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Research Article

ANTIMICROBIAL ACTIVITY OF A PROTEASE INHIBITOR ISOLATED FROM THE RHIZOME OF *CURCUMA AMADA*

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ABSTRACT

Objective: Protease inhibitors (PIs) are effective antimicrobial agents, and this study was aimed to study the antibacterial efficacy of a PI isolated from the rhizome of *Curcuma amada*.

Methods: A proteinaceous protease inhibitor was isolated from the rhizome of *C. amada* and purified by Sephadex G-50 gel permeation chromatography. The purified inhibitor was denoted as Curcuma amada protease inhibitor (CAPI). The antibacterial effect of CAPI against Gram-positive and Gram-negative bacteria, the minimal inhibitory concentration (MIC), and minimal bactericidal concentration (MBC) of CAPI was studied *in vitro,* and the membrane disruption activity of CAPI was also analyzed.

Results: CAPI was effective against both Gram-positive and Gram-negative bacteria, with slightly higher concentrations required for Gram-negative bacteria. The MIC ranged from 75 to 100 µg/ml and the MBC ranged from 100 to 125 µg/ml of CAPI. The study of membrane disruption by CAPI revealed the release of cell contents, namely, reducing sugars and proteins from the bacterial cell.

Conclusion: A PI was effectively isolated from the rhizome of *C. amada*, and the isolated inhibitor proved to be a promising antibacterial agent.

Keywords: *Curcuma amada* rhizome, Protease inhibitor, Antibacterial activity, Minimal inhibitory concentration, Minimal bactericidal concentration.

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INTRODUCTION

Antimicrobial peptides are present in a wide variety of organisms, including animals, plants, microbes, and insects. These peptides are crucial components of defense strategy adopted by organisms to combat pathogens. These peptides belong to several subfamilies, the most prominent being protease inhibitors (PIs) [1].

PIs are ubiquitous in nature and play multiple roles in plants and animals. These compounds help to regulate metabolic pathways and maintain homeostasis. Plants produce these compounds that act as natural defenses against pests and pathogens. Plant PIs (PPIs) are generally small proteins or peptides present in tubers, seeds, and also in the aerial parts of plants [2]. The defensive capabilities of PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development [3]. PPIs are being researched for their medicinal values such as antimicrobial potential, anticarcinogenic activity, inhibition of blood coagulation, and anti-inflammatory potential [4].

Mango ginger (*Curcuma amada*) is a perennial herb with modified stem called rhizome below the ground. It has morphological and phylogenic resemblance with ginger (*Zingiber officinale*) but imparts mango (*Mangifera indica*) flavor [5]. The mango ginger rhizome has been extensively used as appetizer, antipyretic, aphrodisiac, and laxative and to cure itching, skin diseases, bronchitis, asthma, and inflammation [6].

The objective of this study was to isolate and partially purify a PI from the rhizome of *C. amada* and to investigate the antibacterial potential of the purified inhibitor.

METHODS

All chemicals used in this study were of analytical grade.

Plant material

Fresh and healthy mango ginger (*C. amada* Roxb.) rhizomes were procured from the local market in Chennai, India. Rhizomes were washed and weighed. The rhizome was authenticated by Dr. J. Jayaraman, Plant Anatomy Research Center, Tambaram, Chennai, India (Voucher No. PARC/2016/1005).

Preparation of crude extract

10 g of mango ginger was weighed and cut into small pieces using a sterile knife. The crude extract was obtained by grinding the small pieces using 30 ml of 0.2 M sodium phosphate buffer, pH 7.0 in a mortar and pestle. It was mixed well using magnetic stirrer and then centrifuged at 2500 rpm for 15 minutes. The supernatant was harvested and stored at 4°C.

Purification of PI

Ammonium sulfate precipitation

Solid ammonium sulfate was added at various concentrations to the crude extract, and the precipitates were recovered by centrifuging at 20000 g at 4°C for 10 minutes. The precipitates were resuspended in 0.2 M sodium phosphate buffer (pH 7.0). The proteolytic activities in the precipitates were determined. The precipitates exhibiting low protease activities were resuspended in 0.2 M sodium phosphate buffer (pH 7.0) and pooled for further use.

Dialysis

The resuspended precipitate was dialyzed extensively using 0.2 M sodium phosphate buffer (pH 7.0). The protein content and proteolytic activities in the dialysate were determined.

Gel filtration chromatography

Ten milliliters of the dialysate was loaded onto a Sephadex G-50 column which had been pre-equilibrated with 0.5 M Tris-HCl buffer of pH 7.0 containing 0.5 M NaCl and eluted with the same buffer at a flow rate

of 30 ml/hr. All eluted fractions (2 ml) were assayed for enzyme activities, and the fractions with high activities were pooled. The pooled fractions were subjected to dialysis against Tris-HCl buffer, and the enzyme activities in the dialysates were determined. The dialysate was lyophilized, denoted as curcuma amada protease inhibitor(CAPI), and stored at 4°C for further use.

Trypsin and chymotrypsin assay

Trypsin and chymotrypsin activity was assayed by the method of Rahman *et al.* [7]. A portion of the purified incubator was incubated for 10 minutes at 30°C with trypsin and chymotrypsin (250 µg/ml in phosphate buffer, pH 7). 2 ml of 1% casein (w/v) was then added and incubated for 10 minutes at 30°C. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid. The reaction mixtures were centrifuged for 10 minutes at 10,000 g. The supernatants were harvested. To 1.0 ml of supernatant, 5.0 ml of 0.4 M sodium carbonate and Folin-Ciocalteu reagent (1:3 dilution) was added, and the resulting solution was read at 660 nm.

Determination of antibacterial activity

Antibacterial activity of sample was determined by disc diffusion method on Muller-Hinton Agar (MHA) medium. The inoculums were spread on the solid plates with a sterile swab moistened with the bacterial suspension.

The discs were placed in MHA plates, and 20 µl of crude extract, 20 µl of partially purified inhibitor (100 µg), and different concentrations of the purified inhibitor (20-100 µg), dimethyl sulfoxide (negative control), and ampicillin (positive control) were placed on the discs. The plates were incubated for 24 hrs at 37°C. Then, the microbial growth was determined by measuring of the zone of inhibition.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Different concentrations of the purified inhibitor (25, 50, 75, 100, and 125 µg/ml) were added to 20 µl of bacterial inoculum in the wells of microtitre plate and incubated at 37°C. The control well was devoid of inhibitor. The absorbance at 490 nm was measured at the end of the incubation period. MIC is the concentration of the inhibitor in the well which exhibits ≥50% reduction in absorbance of the control. For the determination of MBC, wells with concentrations of inhibitor which exhibited ≥50% reduction were chosen. Aliquots from those wells were transferred to Petri dishes containing nutrient agar and incubated at 37°C. MBC corresponds to the concentration of the inhibitor which shows no bacterial growth.

Study of bacterial membrane disruption

Varying concentrations of the purified inhibitor (25, 50, 75, 100, and 125 µg/ml) were added to bacterial cultures (10^9 CFU/ml) and incubated at 37°C. After 24 hrs, 1 ml of the culture was harvested and centrifuged, and the supernatant was analyzed for the presence of reducing sugars and proteins.

RESULTS

The crude extract was obtained from the rhizome, and the presence of PI was studied by analyzing the inhibition of the activity of trypsin and chymotrypsin. About 500 mg of protein was present in the crude extract, and the inhibition of proteolytic activity was determined to be 7%. Purification using ammonium sulfate fractionation followed by dialysis was performed. The dialysate thus obtained exhibited maximum inhibitory activity of 24%. The partially purified inhibitor may contain other proteinaceous contaminants, and hence the inhibitor was subjected to gel filtration chromatography. The dialysate was further purified on Sephadex G-50 column, and the purified inhibitor exhibited 80% activity (Table 1).

The antibacterial activity of CAPI was evaluated against few Gram-positive and Gram-negative bacteria such as *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus.* The efficacy of the purified inhibitor was comparable to the reference standard, ampicillin.

The crude extract was minimally effective on *S. aureus* and *B. subtilis* and absolutely no effect on the other tested bacteria. When compared to purified CAPI, the partially purified exhibited 50-60% of inhibitory activity. The purified CAPI showed significant activity against *S. aureus, E. coli, B. subtilis, S. typhi*, and *P. aeruginosa.* The highest activity was shown against *S. aureus* and *B. subtilis,* and the least activity was against *S. typhi* by both the partially purified and purified CAPI (Table 2).

The MIC and MBC of CAPI for different bacterial species were determined. The MIC and MBC were found to be 75 and 125 µg/ml, respectively, for Gram-positive bacteria and 100 and 125 µg/ml, respectively, for Gram-negative bacterial species studied. The difference in MIC and MBC of Gram-positive and Gram-negative bacteria can be attributed to the presence of lipopolysaccharide capsule in Gram-negative bacteria which offers resistance to the PI [8].

Disruption of membrane activity was studied by analyzing the release of cell components in the cultures treated with different concentrations of purified CAPI. The release of reducing sugars and proteins were

Table 1: Purification profile of PI

Purification steps	Extent of inhibition of trypsin (%)	Protein (mg)	Yield (%)	Purification (fold)
Crude extract	7	325	100	1
Ammonium sulfate fractionation	24	180	55.38	3.4
Sephadex G-50 gel permeation chromatography	80	85	26.15	11.4

Values expressed as the mean of three independent experiments. PI: Protease inhibitor

Table 2: Antibacterial activity of CAPI

Bacteria	Zone of inhibition (mm)*					
	CE	PP CAPI	CAPI (µg/ml)			
			25	50	75	100
Salmonella typhi	-	06±1	08±0.3	09±0.6	10±0.8	12±0.5
Escherichia coli	-	06±0.5	08±0.5	10±0.2	11±0.5	13±0.5
Pseudomonas aeruginosa	-	07±0.3	08±0.3	10±0.6	11±0.7	12±0.8
Bacillus subtilis	03±0.3	10±0.2	11±0.2	12±0.5	14±0.4	16±0.5
Staphylococcus aureus	03±0.6	11±0.4	13±0.5	15±0.3	16±0.6	18±0.8

*Data represent mean±SD. $p<0.05$, -: No zone of inhibition, CE: Crude extract, PP CAPI: Partially purified curcuma amada protease inhibitor

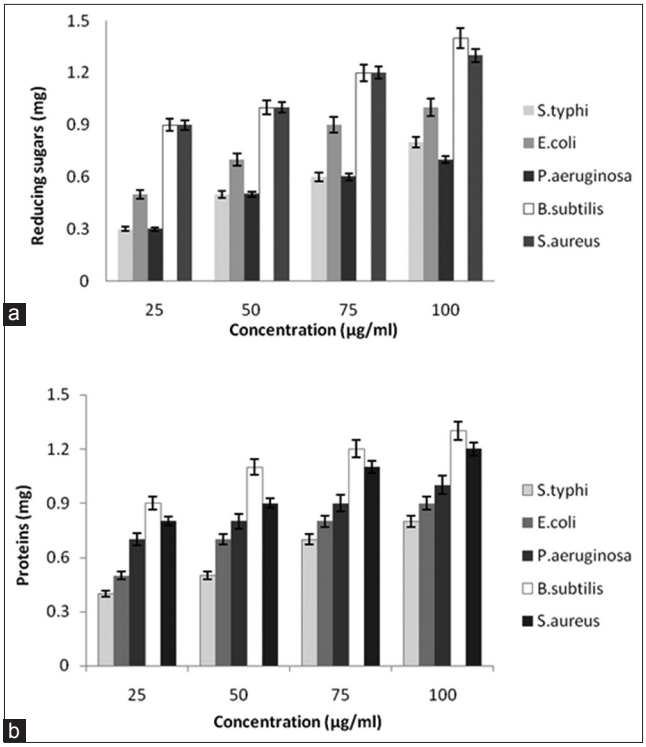

Fig. 1: Bacterial membrane disruption by curcuma amada protease inhibitor at the end of 24 hrs of incubation. (a) Release of reducing sugars, (b) release of proteins. Values represented are mean±standard deviation

studied at 0 and 24 hrs of incubation. The results recorded at the end of 24 hrs of incubation are presented in Fig. 1.

DISCUSSION

PPIs are small peptides which are an integral part of the plant defense system. These inhibitors have several biomedical and clinical applications, and the biological activities of these inhibitors in animal models have been reported [9-11]. *C. amada* has several medicinal values as claimed traditionally, and most of these values have been confirmed scientifically [6]. The present study was intended to isolate a PI from *C. amada* and assesses its antibacterial activity. A novel PI, denoted as CAPI, was isolated and purified from the rhizome of *C. amada.* The inhibitor being proteinaceous in nature was satisfactorily purified using gel permeation chromatography. This was established by the yield and purification fold obtained at the end of the purification process.

PIs have been reported as antibacterial agents, the activity being attributed to the interaction of these inhibitors with the physiologically important proteases as well as with the proteins present in the cell wall or membrane. These interactions culminate in changes in membrane permeability and the death of the bacteria [1,12].

CAPI exhibited antibacterial activity as effectively as the standard under the study, ampicillin. The antibacterial activity of the inhibitor improved with its purity. The elimination of coexisting proteins by gel filtration chromatography increased the antibacterial potential of the isolated inhibitor. The antibacterial activity of CAPI could be attributed to the effect of the inhibitor on the bacterial cell wall and membrane. Disruption of bacterial cell wall and membrane results in release of cellular contents such as reducing sugars and proteins. The gradual increase in the release of these components from the bacteria indicated that CAPI had disrupted the bacterial membrane during the incubation process, thereby releasing cell contents. The release of reducing sugars and proteins was directly proportional to the concentration of CAPI and the duration of incubation. Membrane disruption was prominent in *B. subtilis*, *S. aureus*, and *P. aeruginosa* when compared to *S. typhi* and *E. coli*. PPIs exhibit a dual role, wherein they suppress the activity of the pathogenic microorganisms' protease and also alter its membrane permeability, thus proving to be effective antimicrobial agents [13].

The results of the present investigation reveal that the PI CAPI isolated from the rhizome of *C. amada* has potential antibacterial activity. Further studies have been initiated to study the efficacy of CAPI *in vivo*. These studies will ascertain the application of CAPI as clinical agents. A similar study by Sukandar *et al.* has established the use of extracts of fingerroot rhizome as effective topical antibacterial agents [14].

CONCLUSION

A PI was isolated and partially purified from the rhizome of *C. amada*. The antibacterial activity of the inhibitor was studied, and the minimum inhibitor concentration required for its activity against Gram-positive and Gram-negative bacteria was identified. The effect of the inhibitor in altering the stability of the bacterial membrane was also studied and proposed that the inhibitor exerted its antibacterial effect through bacterial membrane disruption.

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