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Dolichin, a New Chitinase-like Antifungal Protein Isolated from Field Beans (*Dolichos lablab*)

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An antifungal protein, possessing a molecular weight of 28 kDa and an N-terminal sequence resembling chitinases, has been purified from the seeds of the field bean *Dolichos lablab*. The procedure involved extraction with aqueous buffer, affinity chromatography on Affi-gel blue gel, and ion exchange chromatography on CM-Sepharose. The protein, designated dolichin, exhibited antifungal activity against the fungi *Fusarium oxysporum*, *Rhizoctonia solani*, and *Coprinus comatus*. Dolichin was capable of inhibiting human immunodeficiency virus (HIV) reverse transcriptase and α- and β-glucosidases which are glycohydrolases implicated in HIV infection. It had very low ribonuclease and cell-free translation-inhibitory activities. © 2000 Academic Press

Key Words: **antifungal protein; seeds; *Dolichos lablab*.**

Antifungal proteins are known to be elaborated by leguminous species. Recently a protein, with an N-terminal sequence identical to that of chick pea thaumatin-like protein (1) and homologous to that of soybean thaumatin-like protein (2), has been isolated from legumes of the French bean *Phaseolus vulgaris* cv *Kentucky wonder* (3). The thaumatin-like protein from seeds of *Phaseolus vulgaris* cv *greensleeves* (4) exhibited an N-terminal sequence same as that from French bean legumes which was demonstrated to exert antifungal activity (3). Structural homology (58–80% identitiy) is detected between the leguminous thaumatin-like proteins and other dicot and monocot thaumatin-like proteins including those from grape berries (5), *Diospyros texana* (6) and tomato fruits (7), tobacco leaves (8), maize seeds (9), oat, barley, wheat and sorghum (10, 11). The aforementioned thaumatin-like proteins all bear remarkable structural resemblance to the sweet protein thaumatin from *Thaumatococcus danielli* (12). Thaumatin itself had only weak or indiscernible effects on the growth of the fungi *Fusarium oxysporum*, *Pleurotus ostreatus* and *Coprinus comatus* although the French bean thaumatin-like protein manifested a significant inhibitory action on the growth of the same fungal species (3).

We present herein results of an investigation to isolate an antifungal protein, which has an N-terminal sequence structurally disparate from the previously described leguminous thaumatin-like proteins (1–4), from the seeds of the field bean *Dolichos lablab*. Despite structural dissimilarity from the French bean thaumatin-like antifungal protein (3), the field bean protein, designated dolichin, was able to effectively inhibit fungal growth. Dolichin shows structural resemblance to chitinases which are known to have antifungal activity (13). In addition, dolichin was endowed with the ability to inhibit human immunodeficiency virus (HIV) reverse transcriptase and the glycohydrolases α- and β-glucosidases.

MATERIALS AND METHODS

(*Dolichos lablab*) seeds were obtained from a local market. The seeds were homogenized in distilled water. The homogenate was centrifuged and the supernatant, designated crude extract, was dialyzed against distilled water and then Tris-HCl buffer (pH 7.2) was added until the final concentration of Tris in the crude extract was 10 mM. The crude extract was then chromatographed on a column of Affi-gel blue gel (2.5 × 10 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 7.2. After elution of a large amount of unadsorbed proteins, the column was eluted with a linear gradient of NaCl (0–500 mM) in the same buffer. The desorbed material was subsequently applied to a column of CM-Sepharose (1.5 × 18 cm) which had been equilibrated with 10 mM Tris-HCl buffer, pH 7.2. Following removal of a large quantity of unadsorbed materials, the column was eluted with a gradient of NaCl (0–500 mM) in the buffer to yield three peaks. LL-2 represents the purified antifungal protein designated dolichin. N-terminal sequencing of dolichin was carried out using a Hewlett-Packard (HP) G-1000A Edman degradation unit and an HP 1000 HPLC System. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dolichin was conducted according to the method of Laemmli and Favre (14).

Assay of dolichin for antifungal activity. The assay for antifungal activity was conducted using 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. Around and at a distance of 1 cm away from the central disk (0.625 cm in diameter) were placed sterile

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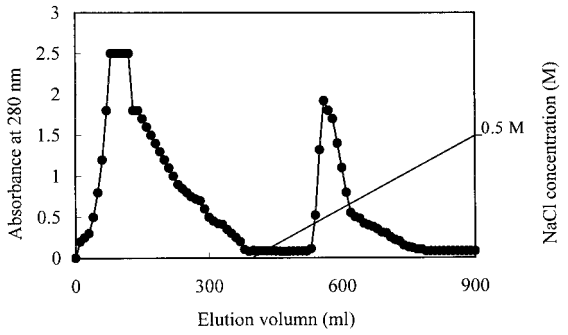

FIG. 1. Fractionation of the crude extract of field beans on an Affi-gel blue gel column equilibrated with the binding buffer (10 mM Tris–HCl, pH 7.2). The gel was washed with the binding buffer and eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer.

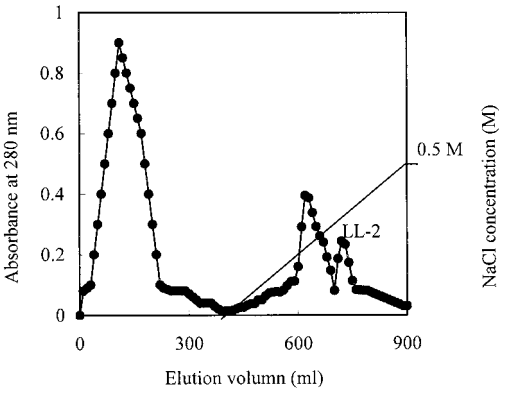

FIG. 2. Elution profile from the CM-Sepharose column. After chromatography on Affi-gel blue gel, the adsorbed fraction was dialyzed and then applied to CM-Sepharose column in 10 mM Tris–HCl buffer (pH 7.2). The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 500 mM in the same Tris–HCl buffer, pH 7.2.

blank paper disks of the same size. An aliquot (6 μl) of dolichin in 10 mM sodium acetate buffer (pH 5.5) containing 130 mM NaCl was introduced to a disk. The plates were incubated at 23°C for 72 h until mycelial growth from the central disk had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungal species used comprised *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus* (3).

Assay of dolichin for HIV reverse transcriptase inhibitory activity. Reverse transcriptase activity was measured by ELISA as described by Collins *et al.* (15) using a non-radioactive kit from Boehringer Mannheim (Germany). The inhibition assay was performed as described in the protocol included with the kit, except that each well contained 2 ng recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 μl.

Assay of dolichin for glycohydrolase inhibitory activity. β-Glucosidase, α-glucosidase and β-glucuronidase and their corresponding p-nitrophenyl glycoside substrates were used to set up the enzymatic reaction in pH 6.4 10 mM MES buffer. The enzyme inhibition assay has been adapted for use in a 96-well microplate allowing greater convenience, speed and reproducibility and enabling rapid screening of samples (15, 16). Substrates and enzymes were dissolved in 50 mM buffer appropriate for each enzyme (MES-NaOH, pH 6.5 for α-glucuronidase, sodium acetate pH 5.5 for β-glucosidase and sodium acetate pH 5.6 for β-glucuronidase). To test enzyme inhibition by dolichin, each well of the microplate contained 2 mM substrate, 40 mM buffer, dolichin and sufficient enzyme to cause a measurable change in absorbance at 405 nm (0.2 unit/well for α-glucosidase, 0.01 unit/well for β-glucosidase and 100 units/well for β-glucuronidase). Dolichin was allowed to interact with the enzymes for 5 minutes before the reaction was initiated by addition of substrate. The total reaction volume was 0.2 ml. The plate was incubated at room temperature for 15 min before the reaction was terminated by addition of 59 μl 2 M glycine-NaOH, pH 10. The plate was then read on BioRad microplate reader at 405 nm.

Assay of dolichin for ribonuclease activity. Yeast tRNA was used as substrate. Dolichin was incubated with 200 μg tRNA in 150 μl 100 mM Tris-HCl buffer (pH 7.5) before 350 μl ice-cold 3.4% perchloric acid was added to terminate the reaction. After standing an ice for 15 min the reaction mixture was centrifuged and the absorbance of the supernatant was read after suitable dilution. One unit of ribonuclease activity is defined as the amount of enzyme which produces an absorbance increase of one per minute in the acid-soluble supernatant per ml of reaction mixture under the specified conditions.

Assay of dolichin for cell-free translation-inhibitory activity. Rabbit reticulocyte lysate was prepared as described in (17). The ability of dolichin to inhibit [^{3}H]leucine into proteins in the rabbit reticulocyte lysate system was assessed as detailed in (18).

RESULTS

Following removal of unadsorbed materials, adsorbed proteins including the antifungal protein could be desorbed from the Affi-gel Blue gel column with a linear NaCl concentration gradient (Fig. 1). The adsorbed peak was subsequently chromatographed on a CM-Sepharose column. The unadsorbed material from the column did not possess antifungal activity. Antifungal activity resided in peak LL-2 in the adsorbed area (Fig. 2). The protein yields throughout the different stages of purification are shown in Table 1. The SDS-polyacrylamide gel electrophoretic pattern of peak LL-2 is shown in Fig. 3. The single band, which attests to the homogeneity of the antifungal protein preparation, possesses a molecular weight of 28 kDa as estimated from its electophoretic mobility relative to those of molecular weight markers. Table 2 presents the N-terminal amino acid sequence of the purified *Dolichos lablab* protein, designated dolichin, in comparison with chitinases and chitinase precursors obtained from a BLAST search. Considerable sequence

TABLE 1

Summary of Purification of Antifungal Protein from *Dolichos lablab*

Fraction	Protein (mg[a])
Crude extract	18585
Affi-Gel Blue Gel	110.2
CM-Sepharose	8.4

[a] Protein obtained from 150 g *Dolichos lablab.*

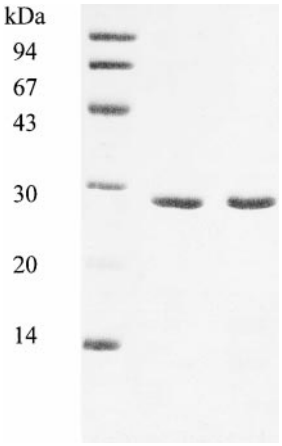

FIG. 3. SDS-polyacrylamide gel electrophoresis of dolichin. From left to right: lane 1, Pharmacia molecular weight standards (from top downward, phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa and lactalbumin 14 kDa); lanes 2 and 3, 25 μg dolichin.

identity was detected. Figures 4 to 6 present the antifungal activity of dolichin on three fungal species. The effect was very prominent on *Coprinus comatus* and *Rhizoctonia solani* but less so on *Fusarium oxysporum*.

The purified antifungal protein inhibited HIV-1 reverse transcriptase, α-glucosidase and β-glucosidase but not β-glucuronidase (Table 3). It inhibited cell-free translation with an IC_{50} of about 30 μM (Table 4). It had minimal RNase activity (data not shown).

DISCUSSION

A procedure which has been successfully employed for isolating French bean thaumatin-like antifungal protein (3) was also applicable for purifying a chitinase-like antifungal protein from *Dolichos lablab* seeds. The procedure entailed adsorption of antifungal protein on the affinity chromatographic medium Affi-gel blue gel. This step enabled removal of an appreciable amount of proteins without antifungal activity in the unadsorbed fraction. The next step involved fractionation on the ion exchanger CM-Sepharose. A portion of the inactive materials was eliminated in the unadsorbed fraction. The antifungal protein was separated from extraneous proteins devoid of antifungal activity by means of a linear NaCl gradient. In spite of structural dissimilarity between French bean thaumatin-like antifungal protein and *Dolichos lablab* antifungal protein, the same purification protocol could be used for purifying both proteins. Whether the procedure is also efficacious for isolating antifungal proteins from other leguminous species remains to be established.

Different molecular weights have been found for thaumatin-like proteins: 20 kDa for French bean, 22 kDa for maize, 23 kDa for tomato, 24 kDa for grape and

TABLE 2

Comparison of N-Terminal Sequence of Dolichin (*Dolichos lablab* Antifungal Protein) with Those of Chitinases and Chitinase Precursors

	Length	Residue		Residue	% Identity
Dolichos lablab		1	GAVGSVINASLFEQLLKHRNDQDPEGKG · FYSYNAFITA	38	100
Canavalia ensiformis	270	30	VGSVIDASLFDQLLKHRNDPACEGKG · FYSYNAFVTA	65	83
Nicotiana tabacum	324	75	LGSIISSSMFDQMLKHRNDNACQGKG · FYSYNAFINA	110	66
Musa acuminate (RAP)	122	67	SVGSIISSSLFEXMLKHRNDAACPGKG · FYTYNAFIAA	103	67
Cicer arietinum	328	69	VGSIISRDTFNQMLKHRDDSGCEGKG · FYTYRAFIAA	104	61
Zea mays	275	19	VASIITEDLFERMLKHRNETDCKARG · FYTYDDFITA	54	58
Persea americana	326	75	VASLISQSVFNQMLKHRNDAACQAKG · FYTYNAFIAA	110	61
Vitis vinifera	325	84	ISSLISKSLFDEMLKHRNDAACPGKG · FYTYEAFISA	119	58
Solanum tuberosum	316	66	LGGVISNSMFDQMLNHRNDNACQGKGNFYSYNAFISA	102	62
Humulus lupulus	316	76	VSSVISSALFEEMLKHRNDGGCPGRG · FYTYDAFLTA	111	61
Castanea sativa	316	77	VGSLISASLFDQMLKYRNDPRCKSNG · FYTYNAFIAA	112	63
Oryza sativa	333	84	AVEAVVSKELFEQLLLHRNDAACPARG · FYTYDAFVTA	120	56
Gossypium hirsutum	263	22	ISSLISQDMFNEMLKHRNDGNCPGKG · FYTYDAFIAA	57	52
Elaeagnus umbellata	317	68	IESVISSNIFNQMLKHRNDGACKAKG FYTYDAFIKA	103	55
Pinus strobilus	265	35	QGVASIISEDVFHQFLKHRNDDACSAKG FYTYSAFIAA	72	52
Lycopersicon esculentum	246	5	ISSLISKNLFERILVHRNDRACGAKG FYTYEAFITA	40	55
Arabidopsis thaliana	335	86	LSGIISSSQFDDMLKHRNDAACPARG FYTYNAFITA	121	52
Pisum sativum	320	80	VGRLVPSSLFDQMLKYRNDGRCAGHG FYTYDAFIAA	115	55
Cucurbita Sp.	311	71	SVGSIINEALYNQMLKYSKDPRCPSNG FYRYNAFITA	107	54
Capsicum annum	253	27	IGSIVTRDLFERMLSFRNNAACPGKG FYTYEAFITA	62	52
Petroselinum crispum	267	26	VGSLISKAMFEDMLKHRNDANCPAKG FYTYEAFIDA	61	58
Triticum aestivum	320	79	VSSIISQSLFDQMLLHRNDAACQAKG FYNYGAFVAA	114	55

Note. RAP, ripening-associated protein. Residues identical to corresponding residues of *Dolichos lablab* antifungal protein are underlined.

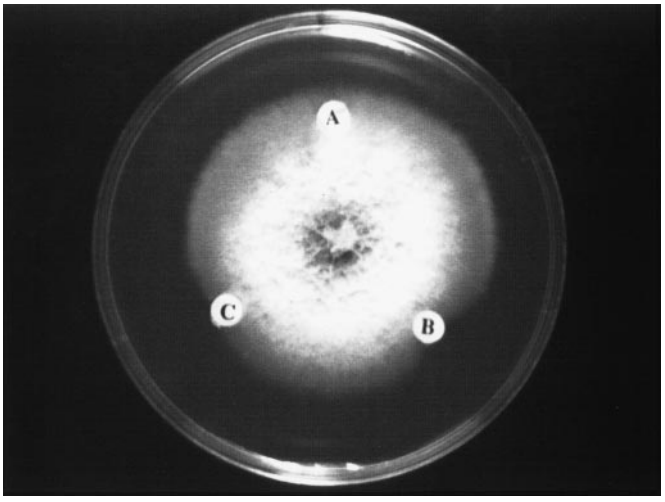

FIG. 4. Inhibitory activity of dolichin toward *Fusarium oxysporum*. (A) 10 mM NaOAc buffer, pH 5.5, (B) 300 μg dolichin, and (C) 60 μg dolichin.

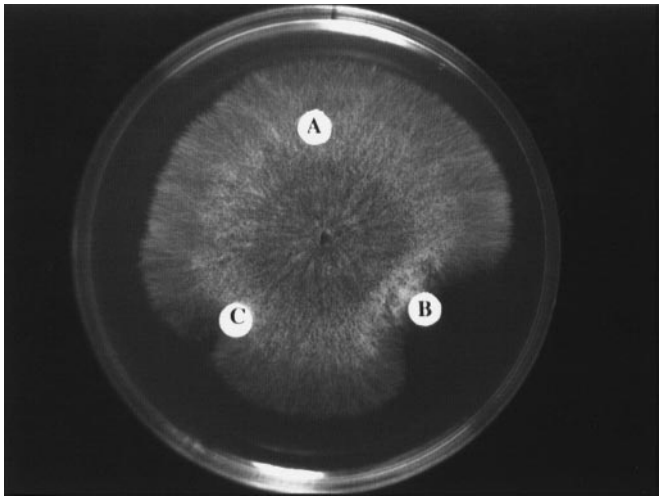

FIG. 6. Inhibitory activity of dolichin toward *Rhizotonia solani.* (A) 10 mM NaOAc buffer, pH 5.5, (B) 300 μg dolichin, and (C) 60 μg dolichin.

27 kDa for *Diospyros texana* (3, 5–7, 9). The *Dolichos lablab* chitinase-like antifungal protein isolated in the present study possessed a molecular weight of 28 kDa which, though within the range of molecular weights reported for thaumatin-like proteins, was distinctly higher than that of French bean thaumatin-like antifungal protein. The molecular weights of chitinases (exceeding 30 kDa) are in general higher than those of the aforementioned thaumatin-like proteins. Compared with chitinases from the leguminous plants *Canavalia ensiformis, Cicer arietinum* and *Pisum sativum,* dolichin had a molecular weight slightly lower than that of the first but considerably lower than those of the second and third. The sequence similarity was also more pronounced between dolichin and *C. ensiformis* chitinase (83% identity) than that between dolichin and *P. sativum* chitinase or between dolichin and C. *arietinum* chitinase (50–65% identity). The sequence analogy between dolichin and chitinases with a molecular weight exceeding 30 kDa, including the *Cicer arietinum* and *Pisum sativum* proteins, commences after the 65th amino acid. This is unlike the situation in thaumatin-like proteins: some demonstrate sequence identity with French bean antifungal protein starting at the first N-terminal amino acid while others begin to show sequence identity after the 20th residue. There are also residues encountered in the N-terminal sequence of dolichin which are rarely observed or not found in the corresponding position in the sequence of any other chitinase. They include the 8th (N), 13th (L) and 22nd to 25th (Q to E) residues. It seems that dolichin is the closest in molecular weight and N-terminal sequence to the chitinase from *C. ensiformis* even though sequence similarity is not evident until the 30th residue of the *C. ensiformis* protein.

Dolichin represents the first example of chitinase-like proteins demonstrated to have anti-HIV activity.

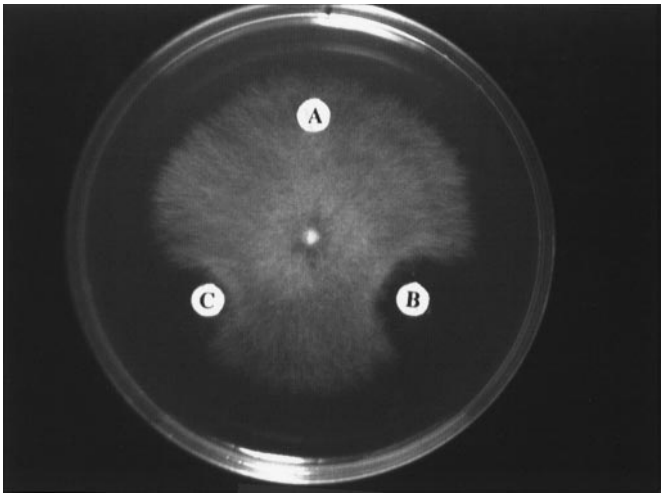

FIG. 5. Inhibitory activity of dolichin toward *Coprinus comatus.* (A) 10 mM NaOAc buffer, pH 5.5, (B) 300 μg dolichin, and (C) 60 μg dolichin.

TABLE 3

Inhibition of HIV-Reverse Transcriptase and Glycohydrolases Caused by Dolichin (*Dolichos lablab* Antifungal Protein)

% Inhibition (mean ± SD, n = 2)			
HIV reverse transcriptase	α-Glucosidase	β-Glucosidase	β-Glucuronidase
65.8 ± 0.8	23.4 ± 1.7	14.4 ± 1.3	Activation (12.6 ± 1.5)

Note. The concentration of the *dolichin* used was 5 mg/ml.

TABLE 4

Inhibition of Cell-Free Translation

Protein	Concentration (μM)	Inhibition (%)
Dolichin	142.9	91.2
	35.7	60.6
	28.6	43.0
	5.7	10.5

It exerted a much more notable inhibitory action against HIV-1 reverse transcriptase (with an IC_{50} less than 180 μM). Its suppressive effect on α- and β-glucosidases was only marginal while there was no inhibition on β-glucuronidase.

Chitinases have been purified from chickpea (*Cicer arietinum*) cell-suspension cultures (13) and ethylene-treated bean leaves (19). In the first case only one of the chitinases, the basic chitinase with a blocked N-terminal, possessed antifungal activity (13). The acidic class III chitinase was devoid of activity on fungi including *Rhizoctonia solani* and showed N-terminal sequence identity with class III acidic chitinases from *Cucumis sativis, Parthenocissus quinquifolia, Hevea brasiliensis* and *Arabidopsis thaliana*. In the latter case the enzyme has been evaluated in detail for its antifungal effect. Light microscopic examination disclosed chitinase-induced swelling of hyphal tips and hyphal distortions in the fungus *R. solani*. Wall disruption, release of chitin oligosaccharides from cell walls and cytoplasm leakage were observed in ultrastructural and cytochemical studies (19). In the present investigation a chitinase-like protein was isolated from untreated seeds and its antifungal activity displayed in three different fungal species including *R. solani, F. oxysporum* and *C. comatus*. Its inhibitory action on the growth of *R. solani* was much more potent than that of the French bean thaumatin-like protein (3).

A scrutiny of sequence data in Table 2 reveals that there is 50–60% sequence identity among chitinases from the legumes *C. ensiformis, C. arietinum* and *P. sativum*. This extent of sequence resemblance among the legume chitinases is not substantially different from that existing among legume chitinases and non-legume chitinases. Nor is it vastly incomparable to that found among non-legume chitinases. The high level (83%) of sequence identity between dolichin and *C. ensiformis* lectin is therefore outstanding. There are three blocks of amino acids which are conserved or nearly so throughout the sequences of the species examined in Table 2: LKHRND, GFYTY and AFITA.

Dolichin possessed minimal cell-free translational and ribonucleolytic activities and is thus unrelated to ribosome inactivating proteins and ribonucleases. It is a novel antifungal and anti-human immunodeficiency virus protein.

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