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

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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 phylogenetic 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.

RESULTS

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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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 37°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) were added to the plates. The plates were incubated for 24 hrs at 37°C. Then, the microbial growth was determined by measuring 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 microtiter plate and incubated at 37°C. The control well was devoid of inhibitor. The absorbance at 660 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 exhibits ≥50% reduction. The MIC and MBC were found to be 75 and 125 µg/ml, respectively, for Gram-positive bacteria such as *S. aureus* and *E. coli*, and 80% activity (Table 1).

**Study of bacterial membrane disruption**

Varying concentrations of the purified inhibitor (25, 50, 75, 100, and 125 µg/ml) were added to bacterial cultures (10⁶ 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).

**DISCUSSION**

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 bacteria. 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 chromography | 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*        | -                        | 00±0.3 | 09±0.6 | 10±0.8       | 12±0.5       |
| *Escherichia coli*        | -                        | 06±0.5 | 08±0.5 | 10±0.7       | 13±0.5       |
| *Pseudomonas aeruginosa*  | -                        | 07±0.3 | 10±0.6 | 11±0.7       | 12±0.8       |
| *Bacillus subtilis*       | 03±0.3                   | 10±0.2 | 12±0.5 | 14±0.4       | 16±0.5       |
| *Staphylococcus aureus*   | 03±0.6                   | 11±0.4 | 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*
Kunitz-type serine protease inhibitor from potato (Solanum) with Protease inhibitor from a (Roxb.) rhizomes against antibacterial activity of Curcuma L in . ISRN Pharm

increase in the release of these components from the bacteria indicated cellular contents such as reducing sugars and proteins. The gradual Disruption of bacterial cell wall and membrane results in release of to the effect of the inhibitor on the bacterial cell wall and membrane. Isolated inhibitor. The antibacterial activity of CAPI could be attributed filtration chromatography. CAPI exhibited antibacterial activity as effectively as the standard 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.

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.

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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