Journal of Animal and Veterinary Advances 11 (21): 3876-3881, 2012
ISSN: 1680-5593

Swainsonine Accumulation by Endophytic Undifilum Fungi in Liquid Media and Determined by Means of a Modified Enzymatic Assay

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Abstract: Endophytic *Undifilum oxytropis* found within locoweeds produces the poisonous alkaloid swainsonine which is responsible for locoism in grazing animals. A modified enzymatic assay for swainsonine in the mycelia of Undifilum fungi was developed. The standard equation was Y = 0.5169X- 0.1129 (regression coefficient r = 0.9985). The calibration graph of swainsonine was linear in the range 0.05-0.5 μg mL^{-1}. The method was validated for linearity, precision and accuracy. The modified method has been applied to explore the swainsonine accumulation properties in the endophytic Undifilum fungi in liquid media. Under the described culture condition, the mycelia weight reached a maximum value on the 20th day whereas the peak of swainsonine biosynthesis took place on the 28th day. The result indicated that the increment of mycelium weight and the swainsonine accumulation in the endophytic Undifilum fungi is not completely synchronous.

Key words: Enzymatic assay, Undifilum fungi, swainsonine accumulation, locoweeds, animals

INTRODUCTION

Locoweeds are toxic plants of the genera *Astragalus* and *Oxytropis* sp. that specifically contain the trihydroxy indolizidine alkaloid swainsonine (Cook *et al*., 2012; Ralphs *et al*., 1988). Consumption of locoweeds by grazing animals can result in chronic neurological disease-locoism which causes great economic losses to the pasture in China, the United States and other countries. Locoweed poisoning is the most widespread poisonous plant problem in China and the Western United States (James and Panter, 1989; Li and Wang, 2003). Swainsonine (Fig. 1) is considered as locoweed toxin and responsible for inducing locoism in animals (Molyneux and James, 1982; Zhao *et al*., 2009).

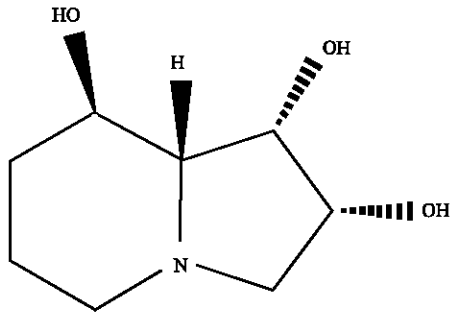

Fig. 1: Structure of the alkaloid swainsonine

Plant endophytic fungi are defined as the fungi which spend the whole or part of their lifecycle colonizing inter-and intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease (Zhao *et al*., 2011). In recent years, a swainsonine-producing endophytic fungi, *Undifilum oxytropis* has been isolated from locoweeds (Braun *et al*., 2003; Yu *et al*., 2010). Several studies have shown that there is a positive correlation between swainsonine concentration and the presence of *U. oxytropis* in locoweeds (Gardner *et al*., 2004; Ralphs *et al*., 2008). Another study has indicated that Undifilum endophytes of locoweed are within the coat and not the embryo of locoweed seed. The endophytic fungus is passed to the next generation through the locoweed's seed coat. When the seed coat is removed and the embryo germinated alone, the locoweed does not contain swainsonine or *Undifilum oxytropis* (Oldrup *et al*., 2010). Consumption of dried endophyte mycelia by rats induces toxic symptoms identical to those caused by consumption of locoweeds (McLain-Romero *et al*., 2004). Results of the above studies suggest that the endophyte Undifilumis responsible for swainsonine production in locoweed plants (Cook *et al*., 2012; Oldrup *et al*., 2010; Ralphs *et al*., 2008).

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Swainsonine was found to inhibit the α-mannosidase activity. Based upon its specific inhibitory properties, enzymatic assay procedures have been developed to determine swainsonine in *Metarhizium anisopliae*, serum and milk of the ewes (Sim and Perry, 1995, 1997; Taylor and Strickland, 2002). Several methods have been reported for the analysis of swainsonine including Thin-Layer Chromatography (Molyneux *et al*., 1991), capillary gas chromatography (James, 1989), LC-MS/MS Method (Gardner *et al*., 2001), HPLC-ELSD Method (Yang *et al*., 2012). Compared with the above methods, the enzymatic assay procedure has the advantage that it is convenient to carry out the enzyme inhibition assay in 96 well microtitre plates using a plate reader to measure the absorbance values. It is suitable for quantification of swainsonine in a large number of samples. In the process of optimization of the fermentation parameters of locoweed's endophytic Undifilum fungi and study its growth characteristic in liquid medium, a lot of Undifilum fungi samples need to be treated. It is convenient to determine swainsonine in a large quantity of Undifilum fungi samples using the enzymatic assay procedure. In the preliminary test, researchers constructed the standard curve according to the reported enzymatic assay procedure. researchers found that the regression coefficient R^2 of the standard curve was about 0.95. The degree of fitting is not very good. In addition, when the fungi strain of sample is different or the medium component is changed, it is necessary to verify the suitability of the enzymatic assay procedure or to modify the procedure. Therefore, the aim of the current study is to establish a modified enzymatic assay for the determination of swainsonine in the endophytic Undifilum fungus and explore the swainsonine accumulation properties in this endophytic fungus.

MATERIALS AND METHODS

Fungi and culture conditions: Fungal Endophytes of Locoweeds (FEL4-F5), isolated from locoweed sample and identified as *Undifilum oxytropis* in a earlier study (Yu *et al*., 2010) were used in this experiment. The fungal endophytes grew on Potato Dextrose Agar (PDA) plates at 20°C for 30 days for inoculation of liquid cultures. Liquid medium contained (per litter): 0.34 g peptone, 15.6 g sucrose, 0.6 g $MgSO_4$. The $7H_2O$, 0.6 g KCl, 0.02 g $FeSO_4$, 0.62 g K_2HPO_4. Prior to use the medium was autoclaved at 121°C for 30 min.

Reagents: Swainsonine, p-nitrophenyl-α-D-mannopyranoside substrate and α-mannosidase from jack bean were supplied by Sigma Aldrich (Sigma-Aldrich Co., LLC, USA). All other chemicals were of analytical grade where possible.

Dose-effect relationship between swainsonine concentration and α-mannosidase activity: Stock solution of swainsonine was prepared by dissolving 1.73 mg swainsonine in 1 mL dH_2O (10^{-2} mol L^{-1}). The standard solutions were prepared by dilution of stock working solution with the dH_2O to reach the concentration range of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} mol L^{-1} for swainsonine. The α-mannosidase activity was determined by the method described in the Enzymatic assay for swainsonine. The line chart of dose-effect relationship between swainsonine concentration and α-mannosidase activity was obtained by plotting the ratio of inhibited reaction rate to control mannosidase reaction rate versus swainsonine concentration.

Standard curve: Calibration graphs were constructed using six different concentrations ranging between 0.05 and 5 μg mL^{-1}. Each concentration was repeated three times. The assay was performed according to experimental conditions previously described. The regression curve was obtained by plotting the ratio of inhibited reaction rate to control mannosidase reaction rate versus negative logarithm (-log) of the swainsonine concentration.

Measurement of mycelia dry weight: Culture samples were washed with distilled water (triple the sample volume) and filtered through preweighed filters which had been dried at 80°C. The mycelia and filter paper were thereafter dried in an oven at 80°C until there were no further weight changes then cooled in a desicator to room temperature and re-weighed; the difference gave the mycelia dry weight.

Extraction of swainsonine from mycelia: Mycelium was dried at 80°C until no further weight loss was observed. Approximately 0.5 g of the dried mycelia was suspended in 10 mL of double distilled water in an Eppendorf tube. This was submerged in boiling water for 30 min and spun at 12000 rpm, 4°C for 15 min. The supernatant was collected and labeled as supernatant 1. The pellet was resuspended in dH_2O (same volume as first used), treated with ultrasonic (50 Hz, 10 min) then submerged in boiling water for 30 min and spun at 12000 rpm, 4°C for 15 min and this process repeated to produce supernatants 2-4. Supernatants 1-4 were pooled, freeze-dried and redissolved in 1 mL dH_2O.

Enzymatic assay for swainsonine: Supernatants were diluted as appropriate to ensure absorbance readings within the swainsonine calibration range. About 10 μL of the supernate (or swainsonine standard solutions) was transferred to aeppendorf tube (volume 1.5 mL) with 50 μL of citrate buffer (50 mM, pH = 4.5) and 20 μL of α-mannosidase enzyme (0.9 U m L^{-1}; Sigma Chemical Co.,) then incubated for 15 min at 37°C. Following incubation, 20 μL of ρ-nitrophenyl α-D-mannopyranoside (12 mM) was added to each well and then incubated for an additional 90 min. The reaction was stopped and color was developed with 200 μL of borate buffer (200 mM, pH = 9.8) added to each reaction. The final reaction volume (300 μL) was divided into three equal parts. Every part (100 μL) was was transferred to a 96 well plate. Optical density was determined at 405 nm (BIO-RAD Model 680 microplate reader, Bio-Rad Laboratories (UK) Ltd.). Four determinations were carried out for each sample of supernatant and averaged.

Fungal growth curve: Conical flasks (250 mL) containing 100 mL of liquid medium were inoculated with 12 plugs of endophytic Undifilum fungi (5.5 mm diam) taken from stock plates of the fungus growing on potato-dextrose agar (Oxenham *et al*., 2005). Flasks were placed in an orbital incubator set at 100 rpm and 18°C. For determination of the growth curve, three flasks were harvested every 4 days for 32 days. The mycelia dry weight and swainsonine content were determined by the methods earlier described.

RESULTS AND DISCUSSION

Dose-effect relationship between swainsonine concentration and α-mannosidase activity: The inhibition of α-mannosidase by swainsonine is quite complex. Figure 2 shown results obtained by incubating jack-bean α-mannosidase with various concentrations of swainsonine. It shows a sigmoidal relationship between swainsonine concentration and the ratio of inhibited reaction rate to control mannosidase reaction rate in the concentration range of 10^{-7} to 10^{-4} mol L^{-1} for swainsonine. Similar results were obtained by Dorling *et al*. (1980) when they studied the inhibition of lysosomal α-mannosidase by swainsonine. It can be seen from the Fig. 2 that there was a linear correlation within a swainsonine concentration range of 0.5×10^{-5} to 0.5×10^{-6} mol L^{-1}. But in the Fig. 2, the increase extent of the value on the X-axis is uneven. It is exponential growth. Therefore, if researchers suitably transform the value on the X-axis, it is possible to construct a calibration curve for swainsonine assay based upon the linear correlation within a swainsonine concentration range of 0.5×10^{-5} to 0.5×10^{-6} mol L^{-1}.

Standard curve for swainsonine assay: Calibration graphs were constructed using six different concentrations ranging between 0.05 and 5 μg mL^{-1}. Each concentration was repeated 3 times. The assay was performed according to experimental conditions previously described. The regression curve was obtained by plotting the ratio of inhibited reaction rate to control mannosidase reaction rate (V_i/V_c) versus negative logarithm (-log) of the swainsonine concentration. The regression equation was Y = 0.5169 X- 0.1129 and the regression coefficient (r) was 0.9985. Y was the ratio of inhibited reaction rate to control mannosidase reaction rate (V_i/V_c), X was the negative logarithm (-log) of the swainsonine concentration. The precision was evaluated by the repeated determination of a sample for six times. The Relative Standard Deviation value (RSD) of swainsonine concentration was 3.41%. In order to determine accuracy, the known amount of swainsonine was added into the fungal endophyte samples and the resultant solutions were analyzed by the enzymatic assay method. The results were shown in Table 1. The recovery of swainsonine was between 97.79 and 104.14% and RSD was between 2.17 and 5.09%.

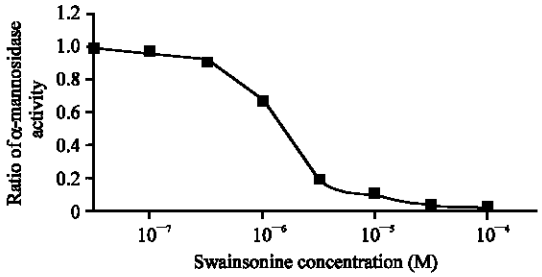

Fig. 2: Effect of swainsonine concentration on α-mannosidase activity

Table 1: Recovery studies of swainsonine added to selected samples of fungal endophyte

Swainsonine in fungi samples supernatant (F5) (μg) (mean±SD, n = 5)	Pure swainsonine added (μg)	Swainsonine (μg)			
		Expected	Actual (mean±SD, n = 5)	Recovery (%)	RSD (%)
0.0823±0.0026	0.1053	0.1876	0.1920±0.0037	104.14	3.39
0.0823±0.0026	0.1447	0.2270	0.2250±0.0073	98.59	5.09
0.0823±0.0026	0.2601	0.3424	0.3366±0.0055	97.79	2.17

SD: Standard Deviation; RSD: Relative Standard Deviation

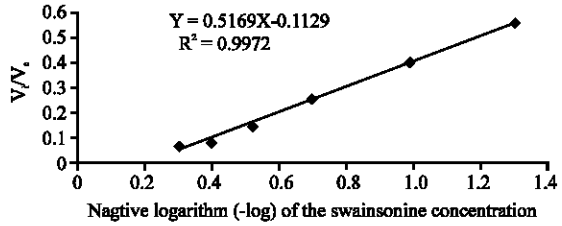

Fig. 3: Calibration curve for swainsonine assay. The calibration curve was obtained by plotting the ratio of inhibited reaction rate to control mannosidase reaction rate (V_i/V_c) versus negative logarithm (-log) of the swainsonine concentration

It can be seen from the Fig. 2 that the range of swainsonine concentrations that can be used in the assay is narrow. The disadvantage of enzymatic assay procedure is that samples need to be diluted properly to give concentrations within the narrow calibration range. Furthermore, the method is very sensitive. Even if a very slight error in operation will have an obvious impact on the test results. In order to obtain a reproducible and accurate test result, accurate pipetting is crucial. Care is required to be taken to reduce the influence of operation errors on the final results. Nevertheless, the enzymatic assay procedure has the advantage that it is convenient to carry out the enzyme inhibition assay in 96 well microtitre plates using a plate reader to measure the absorbance values. It can treat many samples at a time. Therefore, it is suitable for quantification of swainsonine in a large number of samples using the enzymatic assay procedure. In addition, the assay relies on the ratio of two enzyme activity measurements and so variations in ambient temperature, etc. from day to day that might affect the absolute enzyme activities will not affect the ratio of activities (Sim and Perry, 1995). In the current study, the value on the X-axis was transformed as negative logarithm (-log) of the swainsonine concentration based on dose-effect relationship between swainsonine concentration and α-mannosidase activity. The degree of fitting of standard curve is acceptable. The method was validated for linearity, precision, accuracy. Application of the developed enzymatic assay procedure to determine swainsonine content in the endophytic Undifilum fungi sample ensured its suitability.

Fungal growth curve: The growth curve was determined by taking the days of cultivation as the abscissa and the mycelia dry weight and swainsonine concentration in the dry mycelia as the ordinate (Fig. 3). As shown in Fig. 3, endophytic Undifilum fungi FEL4-F5 grew slowly and reached a peak on the 20th day. After this the mycelia

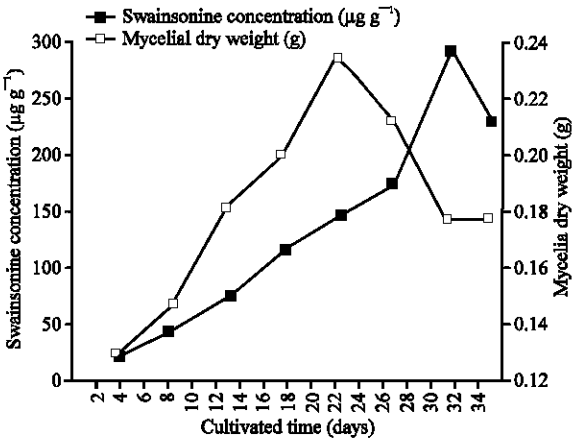

Fig. 4: Relationship between mycelia dry weight and swainsonine concentration during the growth of Endophytic Undifilum fungi FEL4-F5 on rotary shaker (100 rpm) at 18°C over 32 days

weight decreased quickly which might be caused by exhaustion of the nutrients in the medium after such a long period of culture. The fungus began to recycle cellular components of older cells for energy purposes. The curve of the swainsonine concentration in mycelia shows that swainsonine concentration increased slowly during the first 20 days but then increased rapidly to reach a maximum value on the 28th day.

Endophytic Undifilum fungi isolated from locoweeds grew very slowly (0.03-0.34 mm day^{-1}) on solid medium and had a thin (2-8 mm diam) septate mycelium. The color of these fungal cultures change from the initially white to greyish will take 10-15 weeks (Braun *et al*., 2003). Biosynthesis of swainsonine in locoweed's *Undifilum oxytropis* is a complicated and consecutive chemical process that takes place in the fungi cells. Many intermediate metabolites are involved in the formation of swainsonine. The variety and content of intermediate metabolites are in a constant state of flux in the whole metabolic process of fungi. The growth cycle of endophytic Undifilum fungi is quite long. Which phase is the peak of swainsonine biosynthesis in the fungi? From the results described earlier (Fig. 4), researchers can see that the increment of mycelium weight and the swainsonine accumulation in the endophytic Undifilum fungi is not completely synchronous. The mycelia weight reached a maximum value on the 20th day but the peak of swainsonine biosynthesis took place on the 28th day. This research helps us to treat samples, catch and analyze swainsonine and its early metabolic precursors in cultures of locoweed's endophytic fungi at the right time. The following tests have proved that this research laid a

foundation for investigation the key steps in the course of biosynthesis swainsonine in Undifilum endophytes of locoweed by isotopic tracer.

CONCLUSION

A modified enzymatic assay for poisonous alkaloid swainsonine in the mycelia of endophytic Undifilum fungi isolated from locoweeds has been developed. The modified method has been applied to explore the swainsonine accumulation properties in the endophytic fungi in liquid media. The result of the test indicated that the increment of mycelium weight and the swainsonine accumulation in the endophytic Undifilum fungi is not completely synchronous. Under the described culture condition, the mycelia weight reached a maximum value on the 20th day but the peak of swainsonine biosynthesis took place on the 28th day.

ACKNOWLEDGEMENT

The researchers wish to acknowledge the National Natural Science Foundation of China, project number 30871901 for financial support.

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