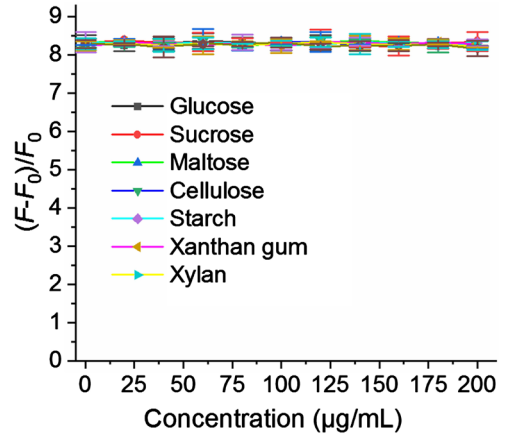

Figure 4. Relative fluorescence response (λ_{ex}= 404 nm, λ_{em}= 492 nm) of aniline blue to 4 μg/mL lentinan in the presence of various saccharides with the concentration from 0 to 200 μg/mL. Data are means ± SD (n = 3).

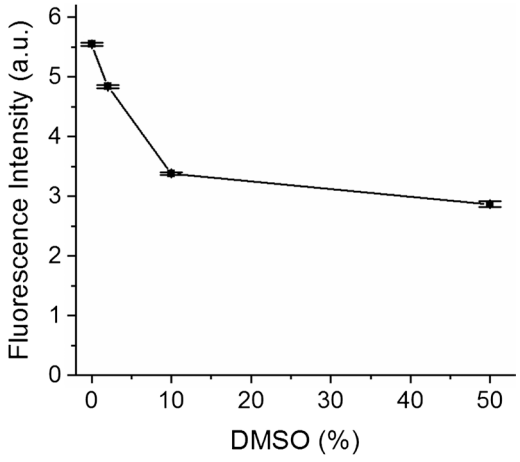

Figure 5. Effect of DMSO on the fluorescence intensity (λ_{ex}= 404 nm, λ_{em}= 492 nm) of aniline blue in the presence of 4 μg/mL lentinan in pH 10.0 glycine-NaOH buffer. Data are means ± SD (n = 3).

the specificity of binding between aniline blue and triple helical structure of lentinan. Glucose, sucrose, maltose, and cellulose were dissociative in aqueous solution without helical structure. The conformations of starch and xanthan gum were single helix and double helix, respectively. Thus, aniline blue could not bind to the cavity of the single or double helical structure. Although xylan possessed triple helical conformation, the glucan was not β-(1,3)-(1,6). Therefore, the excellent selectivity could be attributed to the specificity of complementary structure between aniline blue and the unique triple helical structure formed by β-(1,3)-(1,6)-glucan of lentinan in aqueous solution, which ensured the selectivity of this approach for lentinan products determination.

3.4. Detecting Lentinan in Health Tonic Solution

In order to examine the performance of this method for practical applications, the recovery test in health tonic solution was conducted. The recovery and RSD were analyzed by the standard addition method, and the results were listed in Table 1. The recovery was 98.9% - 103% with an average recovery rate of 101%. The relative standard deviation was 1.73%. These results indicated that the presence of lentinan in health tonic solution could be precisely detected. Furthermore, aniline blue fluorescent method and phenol-sulfuric acid method as a common measurement were used to determine the original lentinan concentration in 10 samples of health tonic solution, respectively. The results showed that the concentrations of lentinan determined by aniline blue method were 197~236 μg/mL with the average concentration of 216 μg/mL (Table S1 in Supporting Information). As a control group, the concentrations were detected to be 3087 - 3766 μg/mL with the average value of 3369 μg/mL. The much higher obtained concentration could be attributed that phenol-sulfuric acid method detected the hydrolyzed glucose content which could not eliminate other interfering substances like oligosaccharide. Aniline blue fluorescent method was suitable for the determination of triple helical lentinan, demonstrating the applicability of this method in complexed lentinan contained products.

4. Conclusion

In summary, we have optimized an aniline blue fluorescent method to quantify the amount of bioactive lentinan with the triple helical conformation. The method

Table 1. Determination of lentinan concentration in health tonic solution using aniline blue fluorescent method (n = 3).

Added lentinan (μg/mL)	Determinedlentinan (μg/mL)	Recovery (%)	Average recovery (%)	Average RSD (%)
0	5.13	100	101	1.73
2.5	7.70	103		
5.0	10.2	101		
7.5	12.5	98.9		

is fast, simple and convenient in operation. The detection limit of this method is lower than or at least comparable to other polysaccharide detection methods. Importantly, the protocol exhibits excellent selectivity for the determination of bioactive lentinan with triple helical structure over other saccharides. The method has been successfully used for the detection of lentinan in health tonic solution, which demonstrate the method has great practicability for quality control of lentinan related healthy products. It should also be noted that the excitation and emission of wavelength of this method is located in the visible region (400 - 600 nm) which may suffer autofluorescence in more complexed biological system. This study should substantially broaden the perspective for further manufacturing a near-infrared (600 - 900 nm) fluorescence probe to achieve precise determination of lentinan in related products.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supporting Information

Table S1. Determination of originallentinan concentration in health tonic solution using phenol-sulfuric acid method and aniline blue fluorescent method, respectively (Data are means ± SD, n = 3).

Samples	Concentration determined by phenol-sulfuric acid method (μg/mL)	Average concentration (μg/mL)	Concentration determined by aniline blue method (μg/mL)	Average concentration (μg/mL)
1	3528 ± 11	3369	228 ± 3	216
2	3269 ± 14		206 ± 2	
3	3108 ± 23		203 ± 2	
4	3192 ± 50		204 ± 2	
5	3087 ± 87		197 ± 2	
6	3367 ± 28		228 ± 4	
7	3766 ± 21		236 ± 2	
8	3647 ± 10		231 ± 1	
9	3409 ± 35		207 ± 5	
10	3318 ± 43		216 ± 10	