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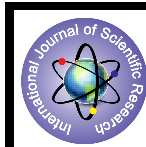
Research Paper

Antifungal Activity Of Double Headed Protease Inhibitors From The Seeds Of *Abelmoschus Moschatus*



Biochemistry

KEYWORDS : Double headed protease inhibitors, trypsin and chymotrypsin inhibitors, Abelmoschus moschatus, antifungal activity.

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ABSTRACT

The main aim of the study was to investigate the antifungal potential of double headed protease inhibitors (AMTI-III & AMTI-IV) isolated from Abelmoschus moschatus seeds on selected pathogenic fungal strains. Both the inhibitors have been purified to homogeneity following the conventional methods of protein purification and they exhibited strong antitryptic and antichymotryptic and moderate antielastase activities. Apparent molecular weights of AMTI-III & AMTI-IV were determined to be 20.8 kDa and 20.2kDa respectively following gel filtration and SDS-PAGE. When tested in vitro, both the inhibitors significantly affected the growth of Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae,Candida glabrata and Asperigillus niger and were ineffective against Fusarium oxysporum, Alternaria alternata, Mucor indicus and Penicillium chrysogenum. Results obtained in this study suggest that double headed protease inhibitors may serve as excellent candidates for the development of novel antimicrobial agents against human pathogenic diseases.

INTRODUCTION

Protease inhibitors (PI's), proteins capable of inhibiting activities of proteases, are abundant in nature. They have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Rakashanda *et al*., 2012). In plants, they also involved in the defensive mechanisms displayed against phytophagous insects and microorganisms (Huma and Khalid, 2007).

Recently, the rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in the isolation and investigation of the mode of action of antimicrobial proteins (Alasbahi and Melzig, 2008).

Many phytopathogenic bacteria and fungi are known to produce extracellular proteinases (Kalashnikova *et al*., 2003) which may play an active role in the development of diseases (Sara and Heale, 1990). In response to such attack by proteinases, plants synthesize inhibitory polypeptides that can suppress the enzyme activities.

Some of the serpins, cystatins, pepstatins and metallo protease inhibitors have been reported to possess antimicrobial activities. (Kim *et al.,* 2009). Double-headed inhibitors from broad beans and potato tubers showed antifungal activity (Ye *et al*., 2001; Kim *et al*., 2005). Proteinase inhibitors, Mungoin from mung bean and Potide G from potato tubers exhibited both antifungal and antibacterial activities (Wang *et al.,* 2006; Kim *et al.,* 2006).

Abelmoschus moschatus (L.) Medic, family *Malvaceae*, is an aromatic and medicinal plant popularly known as Mushkdana / Kasturi bhendi. The seeds are rich in protease inhibitors and they are used to check excessive thirst, cure for stomatitis, dyspepsia, urinary discharge, gonorrhea, leucoderma and itchiness and serve as cardiac tonic, aphrodisiac, diuretic and antispasmodic agents.

Although *Abelmoschus moschatus* seed protease inhibitors (AMTI-III and AMTI-IV) were found to be active against trypsin, chymotrypsin and elastase, their differential influence on the growth of fungi is not yet examined. Hence, the present investigation was carried to demonstrate the antifungal potential of double headed protease inhibitors on selected fungal strains.

MATERIALS AND METHODS

Source: *Abelmoschus moschatus* plants bearing pods of uniform size were selected in and around Visakhapatnam district. Pods were collected at the ripening stage and seeds removed from the pods were used for the isolation and purification of trypsin inhibitors.

Chemicals: Bovine pancreatic trypsin (1 x crystallized, DCC-treated, type xi), bovine serum albumin (BSA), chymotrypsinogen A, ovalbumin, lysozyme, phosphorylase b, soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. -N-benzoyl- DL- arginine- p-nitoanilide HCl (BAPNA), DEAE-cellulose were also from Sigma Chemical company, St. Louis, Missouri, U.S.A. Sephadex G-100 and G-200 were purchased from Pharmacia Uppsala, Sweden. Potato dextrose agar (PDA) was purchased from Himedia Pvt Ltd, Mumbai, India.

All other chemicals used were of analytical grade.

Test organisms:
The fungal strains, *Asperigellus niger* (MTCC 2723), *Asperigillus flavus* (MTCC 4633), *Fusarium oxysporum* (MTCC 1755), *Alternaria alternata* (MTCC1362), *Candida albicans* (MTCC 227), *Candida glabrata* (MTCC 3016), *Candida tropicalis* (MTCC 184), *Mucor indicus* (MTCC 6333), *Penicillium chrysogenum* (MTCC 161) and *Saccharomyces cerevisiae* (MTCC 2918) were collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Purification of double headed protease inhibitors:
The double headed protease inhibitors (AMTI-III&AMTI-IV) have been isolated and purified from the seeds of *Abelmoschus moschatus* following the procedure described in our previous paper (Muni Kumar *et al*., 2015).

Protein estimation: Protein was estimated by the method of Lowry *et al*., (1951) using bovine serum albumin as the standard.

Determination of molecular weight Molecular weight of the inhibitor was determined by SDS-PAGE using the method of Laemmli (1970) and also by gel filtration on Sephadex G-200 column.

Measurement of trypsin and trypsin inhibitory activity
Trypsin activity and trypsin inhibitory activity was assayed by the method of Kakade *et al.,* (1969) using BAPNA as the substrate.

Measurement of chymotrypsin and chymotrypsin inhibitory activity
Esterolytic activity of chymotrypsin and chymotrypsin inhibitory activity was assayed by the method of Prabhu and Pattabiraman (1977) using ATEE as the substrate.

Assay of elastase and elastase inhibitory activity
Esterolytic activity of elastase was assayed according to the method of Naughton and Sanger (1961) using elastin congo red as substrate. The method is based on measuring the release of the dye from elastin congo red by elastase at 495 nm.

One enzyme inhibitory unit is defined as the number of enzyme units inhibited under these conditions.

Antifungal activity: Antifungal activity of AMTI-I and AMTI-II was performed using the agar well diffusion method of Perez *et al.,* (1990).The cultures of 48 h old grown on potato dextrose agar (PDA) were used for inoculation of fungal strains on PDA plates. An aliquot (0.2 ml) of inoculum was introduced to molten PDA and poured into a petri dish by pour plate technique. After solidification, the appropriate wells were made and they were filled with the buffer containing 50 - 100 μg of each of the inhibitor and allowed for diffusion of inhibitors for 45 min. The plates were incubated at 25°C for 48 h. The fungicides, Flucanazole and Ketoconazole replaced the inhibitors in the positive control. The inhibition zones were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates.

Minimum inhibitory concentration (MIC) assay:
Minimum Inhibitory Concentrations (MIC) of both the inhibitors were determined according to the method of Elizabeth (2001).

A series of two fold dilution of each inhibitor, ranging from 500-2000 μg/ml, was prepared. After sterilization, the medium was inoculated with the aliquots of culture containing spores/ slant cultures and incubating for 48 h in aseptic condition and transferred into sterile 6 inch diameter petri dishes and allowed to set at room temperature for about 10 min and then kept in a refrigerator for 30 min. After the media was solidified, wells were made and different concentrations of each inhibitor ranging from 25-2000 μg/ml were added to the wells of each petri dish. The blank plates were without inhibitors. Inhibition of the growth of the organism in the plates containing inhibitor was judged by comparison with the growth in the control plates. The MICs were determined as the lowest concentration of the AMTI inhibiting visible growth of each organism on the agar plate.

RESULTS AND DISCUSSION
AMTI-III and AMTI-IV from *Abelmoschus moschatus* seeds were purified to homogeneity following conventional methods of protein purification such as thermal denaturation, ammonium sulphate fractionation and ion exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-100.

The purification of these inhibitors is summarized in Table-1. Yields of AMTI-III and AMTI-IV were 7.63% and 7.20% respectively. During the purification, the ratio of trypsin inhibitory activity (TIA) to chymotrypsin inhibitory activity (CIA) in the two inhibitors remained nearly constant suggesting that the two inhibitory activities might reside in the same protein molecule.

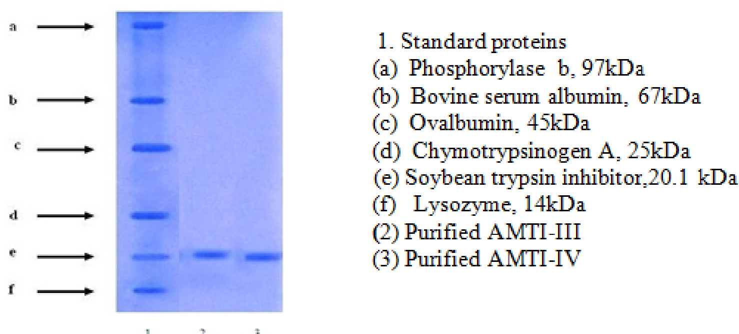
The molecular weights of AMTI-III and AMTI-IV, as determined by SDS-PAGE were found to be 20.4kD and 20.8kDa respectively (Fig-1). These values were close to those obtained with gel filtration on Sephadex G-200. Both the inhibitors gave a single sharp band on SDS-PAGE even in the presence of 2-mercaptoethanol supporting the monomeric nature of the protein.

TABLE-1: SUMMARY OF PURIFICATION OF DOUBLE HEADED TRYPSIN INHIBITORS FROM *ABELMOSCHUS MOSCHATUS* SEEDS

Preparation	Volume (ml)	Total protein (mg)	Total activity units		Specific activity Units/mg protein		TIA/CIA	Yield%	Fold purification
			TIU $\times 10^3$	CIU $\times 10^3$	TIA $\times 10^2$	CIA $\times 10^2$			
Crude extract	250	2087.5	788.4	421.1	3.77	2.01	1.90	100	1.00
Acetone Treatment	230	1988.2	771.5	414.4	3.89	2.08	1.86	97.86	1.03
Heat treatment	215	1016.4	626.4	328.4	6.16	3.23	1.94	79.45	1.63
Ammonium sulphate(60%) Fractionation	60	424.8	482.8	238.2	11.36	5.61	1.96	61.24	3.01
DEAE-Cellulose NaCl gradient elution									
PI-III (0.25M NaCl)	152	44.2	64.4	34.8	14.57	7.8	1.87	8.17	3.86
PI-IV (0.5M NaCl)	184	42.8	68.6	35.4	16.02	8.27	1.95	8.70	4.25
Sephadex G-100									
PI-III	46	31.6	60.2	32.4	19.05	10.25	1.86	7.63	5.05
PI-IV	50	30.5	56.8	30.2	18.62	9.90	1.88	7.20	4.94

*Yield and fold purification were calculated on the basis of TIU and TIA respectively. TIU – Trypsin inhibitory units TIA – Trypsin inhibitory activity

FIGURE -1: SDE-PAGE on 5 - 20% gradient slab gels



The purified double headed inhibitors were tested for their antifungal potential against *Asperigellus niger, Asperigillus flavus, Fusarium oxysporum, Alternaria alternate, Candida albicans, Candida glabrata, Candida tropicalis, Mucor indicus, Penicillium chrysogenum and Saccharomyces cerevisiae* in the range 500-2000 µg/ml along with the positive control containing the fungicides, Flucanazole and Ketoconazole.

Table - 2 shows the effect of AMTI-III and AMTI-IV on the growth of fungal strains. Both the inhibitors significantly affected the growth of *Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae, Candida glabrata and Asperigellus niger* with zones of inhibition recorded as 23 mm, 21 mm, 19 mm, 19 mm, 24 mm and 22 mm for AMTI-III and 21 mm, 20 mm, 17 mm, 18 mm, 23 mm and 21 mm for AMTI-IV respectively. The inhibitors did not exhibit any inhibitory effect on the growth of other fungal strains tested. The fungicides, Flucanazole (20µg) and Ketoconazole (20µg), on the other hand, produced an inhibition zone of 32 – 34 mm in the control (Fig 2).

Minimum inhibitory concentrations of both inhibitors for antifungal activity were presented in Table-3.

The inhibitors, AMTI-III and AMTI-IV, exhibited antifungal activity with varying degrees against pathogenic fungal strains tested. The two inhibitors have no inhibitory effect on the growth of fungi- *Fusarium oxysporum, Alternaria alternata, Mucor indicus and Penicillium chrysogenum* tested.

These inhibitors are similar to proteinase inhibitors from broad beans and buckwheat) seeds in their antifungal activity (Ye and Ng, 2002; Dunaevsky *et al.*, 2001).

Trypsin inhibitors exhibiting antifungal activity include those from seeds of the pearl millet, chili pepper, *Capsicum annuum*, and *Clausena lansium* (Joshi *et al.*, 1998; Ribeiro *et al.*, 2007; Ng *et al.*, 2003) and from seeds of malaytea scurfpea (Yang *et al.*, 2006), *Acacia plumosa* (Lopes *et al.*, 2009), limenin, from large lima beans (Wang and Rao, 2010) and *Mucuna pruriens* (Chandrashekharaiah, 2013).

TABLE-2: EFFECT OF AMTI-III AND AMTI-IV ON FUNGAL GROWTH

Name of the fungal strain	Zone of Inhibition (Diameter in mm)					
	AMTI-III		AMTI-IV		Positive controls	
	50 µg	100 µg	50 µg	100 µg	Flucanazole (20 µg)	Ketoconazole (20 µg)
Asperigillus niger	12	22	10	21	34	33
Asperigillus flavus	9	19	8	17	34	32
Fusarium oxysporum	-		-		32	31
Alternaria alternate	-		-		31	34
Candida albicans	11	23	10	21	34	33
Candida glabrata	12	24	11	23	32	34
Candida tropicalis	10	21	10	20	34	32
Mucor indicus	-	-	-	-	32	31
Penicillium chrysogenum	-	-	-	-	31	32
Saccharomyces cerevisiae	9	19	9	18	32	34

Fungal strains were spread on potato dextrose agar plates. Different amounts of the inhibitors (50 µg and 100 µg) were placed in the wells and allowed for diffusion. Controls contained Flucanazole (20 µg) and Ketoconazole (20 µg) in place of inhibitors. The incubation period was 48 h at 25ºC. Zone of inhibition was measured and minimum inhibitory concentration of each inhibitor was determined.

FIGURE-2: EFFECT OF AMTI-III AND AMTI-IV ON FUNGAL GROWTH CONCENTRATIONS OF AMTI

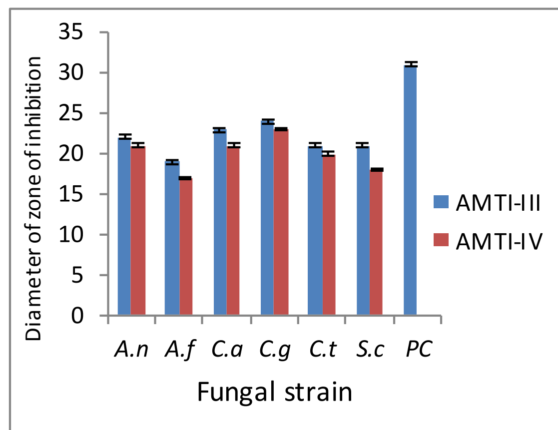


TABLE-3: MINIMUM INHIBITORY

Name of the fungal strain	MIC(µg/ml)	
	AMTI-III	AMTI – IV
Asperigillus niger	250	250
Asperigillus flavus	250	250
Fusarium oxysporum	-	-
Alternaria alternate	-	-
Candida albicans	250	250
Candida glabrata	250	250
Candida tropicalis	250	250
Mucor indicus	-	-
Penicillium chrysogenum	-	-
Saccharomyces cerevisiae	500	500

P.C-Positive control (Flucanazole/Ketoconazole).
A.n - Asperigillus niger, A.f- Asperigillus flavus,
C.a- Candida albicans, C.g-Candida glabrata,
C.t- Candida tropicalis, S.c- Saccharomyces cerevisiae.

Some proteinase inhibitors have shown both antibacterial and antifungal activities. A protease inhibitor from the leaves of *Coccinia grandis* strongly inhibited the growth of pathogenic microbial strains including both bacterial and fungal strains (Satheesh and Murugan 2011). A trypsin inhibitor from soap nut seeds (SNTI) have been reported to exert potent antifungal activity against dermatophytic fungi, *Trichophyton rubrum* and *Malassezia fur fur* in addition to its antibacterial activity (Rachel *et al.*, 2013).

Microbes are known to elaborate proteases into extracellular medium for gaining entry into the host and protease inhibitors by binding to such extracellular proteases could exert antimicrobial effect. The antifungal role of trypsin inhibitors has also been attributed to their ability to interfere with chitin biosynthetic process during fungal cell wall development by inhibiting the proteolytic activation of chitin synthase zymogen (Adams *et al.*, 1993).

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CONCLUSION

The results of the present investigation clearly demonstrate that the double headed protease inhibitors, AMTI-III and AMTI-IV, of *Abelmoschus moschatus* may serve as potential antifungal agents and find application in the medical front as therapeutic agents for infections caused by specific pathogenic fungal strains.

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