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Isolation of Vulgin, a New Antifungal Polypeptide with Mitogenic Activity from the Pinto Bean

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Abstract: An antifungal polypeptide bearing an *N*-terminal sequence with some homology to chitinases was purified from an extract of pinto beans. The polypeptide, designated vulgin, exerted antifungal activity toward *Mycosphaerella arachidicola*, *Coprinus comatus*, *Fusarium oxysporum* and *Botrytis cinerea*. Vulgin inhibited translation in a rabbit reticulocyte lysate system with an IC_{50} of 4.3 μM and HIV-1 reverse transcriptase activity with an IC_{50} of 58 μM. Vulgin stimulated *in vitro* incorporation of methyl [^{3}H] thymidine into mouse splenocytes. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chitinase; antifungal; mitogenic; pinto bean

INTRODUCTION

Leguminous plants have formed a popular subject of research owing to the abundance of polypeptides and peptides with important biological activities that they elaborate. Examples of these proteins are protease inhibitors [1], amylase inhibitors [2], lectins [3], antifungal polypeptides [4,5] and ribosome inactivating proteins [6]. All these polypeptides may have a common function of defence against predators such as insects [7] and pathogens such as fungi [8].

To date a large number of antifungal polypeptides have been reported. Structurally they can be divided into many types that comprise thaumatin-like polypeptides [9], chitinases [4,5,9], glucanases [5], embryo-abundant polypeptides [10], miraculin-like polypeptides [8], cyclophilin-like polypeptides [11], allergen-like peptides [12] and antifungal peptides [13]. Sometimes a combination of antifungal polypeptides is found in a single species [14].

From the adzuki bean an antifungal peptide has been purified [15]. From the pinto bean a 5 kDa antifungal peptide with sequence homology to the 10 kDa cowpea precursor has been isolated [16]. We report herein the isolation of a chitinase-like antifungal polypeptide designated vulgin from the pinto bean. Vulgin manifested activities similar to the pinto bean antifungal peptide [16] including antifungal, mitogenic, HIV reverse transcriptase inhibiting and translation-inhibiting activities.

MATERIALS AND METHODS

Isolation Procedure

Pinto beans (*Phaseolus vulgaris*) from the USA were obtained from a local vendor. They were first soaked in distilled water for a few hours before homogenization. To the supernatant obtained after centrifugation, Tris-HCl buffer (pH 7.2) was added until the final concentration attained 10 mM. The supernatant was then chromatographed on a column of Affi-gel blue gel (2.5 × 18 cm) in 10 mM Tris-HCl buffer (pH 7.2). After elution of unadsorbed protein, adsorbed proteins were eluted

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with a linear gradient of 0–0.5 M NaCl in the starting buffer in two peaks A and B. The first adsorbed peak, A, was then fractionated on a column of SP-Toyopearl (2.5 × 18 cm). The column was initially eluted with 10 mM Tris-HCl buffer to remove unbound material and subsequently with a linear gradient of 0–0.5 M NaCl to desorb bound material. The second adsorbed peak, A2, was further purified by FPLC on a Mono S column (1 ml) in 10 mM Tris-HCl buffer (pH 7.2). After elution of unadsorbed material, the column was eluted with a linear concentration gradient of 0–0.5 M NaCl in the Tris-HCl buffer to elute adsorbed material. The purity and molecular mass of the major peak with antifungal activity were assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli and Favre [17] using 15% gel, and also by FPLC-gel filtration on a Superdex 75 column which had been calibrated with molecular mass standards. *N*-terminal sequencing of the purified antifungal polypeptide was conducted using a Hewlett-Packard G-1000A Edman degradation unit and an HP 1000 HPLC system [6, 15].

Assay for Translation-Inhibiting Activity

The purified antifungal polypeptide was assayed for cell-free translation-inhibiting activity in a rabbit reticulocyte lysate system as described by Lam *et al.* [6]. The antifungal polypeptide (10 µl) was added to 10 µl of radioactive mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine and 1 µCi [4,5-^{3}H] leucine) and 30 µl working rabbit reticulocyte lysate containing 0.1 µM hemin and 5 µl creatine kinase. Incubation proceeded at 37 °C for 30 min before addition of NaOH and H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of a trichloroacetic acid solution (40%) was used to precipitate radioactively labelled protein. The precipitate was collected, washed and dried. The filter was suspended in scintillant and counted.

Assay for HIV Reverse Transcriptase Inhibiting Activity

The assay of the antifungal polypeptide for ability to inhibit human immunodeficiency virus (HIV) reverse transcriptase activity was carried out as detailed by Collins *et al.* [18] using a nonradioactive ELISA kit. The absorbance of the samples at 405 nm can be determined using a microtitre plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of antifungal polypeptide was calculated as the percent inhibition compared with a control without the antifungal polypeptide.

Assay for Mitogenic Activity

The antifungal polypeptide was assayed for mitogenic activity in mouse splenocytes as detailed by Wang *et al.* [19]. Four C57BL/6 mice (20–25 g) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sieve and resuspended to 5×10^6 cells/ml in culture medium. The cells (7×10^5 cells/100 µl/well) were seeded into a plate and serial dilutions of a solution of the antifungal polypeptide in 100 µl medium were added. After incubation of the cells at 37 °C in 5% CO_2 for 24 h, 10 µl methyl [^{3}H]thymidine (0.25 µCi) was added, and the cells were incubated for a further 6 h. The cells were then harvested, and the radioactivity was measured.

Assay for Antifungal Activity

The antifungal activity of the purified polypeptide was assayed using sterile petri plates (100 × 15 mm) containing 10 ml potato dextrose agar. Sterile paper disks (0.625 cm in diameter) were placed at a distance of 1 cm from the rim of the mycelial colony. The test sample was added to a disk, and the plate was incubated at 23 °C until mycelial growth had enveloped disks containing the control (buffer) and had formed crescents of inhibition around disks with samples expressing antifungal activity. The fungi studied comprised *Mycosphaerella arachidicola, Coprinus comatus, Fusarium oxysporum* and *Botrytis cinerea* [20].

For a quantitative assay, different doses of the antifungal polypeptide were added separately to aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into separate 6 cm petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was inoculated. Buffer only without antifungal polypeptide served as a negative control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined [20].

RESULTS

Purification and *N*-terminal Sequence

Affinity chromatography of a crude extract of pinto beans on Affi-gel blue gel yielded an unadsorbed peak and two adsorbed peaks, peaks A and B. Peak A was a sharper peak (Figure 1a). Ion exchange chromatography of peak A on SP-Toyopearl resulted in a large unadsorbed peak and three adsorbed peaks A1, A2 and A3 (Figure 1b), A1 being the smallest. A2 was fractionated by FPLC on Mono S into a small unadsorbed peak and four adsorbed peaks (Figure 1c). The major adsorbed peak contained a purified antifungal polypeptide designated vulgin with a molecular mass of 28 kDa as estimated by SDS-polyacrylamide gel electrophoresis (Figure 2) and gel filtration on Superdex 75 (data not shown). The *N*-terminal sequence of vulgin is presented in Table 1. A certain extent of similarity to partial sequences of some chitinases was apparent. From 100 g seeds, 14.2 g crude extract, 736 mg fraction A from Affi-gel blue gel, 76.8 mg A2 from SP-Toyopearl and 18.0 mg vulgin from Mono S were obtained.

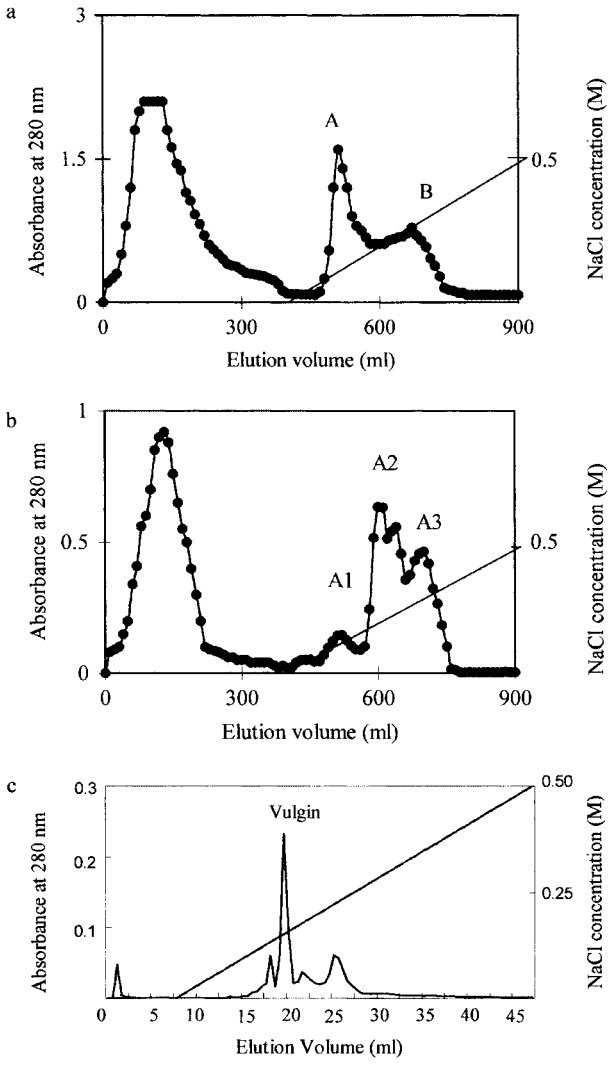

Figure 1 (a) Affinity chromatography of crude extract of pinto beans on Affi-gel blue gel. The slanting lines across the chromatograms indicate application of a linear salt concentration gradient (0–0.5 M NaCl) to elute absorbed protein. (b) Ion exchange chromatography of fraction A (from a) on SP-Toyopearl. (c) Ion exchange chromatography of fraction A2 (from b) by FPLC on Mono S. The purified antifungal polypeptide, vulgin, was eluted as the major adsorbed peak.

Translation-Inhibiting and HIV-1 Reverse Transcriptase-Inhibiting Activities

Vulgin inhibited cell-free translation and HIV-1 reverse transcriptase activity with an IC_{50} of 4.3 μM and 58 μM respectively (Tables 2 and 3).

Antifungal Activity

The antifungal activity of vulgin is shown in Figure 3. Strong activity was exerted against *Mycosphaerella arachidicola* and *Coprinus comatus* and weaker activity against *Fusarium oxysporum*

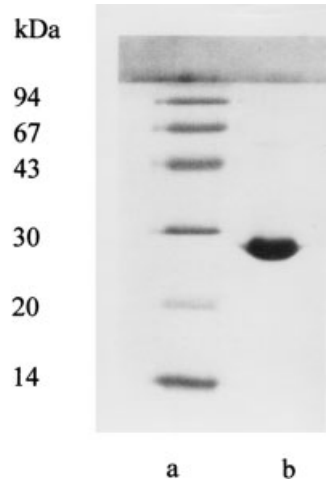

Figure 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of vulgin. Left lane: molecular mass markers, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (MW 14.4 kDa). Right lane: vulgin (15 μg).

Table 1 *N*-Terminal Sequence of Vulgin (Results of a BLAST search)

	Length[b]	Residue[a]	Partial Sequence	%	Identity
Vulgin		1	VDVGTVLTATFIEQFFKHRNDQAPEGKGFYTYNAFISAAR	100	50
Peanut chitinase	264	26	DAGTIITQPLYNEFLKHLTDSRCEAHGFYTYNAFVTAAR	50	100
Arbis alpina chitinase	212	48	DLSGIISSSQFDDMLKHRNDAACPARGFYTYNAFITAAK	42	49
Grape chitinase	325	83	DISSLISKSLFDEMLKHRNDAACPGKGFYTYEAFISAVK	45	44
Chickpea chitinase	328	69	VGSIISRDTFNQMLKHRDDSGCEGKGFYTYEAFIAAAK	45	54
Garden pea chitinase	320	79	DVGRLVPSSLFDQMLKYRNDGRCAGHGFYTYDAFIAAAR	50	49

Residue No. 26 (i.e. residue = 26)[a] in peanut chitinase which has 264 residues (i.e. length = 264)[b] is D.
Residue identical to corresponding residues in vulgin are underlined.

Table 2 Inhibition of HIV-Reverse Transcriptase by Vulgin

Concentration (μM)	Inhibition (%) (mean ± SEM, $n = 3$)
59.5	53.6 ± 1.8
11.9	21.4 ± 0.9
2.3	8.7 ± 2.0

IC_{50} = 58 μM.

Table 3 Inhibition of Cell-Free Translation in Rabbit Reticulocyte Lysate by Vulgin

Concentration (μM)	Inhibition (%) (mean ± SEM, $n = 3$)
108.0	89.2 ± 2.5
21.6	68.4 ± 1.6
4.3	49.5 ± 1.0
0.9	16.7 ± 0.8

IC_{50} = 4.3 μM.

and *Botrytis cinerea*. Vulgin inhibited the growth of *Botrytis cinerea* with an IC_{50} of 7 μM.

Mitogenic Activity Toward Splenocytes

There was an approximately 4-fold stimulation of methyl[^{3}H] thymidine incorporation into murine splenocytes at 3 μM vulgin. The mitogenic activity of vulgin started to decline beyond 3 μM.

DISCUSSION

The pinto bean antifungal polypeptide purified in this investigation designated vulgin exhibited an *N*-terminal sequence with some similarity to chitinases. The polypeptide designated vulgin exerted a distinct antifungal effect toward *Mycosphaerella arachidicola, Coprinus comatus*, *Fusarium oxysporum* and *Botrytis cinerea*. Its antifungal activity against *Fusarium oxysporum* was stronger than many leguminous antifungal proteins [8, 9, 11, 12]. It exerted a more potent inhibitory action on *Mycosphaerella arachidicola* than cowpea chitinase [14], a similar action on *Coprinus comatus* as sativin [8], and a weaker antifungal action than ginkbilobin toward *Botrytis cinerea* [10].

Vulgin inhibited HIV-1 reverse transcriptase activity with an IC_{50} of 58 μM. Some natural products also inhibited the HIV enzyme with a similar potency [21]. The potency of vulgin was higher than some of the leguminous antifungal proteins. Similar to other antifungal polypeptides, vulgin inhibited cell-free translation in the rabbit reticulocyte lysate system with an IC_{50} in the micromolar level. However, the IC_{50} values for other antifungal polypeptides often exceeded 10 μM, indicating that vulgin had a higher activity.

Mungin, a cyclophilin-like antifungal polypeptide from mung bean, exhibited an anti-mitogenic activity toward murine splenocytes [1]. Other antifungal polypeptides including hypogin from peanuts and chrysancorin from *Chrysanthemun, coronarium* seeds [12, 22] had a stimulatory influence on methyl-^{3}H-thymidine uptake by splenocytes. Yet some others such as chive chitinase [20] had no effect at all on splenocyte proliferation. Vulgin

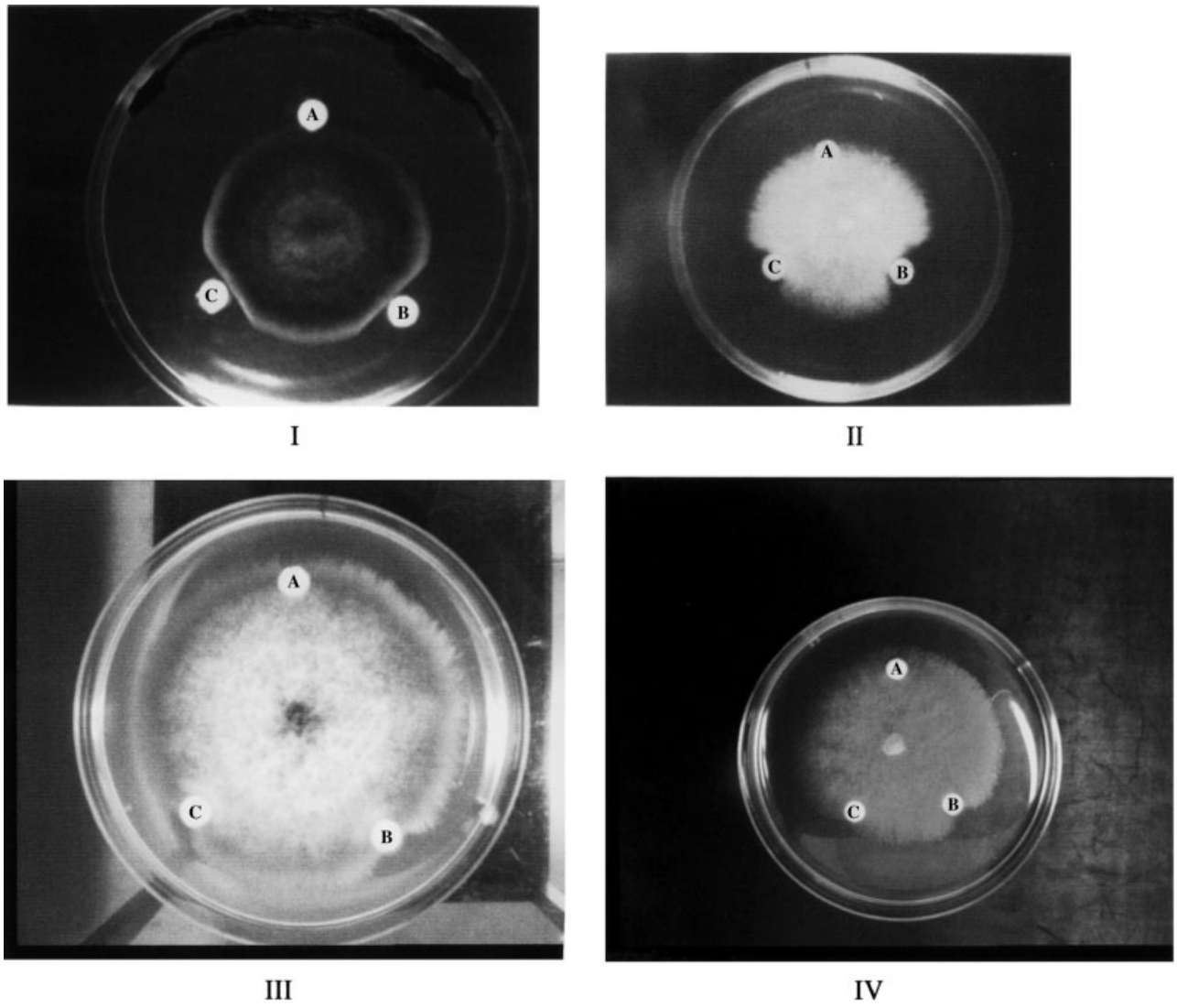

Figure 3 Antifungal activity of vulgin toward (I) *Mycosphaerella arachidicola*, (II) *Coprinus comatus*, (III) *Fusarium oxysporum* and (IV) *Botrytis cinerea*. A, control; B, 300 μg vulgin; C, 60 μg vulgin.

induced an approximately 4-fold higher proliferation of splenocytes.

Vulgin resembled other antifungal polypeptides and peptides [8–12] in that it was adsorbed on Affi-gel blue gel, CM-Sepharose and Mono S. Its molecular mass of 28 kDa is similar to those of jackbean and peanut chitinases but smaller than those of grape, chickpea and garden pea chitinases.

To sum up, a chitinase with potent antifungal, translation-inhibiting and HIV-1 reverse transcriptase inhibiting activity was isolated from the pinto bean.

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