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Muhammad Shahid
Muhammad Tayyab
Farah Naz
Amer Jamil
Muhammad Ashraf

See next page for additional authors

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Authors
Muhammad Shahid, Muhammad Tayyab, Farah Naz, Amer Jamil, Muhammad Ashraf, and Anwar Gilani
Activity-guided Isolation of a Novel Protein from *Croton tiglium* with Antifungal and Antibacterial Activities

Muhammad Shahid¹, Muhammad Tayyab¹, Farah Naz¹, Amer Jamil¹, Muhammad Ashraf² and Anwarul Hassan Gilani³*

¹Molecular Biochemistry Lab., Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan  
²Department of Botany, University of Agriculture, Faisalabad-38040, Pakistan  
³Natural Products Research Division, Department of Biological and Biomedical Science, Aga Khan University Medical College, Karachi, Pakistan

This study describes the activity-guided isolation and purification of a novel antimicrobial protein from the seed of *Croton tiglium* Linn. Purification was carried out by (NH₄)₂SO₄ precipitation, gel filtration and DEAE-cellulose ion-exchange chromatography. Antifungal and antibacterial activities were determined after each purification step. SDS-polyacrylamide gel electrophoresis revealed that the purified protein was a monomer with molecular mass of 50 kDa. This is a first report on purification of a protein from *Croton tiglium*, which possesses a strong and broad spectrum antimicrobial activity. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: *Croton tiglium*; bioactive peptide/protein; antibacterial; antifungal.

INTRODUCTION

*Croton tiglium* Linn (Euphorbiaceae), vernacular name; Jamal gota (Urdu), Croton (English) is mainly used as purgative and antispasmodic (Usmanghani et al., 1997). It is native to tropical Asia from India to New Guinea and Java, Indonesia, China and Philippine Islands, and cultivated to a limited extent in Southern California as an ornamental plant (Duke, 1983).

The plant has not been widely studied for its biologically active constituents. A few studies on the other species of the plant revealed the presence of anti-inflammatory (Nardi et al., 2003), immunomodulatory (Risco et al., 2003), and antioxidant (Lopes et al., 2004), (Tieppo et al., 2006), activities. Phytochemical studies on *Croton tiglium* resulted in isolation of a few compounds (Thonte and Khandelwal, 1993).

Preliminary screening on different medicinal plants for the antifungal activity revealed that *Croton tiglium* was very active against *Aspergillus tamarii*, *Rhizopus solani*, *Mucor mucedo* and *Aspergillus niger* (Jamil et al., 2007). While the other species of *Croton* have been shown to possess some antibacterial activity (Peres et al., 1997; Abo et al., 1999), no systematic study on antibacterial and antifungal activities has been reported for *Croton tiglium*. In the present study, we describe the isolation and purification of an antimicrobial protein from *Croton tiglium* with antifungal and antibacterial activities, which is the first study of such nature.

MATERIALS AND METHODS

Seeds of *Croton tiglium* Linn (Voucher No. 11165, 26-05-1928, Herbarium Punjab Agricultural College, Lyallpur, Shelf No. 6/35) were obtained and taxonomically identified from the Department of Botany, University of Agriculture, Faisalabad, Pakistan.

**Extraction.** A two-step extraction procedure was followed. In the first step the ethanol soluble components were removed by treating the ground seed (200 g) with 80% ethanol. Ethanol was removed by filtration and the residues were air-dried for further extraction. Next, the residue was extracted in 10 mM sodium phosphate buffer (PMSF was added to final concentration of 1 mM as protease inhibitor) in a ratio of 1:2 in pestle and mortar. The extract was blended in a blender (Mamrelax, Fait Common, France) and centrifuged at 10 000 × g for 15 minutes at 4 °C. The residue was discarded and the supernatant was filtered to remove the seed residue present in the supernatant. The resultant filtrate (crude extract) was stored at 4 °C till further analysis (Turrini et al., 2004).

**Purification.** The proteins and peptides in the crude extract were precipitated with (NH₄)₂SO₄ at 80% saturation (Huynh et al., 1996). The precipitated crude extract was centrifuged at 10 000 × g, 4 °C for 10 min. The residue was re-suspended in the extraction buffer. The residue and the supernatant were tested for antifungal and antibacterial activities. The re-suspended residues with maximum antimicrobial activity were purified by gel filtration (Deutscher, 1990) on Sephadex G-100 column (2 × 30 cm), equilibrated and eluted with 10 mM tris-HCl buffer, pH 8. Absorbance of the eluants
was recorded at 280 nm. The fractions with maximum protein contents were pooled and tested for antimicrobial activity. The fractions showing antimicrobial activity were further purified by the ion exchange chromatography on DEAE-cellulose column equilibrated with 10 mM Tris-HCl buffer, pH 8. The elution was carried out in a gradient of sodium chloride (0.2–1 M) in the same buffer (Terras et al., 1993). Antifungal and antibacterial activities were determined for each fraction. Molecular mass of the protein was determined by SDS-PAGE (Laemmli, 1970).

Antimicrobial assay. The antifungal and antibacterial potency of crude extract and different chromatographic fractions were examined against fungal (Aspergillus niger ATCC 10575 and Mucor mucedo local isolate) and bacterial (Bacillus subtilis JS2004 and Pasteurella multocida B2 strain) strains obtained from the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. Potato dextrose agar (PDA) and nutrient agar (NA) media (Oxoid) were used for the growth of fungi and bacteria, respectively. Chloramphenicol solution (10 mM) was added to the medium for fungal strains to avoid the bacterial growth (Emmons et al., 1970).

Fungal spores and bacterial cultures were inoculated in Petri plates containing the respective media, and incubated at 27 °C and 37 °C for fungi and bacteria, for 24 and 48 h, respectively. Small filter paper discs (Beckman Instruments (UK) Ltd, High Wycombe, UK) were then laid flat on the growth medium having fungal or bacterial growth and 100 μL of the test extract was applied on each disc. The Petri plates were incubated again for 48 h, for the growth of microbes. The antimicrobial activity was shown by the appearance of the clear zones around the growth. The zones of inhibition were measured in millimeters with a zone reader (Huynh et al., 1996).

Protein contents determination. The protein contents of the samples were determined by Bradford method (Bradford, 1976) against bovine serum albumin (BSA) as standard.

RESULTS AND DISCUSSION

Antimicrobial activity of the ethanol and crude extracts of Croton tiglium Linn were tested against fungal (Mucor mucedo, Aspergillus niger) and bacterial strains (Pasteurella multocida and Bacillus subtilis) with strong inhibitory effect (Table 1); hence, activity-guided fractionation was carried out to isolate the respective protein. Figure 1 shows antibacterial activity of various extracts and fractions of C. tiglium against Bacillus subtilis.

For purification, crude extract of C. tiglium seeds was subjected to ammonium sulphate precipitation to 80% saturation level. The supernatant did not show any antifungal or antibacterial activity except a weak effect against A. niger (Table 1). Strong antifungal and antimicrobial activities were observed for the residue, showing that the activity was most likely due to some protein or peptide. Treatment of the residue with proteinase K abolished the activity confirming that the activities were...
due to proteins or peptides in the extract and not due to some organic compounds (Jamil et al., 2007).

The residue, re-suspended in buffer after ammonium sulphate precipitation, was applied to gel filtration column (Sephadex-G100). One milliliter fractions were collected and the absorbance was noted at 280 nm (Fig. 2). Activity-directed antimicrobial effect revealed that no or a little effect could be observed for the peaks I, III and IV, while peak II exhibited strong effect against M. mucida, A. niger and B. subtilis. Very strong antibacterial activity was observed against P. multocida.

When all individual fractions of the peak II were subjected to antibacterial activity, it was found that the fraction 13 had maximum effect (data not shown). The proteins obtained from the fraction 13 of the gel filtration column were subjected to DEAE-cellulose chromatography (Fig. 3). A major peak was detected constituting fractions 3–7 that showed a strong antifungal activity against both the test fungi and B. subtilis. A very strong antibacterial activity was found against P. multocida. A minor peak (fraction 13–16) was also observed, but it did not show any significant antimicrobial activity.

The crude extract, fraction 13 of gel filtration and fraction 5 of the ion-exchange chromatographic sample were electrophoresed on SDS-polyacrylamide gel. It was observed that the antimicrobial protein migrated as a single coomassie stain band for the ion-exchange sample.

The estimated molecular mass of the protein was 50 kDa. SDS-PAGE of the crude extract showed many protein bands that disappeared after gel filtration, and finally a single protein band corresponding to 50 kDa was obtained after ion-exchange chromatography. The purified protein, named as Ct-50, exhibited positive antimicrobial activity against the fungal and bacterial strains tested in the study.

As discussed earlier, no report to our knowledge exists in the literature for the antimicrobial proteins or peptides from Croton tiglium. However, antimicrobial proteins with similar molecular mass have been isolated by some workers from other plant species. A 45 kDa antifungal protein has been reported from blast fungus (Magnaporthe grisea)-treated rice leaves (Lee et al., 2007). Another 53 kDa homodimeric protein was purified from American ginseng (Panax quinquefolium) roots exhibiting antifungal, ribonuclease and anti-HIV-1 reverse transcriptase activities, designated as quinqueginsin (Wang and Ng, 2000). A protein with molecular mass of 30 kDa possessing strong and broad-spectrum antifungal activity is reported from the leaf extracts of Engelmannia pinnatifida (Huynh et al., 1996). Similarly, 30 kDa ribosome-inactivating protein possessed antifungal potential, and a 28 kDa antifungal protein, were isolated from dehusked barley grains (Leah et al., 1991).

In conclusion, the results of this study show that Croton tiglium Linn is a good source of antimicrobial proteins. A 50 kDa protein was purified from the plant that exhibited strong and broad spectrum antimicrobial activity. However, further studies like sequence analysis and characterization of the purified protein are necessary before its use as antimicrobial agent against pathogenic fungi and bacteria.

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