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SPECTROFLUORIMETRIC DETERMINATION OF BRASSINOSTEROIDS PLANT HORMONES IN BIO-EXTRACT SAMPLES

(Penentuan spektrofluorimetrik bagi Hormon Tumbuhan Brassinosteroids dalam Sampel Bio-Ekstrak)

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Abstract

A spectrofluorometric method was developed for determination of brassinosteroids plant hormones based on the reaction between brassinolide and *m*-dansylaminophenylboronic acid. 90 µM of *m*-dansylaminophenylboronic acid solution was chosen as the optimal concentration of the reagent. The temperature of the reaction was 30°C (room temperature) for 20 min duration in 5 mM phosphate buffer solution, pH 7. The excitation and emission wavelengths were 380 nm and 505 nm, respectively. Under optimal condition, linearity was found in the range 1-50 µM. Standard addition was used for the brassinosteroids determination in bio-extract samples, without any sample preparation. Limit of detection (3*SD*, n=10) of 0.24 µM and limit of quantitation (10*SD*, n=10) of 0.48 µM were obtained using sample blank with this standard addition method. Result was demonstrated to be a suitable semi-quantitative technique and was applied successfully for the determination of total brassinosteroids plant hormones equivalent to brassinolide.

Keywords: brassinolide, brassinosteroids, *m*-dansylaminophenylboronic acid, bio-extract, spectrofluorometry

Abstrak

Kaedah spektroflourometrik telah dibangunkan bagi penentuan hormon tumbuhan brassinosteroids berdasarkan tindak balas antara brassinolide dan asid m-dansylaminofenilboronik. 90 µM larutan asid m-dansylaminofenilboronik dipilih sebagai reagen pada kepekatan optimum. Suhu tindak balas adalah 30 °C (suhu bilik) untuk tempoh 20 min di dalam 5 mM larutan penimbal fosfat, pH 7. Panjang gelombang pengujaan dan pancaran masing – masing adalah 380 nm dan 505 nm. Di bawah keadaan optimum, kelinearan diperolehi di dalam julat 1-50 µM. Penambahan larutan piawai digunakan bagi penentuan brassinosteroids di dalam sampel bio-ekstrak, tanpa sebarang penyediaan sampel. Had pengesanan (3SD, n = 10) iaitu 0.24 µM dan had kuatifikasi (10SD, n = 10) iaitu 0.48 µM telah diperolehi dengan menggunakan sampel pengosong dan larutan piawai yang ditambah. Keputusan menunjukkan bahawa kaedah semi kuantitatif ini sesuai dan boleh diaplikasikan bagi penentuan jumlah hormon brassinosteroids di dalam tumbuhan yang bersamaan dengan kandungan brassinolide.

Kata kunci: brassinolide, brassinosteroids, asid *m*-dansylaminofenilboronik, bio-ekstrak, spektroflourometri

Introduction

In 1970, Brassinosteroids (BRs) known as “brassins” were first discovered by extraction from rape pollen (*Brassica napus*) [1,2]. At present, more than 60 naturally occurring BR compounds have been discovered and characterized from various plant sources [3]. They are involved in numerous biological processes in plants such as inducing cell elongation and division, and enhancing tolerance to drastic environments [4]. In general, BRs are free steroidal compounds and might be bound to sugar and fatty acid compounds. The BR concentrations in plants are extremely

low compared with other plant hormones. Nowadays organic agriculture has become very popular and bio-extracts are being used to replace chemical fertilizers. Bio-extract is a liquid derived from the fermentation of vegetables and fruits with sugar.

Microanalysis methods have been used for identification and determination of traces of BRs using derivatizing reagents. After that, the reaction product was analysed by LC with UV detection [5], LC with fluorimetric detection [6-10], UHPLC-ESI-QqQ-MS [4] and GC-MS [1]. These methods are capable of determining each individual plant hormone in this BRs group; however, the experimental procedures are complicated and time-consuming. In this research a spectrofluorometric method was proposed for the determination of total BRs in a sample. Brassinolide was chosen for calibration because it is found at the high levels in many plants [11]. It was also the first plant growth hormonal steroid discovered and has higher biological activities than the other steroidal compounds in the group [11-13]. Due to the chemical structure of brassinolide (Figure 1) [13], it has α-oriented hydroxyl groups (*cis* configuration) at C-2 and C-3 in ring A, and C-22 and C-23 in the side chain which can react with m-dansylaminophenylboronic acid. Therefore, it was chosen as a representative of the BRs, most of which have the similar diol groups [4]. The method was based on a derivatization of all BRs with *m*-dansylaminophenylboronic acid as the fluorogenic reagent. It offers a simple, rapid and inexpensive operation and can be applied to real complex samples.

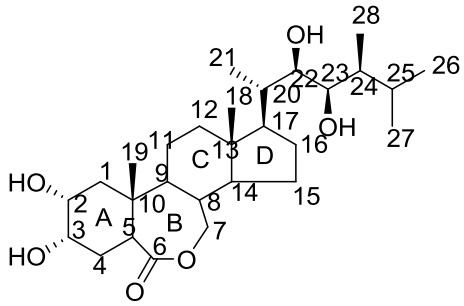

Figure 1. The chemical structure of brassinolide.

Materials and Methods

Instrument

The spectrofluorimetric measurements were recorded using a FP-6200 Jasco spectrofluorophotometer equipped with xenon arc lamp and a micro fluorometer quartz cell (1000 μL); spectral bandwidths of 10 and 5 nm were used for excitation and emission, respectively.

Materials and Reagents

Brassinolide (BL) standard was purchased from Sigma-Aldrich, UK with a purity of 90%, HPLC grade. *m*-dansylaminophenylboronic acid (DABA) was from Sigma-Aldrich, USA. Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12\ H_2O$) and potassium dihydrogenphosphate (KH_2PO_4) were of analytical reagent grade from Carlo Erba, Italy. Sodium hydroxide and dimethylsulfoxide (DMSO) were purchased from Merck, Germany. Methanol (HPLC grade) and hydrochloric acid (AR grade) were purchased from RCI Labscan, Thailand.

Brassinolide standard stock solution

A 100 μM BL stock solution was prepared by dissolving 2.40 mg of BL in 20 mL of methanol and making up to volume with deionized water in a 50 mL volumetric flask. This solution was stable for a month when kept at 4^{o}C in a refrigerator.

General procedure

750 µL of BL stock standard solution was transferred into a small centrifuge tube. The 100 µL of DABA solution was added, followed by 650 µL of phosphate buffer solution. The reaction mixture was left for 20 min at room temperature. The fluorescence intensity of the reaction product was measured at 505 nm after excitation at 380 nm. A blank experiment was carried out using the same procedure but without BL solution.

Standard addition procedure for bio-extract and liquid fertilizer samples

Bio-extract samples were made elsewhere by fermentation of various blended plant materials with sugar and water at a ratio of 1:3:5 plant material: sugar: water in a closed container for 6 months. Liquid fertilizer samples were bought from local agrochemical shops.

Each bio-extract sample was filtered with filter paper (Whatman no.1, pore size 11 µm) and diluted with deionized water. An aliquot of diluted samples in the range 20-700 µL, depending on either analyte concentration or sample color, was pipetted into a small centrifuge tube. The sample was spiked with BL stock standard at four different levels corresponding to 0, 0.5, 1, and 2 times the concentrations of brassinolide presented in the samples. The 100 µL of 1.35 mM of DABA solution (2.50 mg of DABA dissolved in 5 mL of DMSO) was added subsequently. The reaction mixture was made to 1.5 mL with 5 mM phosphate buffer solution (pH 7) and mixed thoroughly. After 20 min the fluorescence intensity was measured as described in general procedure section.

Results and Discussion

Optimization of experimental parameters

The experimental factors affecting the development of the reaction product and its stability were carefully studied and optimized. Five factors; namely pH, phosphate buffer concentration, reaction temperature, reaction time and concentration of the DABA were changed individually while the others were kept constant. BL was found to react with *m*-dansylaminophenylboronic acid at pH 7 forming a fluorescent derivative product. The excitation and emission spectra are shown in Figure 2. The excitation wavelength at 380 nm and emission wavelength at 505 nm were similar to those used in previous research [6].

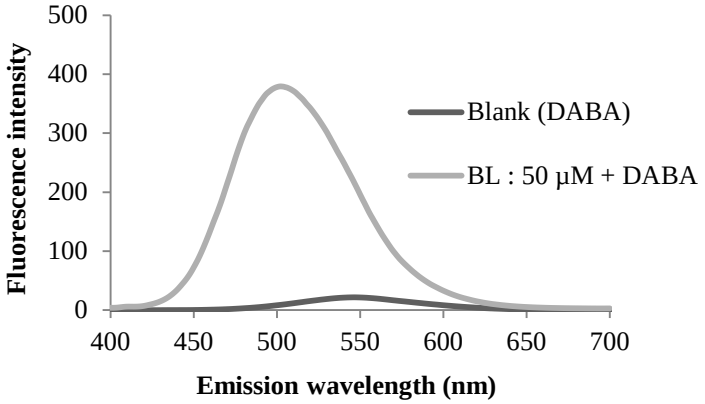

Figure 2. Fluorescence spectra of product (BL: 50 µM and DABA: 160 µM) and reagent blank solution (DABA: 160 µM) at pH 7.

Effect of pH

The influence of pH on fluorescence intensity of the reaction product was studied in the range of pH 5-9. Maximum fluorescence intensity was obtained at pH 7 with 10 mM phosphate buffer solution, when 50 µM of brassinolide was reacted with 160 µM of DABA (Figure 3). The results indicated that at pH less than 7, the fluorescence intensity decreased. The protons in the phosphate buffer solution could lead to the protonation of the dimethylamine group in DABA and cause significantly decreased fluorescence intensity of the reaction product. When the pH is higher than 7, the sulfonamide group of DABA can be deprotonated and the hydroxyl ion in the buffer solution can

be added to the boronic acid group of DABA. The new product caused the peak in the fluorescence spectrum to shift, which resulted in a decrease of the fluorescence intensity at 505 nm [14]. Therefore phosphate buffer solution at pH 7 was chosen in further experiments.

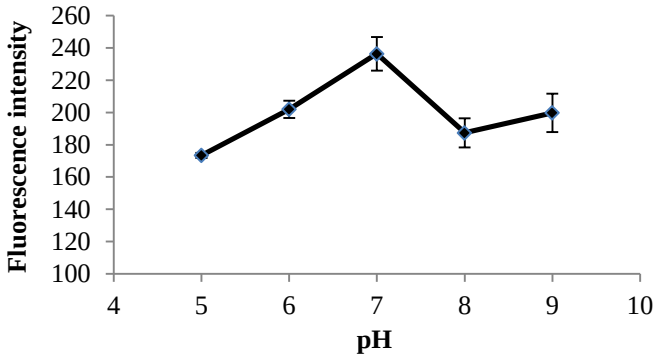

Figure 3. Fluorescence intensity of the reaction product at different pH.

Effect of phosphate buffer concentration

The buffer concentration affects its ionic strength, which resulted in a change of the pK_a value of the buffer acid and consequently, a change of its pH, even though the concentration ratio of the buffer species was kept constant [15]. Thus, the optimized buffer concentration was necessary. The influence of phosphate buffer concentration was studied in the range of 0.1-10 mM using $Na_2HPO_4.12\ H_2O$ adjusted to pH 7 using HCl. Fluorescence intensity of the reaction products of 50 µM brassinolide and 160 µM DABA are shown in Figure 4. Increasing the concentration of the phosphate buffer solution produced a proportional increase in the fluorescence intensity of the reaction product up to 5 mM. The intensity decreased between 5 and 10 mM. This could due to the change of buffer pH or the quenching effect from using high concentration of buffer. Thus, 5 mM of phosphate buffer solution was selected in subsequent experiments.

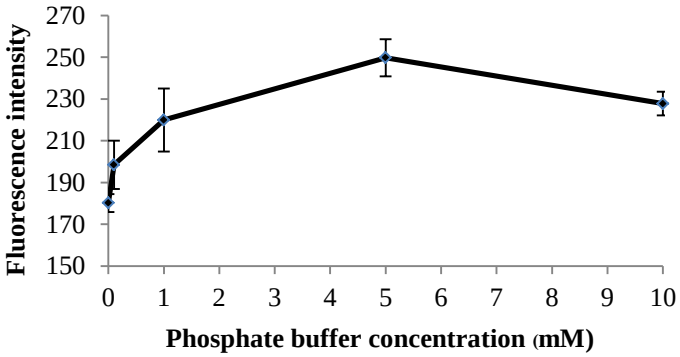

Figure 4. Fluorescence intensity of the reaction product at different buffer concentration.

Effect of temperature

In previous research, a reaction temperature at 70°C was used for derivatization of DABA with brassinosteroids [6]. However, in the other work, room temperature was chosen for derivatization process of DABA with saccharides [14, 16-17]. In this research the effect of reaction temperature was tested within the range 25-90°C. The fluorescence intensity at different reaction temperatures is shown in Figure 5. Increasing the reaction temperature

higher than 40°C resulted in a subsequent decrease in the fluorescence intensity of the reaction product. This could be because in fluorescence high temperatures can cause non-radiative processes via thermal agitation, which results in a decrease of the fluorescence intensity [18]. The fluorescence intensity at 30°C and 40°C revealed no significant differences. Therefore, room temperature (about 30°C in our laboratory) was chosen as the optimal reaction temperature for convenient.

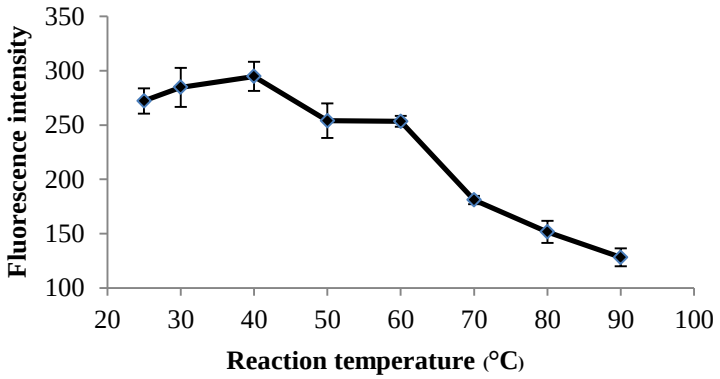

Figure 5. Fluorescence intensity of the reaction product at various reaction temperature.

Effect of reaction time

Different reaction time intervals were investigated in the range 5-70 min at room temperature. The relation between fluorescence intensity and reaction time is shown in Figure 6. It was found that after 20 min, the reaction product reaches its highest fluorescence intensity and remains constant for about 50 min. Thus, the optimal reaction time used in the present work was 20 min. This result was in agreement with previous work [9,10]. The derivatization reaction could be completed in 20 min, therefore no more products to produce the fluorescence intensity.

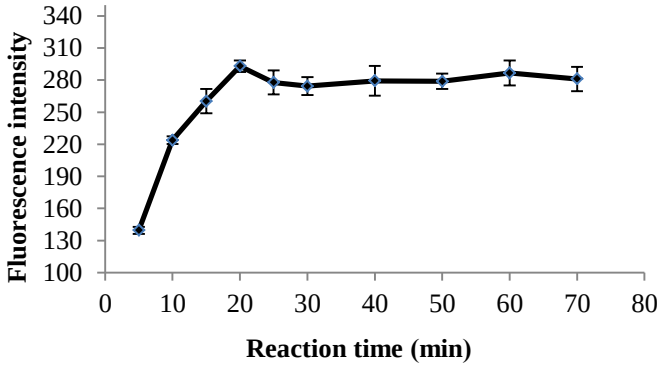

Figure 6. Fluorescence intensity of the reaction product at various reaction time.

Effect of DABA concentration

The influence of the concentration of *m*-dansylaminophenylboronic acid (DABA) was studied in the range of 10-400 µM (Figure 7). The DABA concentration was proportional to the fluorescence intensity up to 90 µM. After that the intensity decreased due to the fluorescence quenching effect of excess DABA in the solution. The DABA concentration was kept constant at 90 µM in this work, which results in a mole ratio during 1: 90 to 1: 2 (BL: DABA).

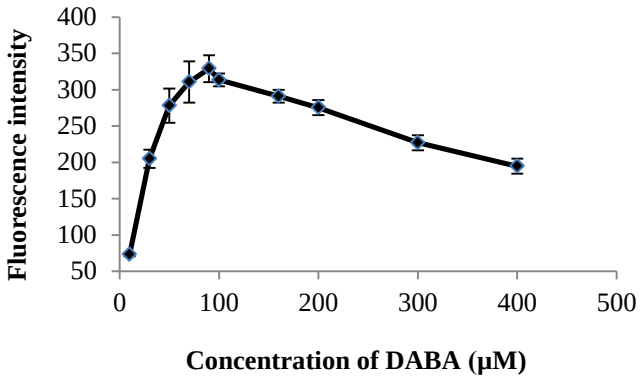

Figure 7. Fluorescence intensity of the reaction product at different DABA concentration.

Evaluation of method performance

By using the above procedure and optimal conditions, the calibration graph for BL was linear in the concentration range of 1-50 µM. The regression equation was found to be $F = 5.92C - 9.18$ (µM), with a coefficient of determination of 0.9966. For the standard addition, leaf debris bio-extract was used as a sample blank to determine the limit of detection (LOD) and the limit of quantification (LOQ) of the standard addition method. This blank solution was used because leaves were reported to contain less BRs (in the range of 0.01–0.1 ng/g), while pollens and immature seeds contain BRs in the range 1–100 ng/g [4]. The determined LOD and LOQ are 0.24 and 0.48 µM, respectively. Rice and rambutan bio-extract samples containing different levels of BL were used to evaluate the precision of the standard addition method. The relative standard deviation of intra-day experiments was determined by repeating the experiment trice in a day. It was found that the relative standard deviations were 8.1% and 11.3% for rice and rambutan bio-extract samples respectively (n=7). Measurements were made once a day for seven days for inter-day precision, their relative standard deviations were 11.8% and 12.9% for rice and rambutan bio-extract samples, respectively (n=7).

One assumption in this analysis method is that the specific fluorescent constant for all the brassinosteroids derivative compounds in the samples are the same and equal to that of the standard brassinolide derivative. The average fluorescent constant (peak height/mass) of 6 reported brassinosteroids was 1.1±0.5 [6]. Although individual fluorescent constants are likely to be similar; some differences are to be expected. For this reason the method should be considered semi-quantitative.

Analysis of bio-extract and liquid fertilizer by standard addition method

The recoveries of these samples based on the determinations of BRs equivalent to brassinolide were found to scatter in a large range (10-52 %). This could indicate that some matrix complex may interfere with the analyte signal (negative interference) causing inaccuracy of the determined concentration. The standard addition method was adopted to solve this problem. The results are shown in Table 1 for various samples.

Table 1. Determination of brassinolide in various samples (n=3) by standard addition method.

Samples	**BRs (µM)**
Rice	17.82 ± 1.09
Curcuma	2.70 ± 0.07
Sweet basil	2.92 ± 0.21
Longkong	4.28 ± 0.15
Star Gooseberry	4.59 ± 0.20
Dragon fruit	32.02 ± 3.61
Ginger	5.07 ± 0.20
Galangal	20.64 ± 1.09
Tomato	18.74 ± 0.71
Bai-ya-nang	13.20 ± 0.73
Rambutan	8.32 ± 0.56
Fertilizer: A	117.40 ± 11.20
Fertilizer: B	4.65 ± 0.76
Fertilizer: C	281.00 ± 28.40
Fertilizer: D	282.70 ± 4.60

Concusion

The proposed method has the advantage of being simple and fast. The consumption of reagents was low compared with chromatographic methods. Furthermore, it could be applied to the determination of brassinosteroids equivalent to brassinolide in bio-extract and liquid fertilizer samples by the standard addition method, which is a convenient strategy to deal with matrix effects in the quantification of fluorescence methods. Complex sample pretreatments were not necessary, requiring only filtration and dilution with deionized water appropriately. Spectrofluorometry was demonstrated to be a suitable semiquantitative technique and was applied successfully for the determination of total brassinosteroids plant hormones equivalent to brassinolide.

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