Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties

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Abstract
A total of 194 fungal endophytes were isolated from wild medicinal plants in Thailand. Cancer-inhibitory asparaginases from 15 selected endophytic strains were tested for their anti-proliferation activity against two cancer cell lines. The endophytic fungi were asparaginase sources which were devoid of glutaminase activity. In vitro experiments showed that asparaginase from the most important strain Colletotrichum sp. E5T9 completely decreased the survival rate of CaCo2 human Caucasian colon adenocarcinoma and decreased the survival rate of HepG2 human Caucasian hepatocyte carcinoma by 70%. When a crude culture of Colletotrichum sp. E5T9 was applied to the L929 connective tissue line, 42.66% and 44.32% cell viability resulted on first and second day, respectively. After the third day, the L929 connective tissue line, treated with Colletotrichum sp. E5T9 were able to grow at normal levels with more than 97% cell viability.

Keywords: endophytic fungi, Thai medicinal plants, asparaginase, anti-cancer, cytotoxicity.

INTRODUCTION
Asparaginase is an enzyme which is used as a chemotherapeutic agent in the treatment of human cancer and tumor diseases (Nakamura et al., 1999; Long et al., 2002; Stams et al., 2003; Lorenzi et al., 2006). Asparaginase catalyses the conversion of asparagine to aspartic acid and ammonia. Asparagine is a nutritional requirement of both normal cells and cancer cells (McCredie et al., 1973). Low levels of the nonessential amino acid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirements for asparagine (Haley et al., 1961; Mitchell et al., 1994) This is because normal cells produce asparagine synthetase, which is able to synthesize asparagine from aspartic acid, whereas, in cancer and tumor cells this enzyme is present at low levels (Nakamura et al., 1999). Asparaginase is produced from a variety of microbial sources including fungi (Gulati et al., 1997; Serquis et al., 2004) yeasts (Dunlop et al., 1980; Ferrara et al., 2006) and bacteria (Maladkar et al., 1993; Geckil and Gencer 2004) Asparaginase from Escherichia coli and Erwinia sp. are usually used as anti-tumor and anti-leukemia agents. Nevertheless, the utilization of asparaginase from these sources was initially limited due to their potential toxicity and several side effects (McCredie et al., 1973). Another need for asparaginase is the frequent requirement of the patient, because of the short half-life and instability of this drug in the treatment process. This results in side effects in patients (Li et al., 2007). Furthermore, asparaginase contaminated with glutaminase activity causes depletion of glutamine in the blood (Spittler et al., 1997). Low glutamine in the blood may lead to serious effects on biochemical functions in the body of patients (Spittler et al., 1997; Derst et al., 2000).

Endophytes from medicinal plants have become a hot topic for metabolite discovery because of their high biodiversity and predicted potential to produce novel compounds (Tomita 2003; Kumar and Hyde 2004; Tajerski et al., 2007; Huang et al., 2008; Hyde and Storey 2008; Raghukumar 2008; Mitchell et al., 2008). In this study we therefore isolated endophytic fungi from Thai medicinal plants and they were tested for their ability to produce asparaginase. The experimental data for asparaginase from these endophytic fungi for in vitro cancer treatment is presented in this paper.
MATERIALS AND METHODS

Sources of endophytic fungi
Endophytic fungi were isolated from fresh material of apparently healthy wild Thai medicinal plants. *Adenanthera microsperma*, *Betula alnoides*, *Cassia alata*, *Hiptage benghalensis*, and *Houttuynia cordata* were collected from a natural forest located at the Queen Sirikit Botanic Garden, Mae Rim District in 2007. *Eupatorium odoratum* and *Stemon tuberosa* were collected from San Sai and Doi Saket in Chiang Mai Province in 2007 and 2008 respectively.

Isolation of endophytic fungi
The protocol for isolation follow methods used in other endophyte studies (Bussabarn et al., 2001; Sánchez et al., 2007; Sánchez et al., 2008; Wei et al., 2007; Rungjindamai et al., 2008; Oses et al., 2008) but adjusted for the specific plant tissues used here following pilot experiments. The plant tissues were washed in running tap water for one hour. Fifty segments of leaves from each plant were cut into 5 mm² pieces, including a vein (25 samples) and intervein (25 samples). 25 segments of branches were then cut randomly to a length of 5 mm. Endophytic fungi were isolated from the bark of the perennial plants *Adenanthera microsperma*, *Betula alnoides* and *Hiptage benghalensis*. Twenty five segments (5 mm long) were cut from the stems and the roots of *Houttuynia cordata*. In total 650 segments of plant material were treated by a triple surface sterilization technique (Taylor et al., 1999; Bussabarn et al., 2001). Each piece was then placed on malt extract agar [malt extract (20 g/l), rose bengal (0.033 g/l), chloramphenicol (50 mg/l), agar (15 g/l)]. All plates were incubated at 30°C until fungal mycelium grew out performing a reverse side of plant tissue. Half strength PDA was used for subculture and stock culture.

Identification of endophytic fungi
Identification was based on colony and hyphal morphology of the fungal cultures, characteristics of the spores (Ellis 1971; Carmichael et al., 1981; Sutton 1971; Von 1981; Hyde et al., 2000) and ITS regions gene homology (Promputtha et al., 2007).

DNA extraction
Total genomic DNA was extracted from fresh mycelium of 3 days old *Colletotrichum* sp. EST9 colony according to modification of the rapid preparation of DNA from filamentous fungi (Jeewon et al., 2003; Jeewon et al., 2004; Lacap et al., 2003; Zhang et al., 2008).

ITS-rDNA amplification and sequence analysis
Primer ITS4 (5'-TCCTCCGCTTATGTAGATGC-3') and ITS1 (5'-TCCGTAAGGTGGAACCTGCGG-3'). ITS-rDNA were referenced by the method of White et al., (1990). The PCR reaction products were examined by electrophoresis in 1% (w/v) agarose gel and the bands stained with ethidium bromide. PCR products were purified using the PCR clean-up gel extraction (NucleoSpin® Extract II) and sequenced by using the primer pair ITS4 and ITS1. The database from National Center for Biotechnology Information website was used to compare with the sequences, the sequences were submitted to GenBank.

Assay for asparaginase and glutaminase
Qualitative analysis of asparaginase
All endophyte isolates were cultured on PDA for 7 days. A 5 mm disk of mycelium was then transferred to the test agar media. The agar plate assay was routinely used for the screening of asparaginase production. The plates contained Modified Czapex Dox’s (MCD) agar [glucose (2.0 g/l), L-asparagine (10.0 g/l), KH₂PO₄ (1.52 g/l), KCl (0.52 g/l), MgSO₄.7H₂O (0.52 g/l), CuNO₃.3 H₂O (0.001 g/l), ZnSO₄.7H₂O (0.001 g/l), FeSO₄.7H₂O (0.001 g/l)] (Saxena and Sinha 1981) supplemented with phenol red (0.009% final concentration) as the indicator. Control plates were MCD agar without asparaginase. All the plates were incubated at 30°C. Pink zone radius and colony diameter were measured after 5 days incubation.

Quantitative analysis of asparaginase and glutaminase
A 5 mm disc of mycelium from agar plate cultures was used as inoculum in each tube. All cultures were incubated at 30°C at 120 rpm for 5 days. The activity of enzyme was determined in MCD culture filtrates by Nesslerization as described by Imada et al., (1973). One unit of asparaginase is the amount of enzyme which catalyzes the formation of 1 μmol of ammonia per min at 37°C. The reaction mixture from each isolate was assayed in triplicate. The glutaminase activity was measured according to modification of Beutler et al., (1985). 0.5 ml of 60mM glutamine in 0.05M tris (hydroxymethyl)aminomethane (tris-HCl) (pH 7.2) was pre-incubated at 37°C for 3 min. Then, 0.5 ml MCD culture filtrates were added and incubated at 37°C for 1 hr. The reaction was stopped by cooling the samples on ice. Glutamate from the reaction mixture was measured by using the colorimetric method (Glutamate assay kit, BioVision, USA). One unit of glutaminase activity is the amount of enzyme which catalyzes the formation of...
Asparaginase production by endophytic fungi

In vitro assay for cytotoxicity activity

Cell lines and culture condition

Cytotoxicity activity of asparaginase from selected endophytic fungi was evaluated in a cell culture system using cells from CaCo2 human Caucasian colon adenocarcinoma, HepG2 human Caucasian hepatocyte carcinoma and L929 mouse connective tissue cell line. Two cancer cell lines, HepG2 and CaCo2 were cultured in MEM (Minimum essential medium eagle) supplemented with 10% fetal calf serum and 40 μg/ml gentamycin. Normal cell line, L929 was cultured in DMEM (Dulbecco’s modified eagle’s medium) supplemented as above media. All cell lines were grown at 37 °C in an atmosphere containing 5% CO2. Viable cell numbers were assessed and counting the number of cells which were not stained by trypan blue using a haemacytometer. The optimal seeding densities of 1 x 10^4 CaCo2 cells, 1 x 10^5 HepG2 cells and 6 x 10^5 L929 cells were seeded into each well of 96 well plates and incubated at 37 °C to allow cell attachment. After 24 hours, 100 μl MCD culture filtrates of selected endophytic fungal isolates containing an asparaginase were added to the plates in six replicates. MCD broth was added as a control well.

Cytotoxicity assay

Cytotoxicity assays against human cancer cell culture in vitro were performed by SRB (Sulphorhodamine) assay (Houghton et al., 2007) This colorimetric assay is based on the uptake of negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) and basic amino acids in the cells. The cells were then treated with asparaginase at selected exposure times of 24, 48, 72 and 96 hours. The cells were fixed with ice-cold TCA for 1 hr at 4°C, the TCA was removed and plates were washed five times in distilled water and allowed to air dry. The cells were incubated with 50 μl SRB dye at room temperature for 30 min. The unbound dye of sulphorhodamine (SRB) solution was then removed by washing the plates quickly with 1% v/v acetic acid five times. The bound SRB were solubilised by adding 100 μl of 10 mM unbuffered Tris Base (pH 10.5) to each well and shaking for 5 min. The plates were read in a 96-well plate reader at wavelength 492 nm. The percentage of cell viability was calculated by using absorbance compared to control (non-treated cell).

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\% \text{ cell viability} = \frac{OD \text{ of treated cells}}{OD \text{ of control cells}} \times 100
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If asparaginase resulted in cell viability of 50% or less, this was defined as a positive test for sensitivity, and more than 50%, was defined as a negative test.

RESULTS

Isolation of endophytic fungi

A total of 194 fungal isolates were obtained from 650 segment samples from seven wild Thai medicinal plants. 15 asparaginase producing isolates were selected and included Colletotrichum sp. (6 isolates), Fusarium sp. (1 isolate), Penicillium sp. (3 isolates), Eupenicillium sp. (1 isolate) and Talaromyces sp. (1 isolate), which were identified based on their morphological characteristics. Non-sporulating endophytic fungi were organized into 3 morphospecies of mycelia sterilia, based on their culture characteristics (Lacap et al., 2003). Sequence analysis of the ITS1-5.8s-ITS2 gene of Colletotrichum sp. EST9 (accession number FJ480405 in GenBank) showed it to be closest in sequence similarity (99%) to Glomerella cingulata isolate P042 (accession number EF423527 in GenBank) (Gibbert and Webb 2007).
Asparaginase production by endophytic fungi

Assay for asparaginase

Qualitative analysis
All endophytic fungi could grow on MCD agar with phenol red, a dye indicator that changes from yellow (acidic condition) to pink (alkaline condition). The pink zone around each fungal colony indicated changes in pH which originated from ammonia accumulation in the medium. 82 isolates gave a positive result in the agar plate assays; 61 isolates of which produced a pink zone around the colonies and 21 isolates produced a pink zone within the colonies. The preliminary selection showed that Colletotrichum sp. E1T9 and mycelia sterilia sp. E3T3 demonstrated high asparaginase activity of 1.523 and 1.530 unit/ml, respectively. 15 asparaginase producing isolates included Colletotrichum sp. E1T6, E1T9, E5T5, E5T9, E5T10 and E5T29, Fusarium sp. E5T28, Penicillium sp. E5T3, E5T4 and E5T24, Eupenicillium sp. E5T6, Talaromyces sp. E5T27 and mycelia sterilia E2T1, E3T3 and E5T12 with which high asparaginase activity (Table 1) were selected as a potential group to test against cancer cell lines. The glutaminase activity from these 15 asparaginase producing isolates were also determined. Penicillium sp. E5T4 and Talaromyces sp. E5T27 had no glutaminase activity and 13 isolates had very slight glutaminase activity in the range of 0.01 to 0.07 unit/ml (Fig. 1).

Figure 2: Effect of asparaginase on the growth of (a) HepG2 and (b) CaCO2. Cells of 1x10^4 were cultured with or without asparaginase at 37 °C for 4 days in a CO2 incubator. 24 hours : 48 hours : 72 hours : 96 hours :

Cytotoxicity activity of asparaginase
After cells were incubated for selected exposure times of 24, 42, 72 and 96 hours, the effect of asparaginase was evaluated for their in vitro cytotoxicity against cancer and normal cell lines by SRB assay (Houghton et al., 2007). The percentage of cell viability based on the optical density value from the incubated control well was calculated. After 96 hours, the treatment of HepG2 and CaCO2 cell lines with asparaginase resulted in 31.43 to 228.57 % and 0 to 69.31 % of cell viability, respectively (Fig 2). Mycelia sterilia E3T3 had the highest enzyme activity but with little anti-proliferation activity. Asparaginase from this isolate induced the growth of cancer cells and stimulated HepG2 cell viability to 134.29% and also demonstrated the lowest inhibition against CaCo2 cell lines. Colletotrichum sp. E5T5 and Colletotrichum sp. E5T9 were positive for HepG2 cell antiproliferation. All selected fungal isolates, except mycelia sterilia E3T3

Quantitative analysis
The 82 asparaginase positive isolates were tested for their asparaginase activity in liquid condition using a Nesslerization assay. Fifty-three of the isolates exhibited asparaginase activity in the range of 0.014 to 1.530 unit/ml (Table 1 [Supplementary data]). Colletotrichum sp. E1T9 and mycelia sterilia sp. E3T3 demonstrated high asparaginase activity of 1.523 and 1.530 unit/ml, respectively. 15 asparaginase producing isolates included Colletotrichum sp. E1T6, E1T9, E5T5, E5T9, E5T10 and E5T29, Fusarium sp. E5T28, Penicillium sp. E5T3, E5T4 and E5T24, Eupenicillium sp. E5T6, Talaromyces sp. E5T27 and mycelia sterilia E2T1, E3T3 and E5T12 with which high asparaginase activity (Table 1) were selected as a potential group to test against cancer cell lines. The glutaminase activity from these 15 asparaginase producing isolates were also determined. Penicillium sp. E5T4 and Talaromyces sp. E5T27 had no glutaminase activity and 13 isolates had very slight glutaminase activity in the range of 0.01 to 0.07 unit/ml (Fig. 1).
Asparaginase production by endophytic fungi

and *Penicillium* sp. E5T6, showed positive activity for CaCO2 cell antiproliferation. *Colletotrichum* sp. E5T9 showed potential to be a good source of asparaginase since it produced 100% inhibition of the proliferation of CaCO2 and inhibited proliferation of HepG2 by 70%. The effect of asparaginases from the 15 isolates on normal cell lines occurred in a similar way. Within 48 hours, asparaginase and normal cell lines had reciprocal effects in several levels until 22.38-97.90% and 19.32-51.14% on first and second day, respectively. Asparaginase from *Colletotrichum* sp. E5T9 reduced normal cell viability to 42.66% and 44.32% on the first and second days respectively. On the third day, L929 cell line recovered to grow at normal levels (Fig. 3).

**DISCUSSION**

Genetic recombination between endophytic fungi and their host plants may occur during evolution (Tan and Zou 2001). Medicinal plants could produce valuable bioactive phytochemicals. Thus, the endophytic fungi isolated from these plants may produce rare and important bioactive compounds as in their host plants. In the past, most information on endophytic fungi was limited to identification and classification (Sánchez et al., 2007; Sánchez et al., 2008; Wei et al., 2007; Rungjindamai et al., 2008; Oses et al., 2008; Hu et al., 2007). It was discovered that endophytic fungus, *Taxomyces andreanae* which was isolated from *Taxus brevifolia* (Pacific Yew) could produce taxol, a product as the host (Stierle et al., 1993; Stierle and Strobel 1995; Strobel et al., 1993). This compound is extensively used to treat human proliferating diseases (Rowinsky and Donehower 1991; Slichenmyer and Von 1991). Taxol and its derivatives are the major group of drugs that are used as anti-cancer treatments. In recent years, medicinal plants have been researched as a potential new source of endophytic fungi that may produce taxol and other anti-cancer agents and medically useful compounds (Kumar and Hyde 2004; Tajesi et al., 2007; Huang et al., 2008; Hyde and Soytong 2008; Raghukumar 2008; Mitchell et al., 2008).

Taxol production was discovered from *Bartalinia robillardoides* Tassi which was isolated from the medicinal plant *Aegle marmelos* (Gangaden and Muthumary 2008). The endophytes *Rhinocladiella* sp. from *Trimerigium wilfordii* were found to produce three new chalasins that inhibited cell division of ovarian and colon tumor cell lines (Wagenaar et al., 2000). Crude extract from endophytic fungi from *Camptotheca acuminata* were found to be cytotoxic towards human tumor cell lines (Lin et al., 2007). Another compound that has anti-proliferation mechanisms against human cancer cell lines is the enzyme asparaginase. There are few reports of asparaginase being produced by endophytic fungi and no observation of such endophytes used in human cytotoxicity *in vitro* test.

In this experiment, agar plate assays and spectrophotometric methods are compared and it was found that some isolates had no enzyme activity despite producing a large positive-pink zone in the agar plate assay. Thirty-two isolates produced a positive zone on agar plates but they had no enzyme activity in the Nesslerization assay. This finding are similar to the earlier work of Hölker et al., (2004) who suggested that the ability of fungi to produce enzyme was different in solid and liquid state. As a consequence of this result we only selected endophytic fungi that had high enzyme activity in liquid substrates to assay against the two cancer cell lines.

Patients treated with asparaginase and having glutaminase activity developed lower glutamine levels in the blood (Spittler et al., 1997). This situation may be problematic and result in serious side effects such as the reduction of DNA synthesis, incomplete liver function and an impaired immune system (Spittler et al., 1997; Derst et al., 2000). Cancer-inhibiting asparaginase should therefore react with asparagine but not glutamine in the blood. The asparaginases produced by the endophytic fungi in this experiment had very low glutaminase activity. When compared glutaminase and asparaginase activity; glutamate were detected at 72 hours : 96 hours.

**Figure 3:** Cytotoxicity of asparaginase against L929 mouse connective tissues. Cell of 1x10³ were cultured with or without asparaginase at 37 °C for 4 days in a CO₂ incubator. 
24 hours : 
48 hours : 
72 hours : 
96 hours.
the nanomole level, while ammonia were detected at the micromole level. Thus, if we applied asparaginase from this fungal source, patients would not develop side effects from glutaminase activity.

The enzyme incubation time against the cancer cell lines showed that asparaginase from some of the endophytes displayed stable inhibition while others were not stable. Only asparaginase from *Colletotrichum* sp. E5T9 could completely inhibit the CaCO2 cell line within 48 hours and still maintain activity until 96 hours. No isolates demonstrated complete inhibition of HepG2 cancer cell lines. Nevertheless, the crude culture from *Colletotrichum* sp. E5T9 resulted in the lowest percentage HepG2 cell viability even though it gave the lowest enzyme activity among the 15 fungal isolates.

The cytotoxicity of crude asparaginases towards L929 mouse connective tissues was evaluated to assess the possibility for development of these enzymes as anti-cancer drugs. Asparaginases from 15 endophytic strains were cytotoxic towards L929 mouse connective tissues and reduced cell viability by more than half on the second day. In this experiment, crude enzymes were used to treat cancer and healthy cells. Due to the fact that in crude enzymes have both asparaginase and several unknown compounds, these compounds may concern with the inhibition of both cancer and healthy cells proliferation. It is therefore recommended that asparaginase from *Colletotrichum* sp. E5T9, a potential drug in cancer anti-proliferation, should be purified. Purified asparaginase can then be assayed and may solve the problems of cancer inhibition and normal cell cytotoxicity. Asparaginase from *Colletotrichum* sp. E5T9 may be relatively thermostable and thus minimize allergic reactions in patients that are continually receiving injections of asparaginase in cancer treatment. These have been no reports on the half life in vivo and in vitro human cancer treatment of asparaginase from fungi. However, the thermostable asparaginase has the advantage in production, storage and denaturation between transportation and treatment (Li et al., 2007).

Understanding plant/endophyte relationships may provide an informed method for isolating endophytic fungi that produce a variety of bioactive compounds (Hyde and Soytong 2008). Plants from unique environments or plants which have medicinal history should be considered as promising sources of novel endophytes and novel compounds (Huang et al., 2008). *Colletotrichum* species are often isolated as endophytes (Photita et al., 2005) and are also plant pathogens (Shenoy et al., 2007; Than et al., 2008). Future studies should target other *Colletotrichum* species or isolates from different habitats to establish if they have similar asparaginase production and whether the asparaginase is medically useful.

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Asparaginase production by endophytic fungi


