Journal of Medicinal Plants Research Vol. 6(44), pp. 5599-5603 17 November, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10-991
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Effect of hexane extract of *Dillenia indica* seed on the activity of secreted aspartyl proteinase of *Candida albicans* and its kinetic studies

K. Himakar Reddy[1]*, Patrick Govender[1], O. V. S. Reddy[2] and P.V. G. K. Sarma[3]

[1]Department of Biochemistry, University of KwaZulu-Natal, Durban-4000, South Africa
[2]Department of Biochemistry, Sri Venkateswara University, Tirupati-517 502, India.
[3]Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences, Tirupati, India

Accepted 31 January, 2011

Secreted aspartyl proteinase (Sap), an extracellular enzyme of *Candida albicans,* is implicated as a pathogenic factor that plays a vital role in invading and colonizing the host tissue under predisposing conditions. The present study was taken up to study the inhibitory effect of hexane extract of *Dillenia indica* seed on Sap and its enzyme kinetics. Sap activity was determined spectrophotometrically at 280 nm using BSA. The enzyme kinetics of Sap showed velocity (Vmax) of 0.058 µg/mg/min and Km 0.174 µM. However *C. albicans* grown in different concentrations of hexane extract of *D. indica* totally inactivated the enzyme, which was further confirmed by SDS-PAGE.

Key words: *Dillenia indica*, *Candida albicans,* secreted aspartyl proteinase, Michaelis-menten, Hanes-Woolf.

INTRODUCTION

Candida infections are problem of growing clinical importance. The incidence of infections has increased dramatically over the past three decades and this trend will inevitably continue in the 21st century, causing responsible for increasing morbidity and mortality in the immunocompromised host (Naglik et al., 2003). *Candida albicans* is a ubiquitous, most common fungal pathogen of humans and etiological agent of candidiasis. It has become the fourth most commonly encountered nosocomial pathogens in blood stream infections in the United States, and candidiasis is associated with mortality rates as high as 60% (Smith, 2005; Pfaller et al., 1998). According to National AIDS Control Organization (NACO, India) the year 2007 statistics, fungal infections occupy 2nd place constituting 57.5% in immunocompromised patients after tuberculosis (65%). Invasive *Candida* infections are serious and often fatal nosocomial disease in immuno-compromised patients.

Virulence factors are pivotal in pathogenesis of most organisms, which includes extra and intra cellular enzymes for their morphological switching and biofilm formation. The virulence factors expressed or required by *Candida* species, and in particular *C. albicans*, to cause infections may well vary depending on the type of infection (that is, mucosal or systemic), the site and stage of infection, and the nature of the host response. In the last few decades, it has been demonstrated that Sap, an extracellular enzyme is important virulence factors for several types of *C. albicans* infections and that inhibition of these proteinases have a protective effect for the host (De Bernardis et al., 2001; Hube and Naglik, 2001). Sap is capable of degrading epithelial and mucosal barrier proteins of host tissue under predisposing conditions. It can inhibit *C. albicans* adherence and attenuate mucosal infections.

Adhesion of *Candida* to host tissues allows the fungus to attain a foothold and to colonize a specific niche environment. Under suitable predisposing conditions when the host is compromised, this colonized site provides the base for candidal proliferation, invasion and, in some instances, dissemination (Naglik et al., 2003). Drug-resistant

*Corresponding author. E-mail: himakarreddybiochem@gmail.com

Candida infections are likely to pose a serious therapeutic challenge over the next few decades. Though research has lead to the development of newer investigational antifungals, only few have been licensed for use. Furthermore, it has been found that treatment with these drugs, especially for extended periods can lead to toxicity or development of resistant organisms during the course of therapy. Consequently, discoveries of novel drug classes are crucial aims of antifungal research. One of the opportunities for the development of new compounds active against *Candida* species includes the development of drugs directed against the *Candida* proteinases (Copping et al., 2005). Medicinal plants have been the basis for the treatment of various infectious diseases in traditional medicine, and possess a variety of compounds of known therapeutic properties. Plant-derived compounds are gaining much importance in pharmaceutical and therapeutically applications as there are usually fewer side effects and are not very toxic (Himakar et al., 2010a). Zhang et al. (2002) showed that the ethanol extract of *Lycopodium cernuum* inhibited the activity of Sap in *C. albicans*. As part of a wider ranging survey of possible links between antifungal exposure and expression of virulence factor in *C. albicans* the present study was taken up.

Dillenia indica plant belongs to family *Dilleniaceae*, commonly called as Dillenia and it is distributed in India, Nepal, Bangladesh and Sri Lanka. The fruit ripens in the month of November and December. Green calyx parts are eaten in various forms of pickles (Abdille et al., 2005). Locally (Telugu name) it is called as *Uvya* or *Kalinga* and its trade name is *Chulta.* The antimicrobial activity and toxicity of hexane extract of *D. indica* seed was reported by the authors (Himakar et al., 2009, 2010b). Preliminary studies showed that hexane extract of *D. indica* seed exhibited anticandidal activity at 0.7 mg/ml concentration by acting on cell wall (unpublished data). The main aim of the present study was to determine the effect of hexane extract of *D. indica* seed on the activity of Sap which plays a major role in invading and colonizing the host tissue. Kinetics of Sap was studied to determine the Vmax and Km of the enzyme.

MATERIALS AND METHODS

Plant material and solvent extraction

Seeds of *D. indica* were procured from Gautam Global, Dehradun (India). The seeds were authenticated by Dr.N.Yasodamma, Department of Botany, S.V.University, Tirupati, India. Hexane extract of *D. indica* seeds was prepared by taking (100 g) seed powder and soxhlated for 3 h with hexane and excess solvent was distilled off. The yield of the extract was 10.6% (v/w).

Secreted aspartyl proteinase (Sap) production

Sap production was according to method of Kwon-Chung et al. (1985). Log phase cultures of C. *albicans* was grown on Yeast extract, Peptone, Dextrose (YEPD) broth. The cell pellets were washed with sterile distilled water, and 10^6 washed cells were then inoculated into flasks (125 ml) containing 20 ml of bovine haemoglobin (BH) broth and incubated on a shaker at 37°C for a total of 65 h. Culture (1 ml) was drawn periodically and centrifuged at 3,000 rpm in a clinical centrifuge for 10 min at 25°C to obtain the culture supernatant. The culture supernatant was the source of the Sap.

Enzyme assay

Sap activity was determined spectrophotometrically following the digestion of bovine serum albumin (BSA) as the substrate described by Morrison et al. (2003). Sap (0.2 ml) was mixed with substrate (0.8 ml of 1% BSA in 0.025 M sodium citrate buffer, pH 2.5) and incubated at 37°C for 3 h. The reaction was halted by the addition of 2.0 ml of 5% trichloro acetic acid (TCA) resulting in precipitation of BSA. The tubes were kept at 4°C overnight and centrifuged at 2000 rpm for 20 min. Proteolysis was determined by measuring the absorbance of the soluble peptides at 280 nm. For control, substrate was added to the culture supernatant and immediately treated with TCA. The absorbance of controls was subtracted from test samples to obtain values for enzyme activity. The experiments were repeated four times and mean (±SD) of the readings was determined.

Effect of hexane extract of *D. indica* seed on Sap

The log phase of *C. albicans* culture treated with different concentrations (0.5 to 2 mg/ml) of hexane extract of *D. indica* seed. Velocity of Sap was determined spectrophotometrically at 280 nm following the digestion of BSA as described by the above protocol.

SDS-PAGE

It was performed according to the method of Laemmli (1970) used to separate proteins according to their size. SDS-PAGE (10%) electrophoresis was performed at 100 v and the gel was stained with 0.125% commassie brilliant blue R250.

Enzyme kinetics of Sap

The effect of substrate concentration on the initial rate of an enzyme-catalyzed reaction is a central concept in enzyme kinetics. Different concentrations of substrates (0.75 to 4.25 µM) were taken and the velocity of the Sap was measured at 280 nm. A graph was plotted by taking the concentrations of substrates on X-axis and velocity of enzyme or reaction rate on Y-axis. Kinetic studies of Sap enzyme was studied using Graph pad Prism 5 software free trial version. Michaelis-Menten and Hanes-Woolf plot were plotted and K_m and V_{max} were determined. Hanes-Woolf best linearizes the Michealis-Menten and subsequently pointed out that the use of linear regression to determine kinetic parameters from this type of linear transformation is flawed, because it generates the best fit between observed and calculated values of $1/v$, rather than v.

RESULTS AND DISCUSSION

Our studies have shown that *D. indica* seed hexane extract inhibits Sap. Velocity of Sap was determined by measuring OD at 280 nm. Sap gradually decreases with

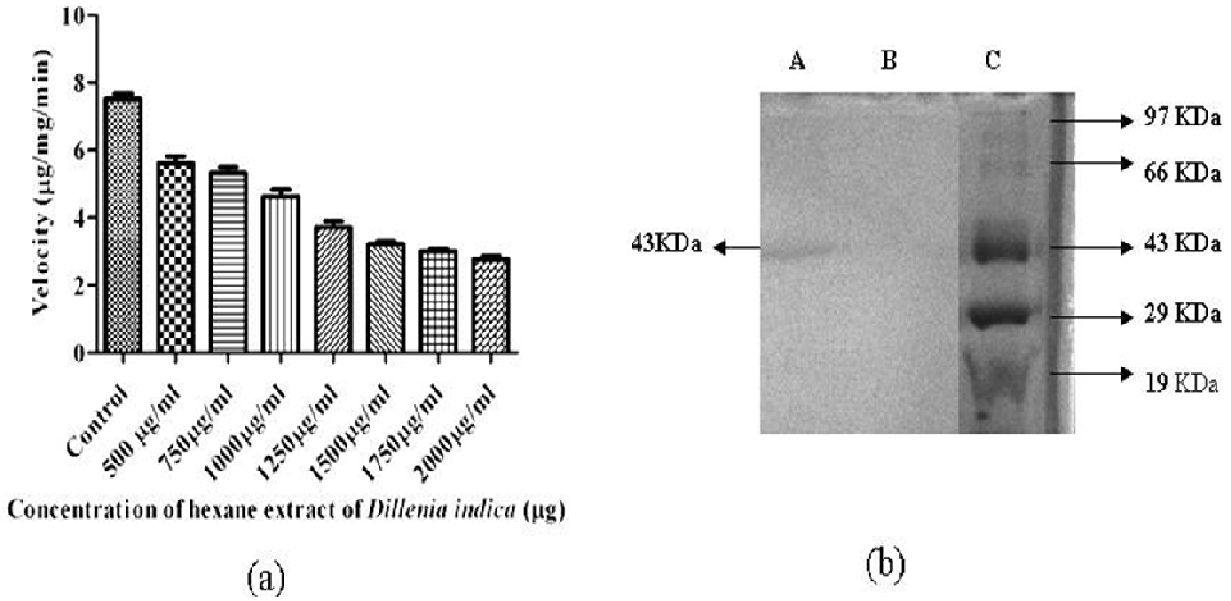

Figure 1. (a) Decreased activity of secreted aspartyl proteinase with the increased concentration of *D. indica* hexane extract, (b) Lane A: Cellular extracts of *C.albicans*, Lane B: *D. indica* hexane extract treated with *C.albicans* culture, Lane C: Molecular weight markers.

the *D. indica* seed hexane extract treated compared to control. Decreased Sap activity was represented in Figure 1a. The antimicrobial activity of hexane extract of *D. indica* seed was reported by the authors (Himakar et al., 2009). *Candida* species developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease, and overcome host defenses (Naglik et al., 2003). The virulence factors expressed or required by *Candida* species, and in particular *C. albicans*, to cause infections may well vary depending on the type of infection (that is, mucosal or systemic), the site and stage of infection, and the nature of the host response (Ferreira et al., 2010). Velocity of an enzyme normally increases with the increase of substrate concentration, but in our studies the velocity of Sap decreased with the increase of substrate concentration, indicating *D. indica* seed hexane extract acts on Sap. When the extracellular proteins were treated with different concentrations of hexane extract of *D. indica* seed and by keeping the substrate concentration constant, there was no change observed in all the treated cultures. It was shown that total enzyme was inhibited by hexane extract of *D. indica* seed, which was confirmed by SDS-PAGE (10%) electrophoresis (Figure 1b). These indicate that *D. indica* seed hexane extract prevented the growth of *C. albicans* by targeting cell wall as well as destroying the Sap, a key virulence factor of *C. albicans.* Some studies have suggested an effect of antifungal agents on expression of virulence factors by *C. albicans*. Zhang et al. (2002) showed the inhibition (IC_{50}) of *C. albicans* proteinases of two natural products from activity guided fraction of ethanol extract of *Lycopodium cernuum* at 20 and 8.5 µg/ml.

Lass-Florl et al. (2003) demonstrated that sertraline, a serotonin reuptake inhibitor, activated monocyte-directed macrophages against *Candida* species and reduced levels of fungal phospholipase and proteinase activities. Wu et al. (2000) showed that exposure of a fluconazole-susceptible *C. albicans* isolate to fluconazole at sub-MIC concentrations led to a reduction in the specific activity of Sap, whereas the same treatment of a fluconazole-resistant isolate led to an increase in extracellular proteinase specific activity.

Kinetic analysis is one of the most basic elements in enzymology. Kinetic studies not only reveals how fast an enzyme can function but also the effect of substrate concentration on the reaction rate, and the sensitivity of the enzyme to specific inhibitors or activators. The Michaelis constant is fundamental and usual procedure for measuring the rate of an enzymatic reaction is to mix enzyme with substrate and observe the formation of product or disappearance of substrate is frequently used in clinical work to define a given enzyme (Wilkinson, 1971). It is expressed in concentration units and is a reciprocal measure of the affinity of an enzyme for its substrate. Vmax was found to be 0.0560 µM/mg/min and Km value was 0.1107 µM (Figure 2a). Hanes-Woolf model best linearizes Michaelis-Menten equation. On X-axis, substrate concentration was taken and on Y-axis [S]/[V] was taken and a linear graph was plotted (Figure

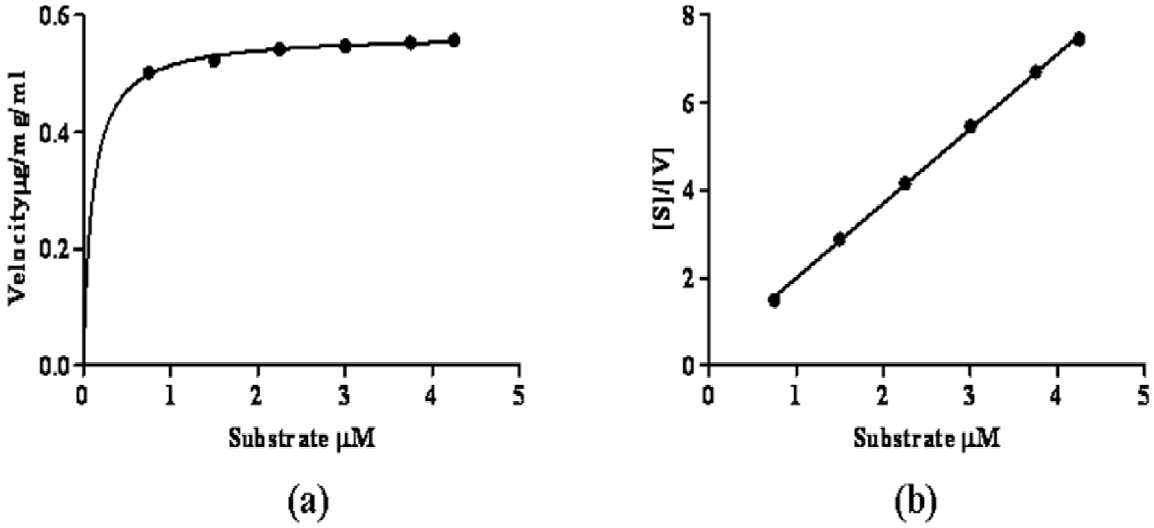

Figure 2. (a) Michaelis-Menten plot, (b) Hanes-Woolf plot.

Table 1. Vmax and Km of Sap in control and treated cells of *C. albicans* with hexane extract of *D. indica* seed.

Culture condition	Activity (μM/ml/min)	Sap activity (μM/mg/min)/V_{max}	Km (μM)
C. albicans extracellular proteins	1.342	0.058	0.174
C. albicans extracellular proteins + hexane extract of *D. indica* seed (1 mg/ml concentration).	No activity	No activity	No activity

2b). Km value was found to be 0.174 μM and V_{max} found to be 0.058 μM/mg/min. The enzyme activity, V_{max} and Km of control and treated culture of *C. albicans* with hexane extract of *D. indica* seed was represented in Table 1. From the studies it is concluded that hexane extract of *D. indica* seed has targeted and destroyed the Sap of *C. albicans.* This resulted Sap disappearance in electrophoresis.

REFERENCES

Abdille MH, Singh RP, Jayaprakasha GK, Jena BS (2005). Antioxidant activity of the extracts from *Dillenia indica* fruits. Food Chem. 90:891-896.

Copping VM, Barelle CJ, Hube B, Gow NA, Brown AJ, Odds FC (2005). Exposure of *Candida albicans* to antifungal agents affects expression of SAP2 and SAP9 secreted proteinase genes. J. Antimicrob. Chemother. 55:645-654.

De BF, Sullivan PA, Cassone A (2001). Aspartyl proteinases of *Candida albicans* and their role in pathogenicity. Med. Mycol. 39:303–313.

Ferreira C, Silva S, Faria OF, Pinho E, Henriques M, Lucas C (2010). *Candida albicans* virulence and drug-resistance requires the O-acyltransferase Gup1p. BMC Microbiol. 10:238.

Himakar RK, Bala NRK, Sarma PVGK, Reddy OVS (2009) *In vitro* studies on antimicrobial and antioxidant activities of *Dillenia indica* seed extract. J. Pure. Appl. Microbiol., 3: 769-776

Himakar RK, Sarma PVGK, Reddy OVS (2010a). A comparative *in vitro* study on antifungal and antioxidant activities of *Nervilia aragoana* and *Atlantia monophylla*. Pharm. Biol. 48:595-602.

Himakar RK, Tharanath V, Bala NRK, Sarma PVGK, Reddy OVS (2010b). Studies on hepatoprotective effect of hexane extract of *Dillenia indica* against CCl_4 induced toxicity and its safety evaluation in wistar albino rats. Res. J. Pharm. Biol. Chem. Sci. 1:441-450.

Hube B, Naglik J (2001). *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiol. 147:1997–2005.

Kwon CKJ, Lehman D, Good C, Magee PT (1985). Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. Infect. Immunol. 49:571-575.

Laemmli UK (1970). Cleavage of structural proteins during the assemblage of the head of bacteriopage T4. Nat. 227:680–685.

Lass-Florl C, Ledochowski M, Fuchs D (2003). Interaction of sertraline with *Candida* species selectively attenuates fungal virulence *in vitro*. FEMS Immunol. Med. Microbiol. 35:11–15.

Morrison CJ, Hurst SF, Reiss E (2003). Competitive binding inhibition enzyme-linked immunosorbent assay that uses the secreted aspartyl proteinase of *Candida albicans* as an antigenic marker for diagnosis of disseminated candidiasis. Clin. Diagn. Lab. Immunol. 10:835-848.

Naglik JR, Challacombe SJ, Hube B (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67:400-428.

Pfaller M.A, Jones RN, Messer SA, Edmond MB, Wenzel RP (1998). National surveillance of nosocomial blood stream infection due to *Candida albicans:* frequency of occurrence and antifungal susceptibility in the SCOPE Program. Diagn. Microbiol. Infect. Dis. 31: 327-332.

Smith A (2005). Clinical yeast and mould infections: isolation and diagnosis. www.cli-online.com

Wilkinson JH (1970). Enzyme kinetics and its relevance to enzyme assay. J. Clin. Pathol. 4: 14-21.
Wu T, Wright K, Hurst SF, Morrison CJ (2000). Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in sub-inhibitory concentrations of fluconazole. Antimicrob. Agents Chemother. 44:1200-1208.
Zhang Z, El SHN, Jacob MR, Pasco DS, Walker LA, Clark AM (2002). Natural products inhibiting *Candida albicans* secreted aspartic proteases from *Lycopodium cernuum*. J. Nat. Prod. 65:979-985.