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First Chromatographic Isolation of an Antifungal Thaumatin-like Protein from French Bean Legumes and Demonstration of Its Antifungal Activity

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A protein, with a molecular weight of 20 kDa, and an N-terminal sequence analogous to those of thaumatin-like proteins (TLPs) and thaumatins, was first isolated from the legume of the French bean *Phaseolus vulgaris* cv *Kentucky wonder* using a simple procedure involving affinity and ion exchange chromatography. The protein was adsorbed on both CM-Sepharose and Affi-gel Blue Gel. It was the first leguminous TLP-like protein demonstrated to exert antifungal activity against *Fusarium oxysporum, Pleurotus ostreatus,* and *Coprinus comatus* but not against *Rhizoctonia solani.*

Key Words: **antifungal protein; thaumatin; French bean.**

In order to combat fungal attack, plants elaborate a variety of inhibitory molecules which comprise phenols, melanins, tannins or phytoalexins and pathogenesis-related proteins which are divided into five classes (1–5). One of the classes is made up of thaumatin-like proteins characterized by a remarkable homology in sequence to the sweet protein thaumatin from *Thaumatococcus danielli* (6).

We report herein the first isolation and characterization of a thaumatin-like protein (TLP), with inhibitory activity on fungal growth, from the legume (pod plus seeds) of the French bean.

MATERIALS AND METHODS

Fresh French beans (*Phaseolus vulgaris*) cv *Kentucky wonder* were purchased from a local market. Authentication was kindly carried out by Professor S. Y. Hu at the Herbarium of the Chinese University of Hong Kong.

The entire legumes 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 applied to 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 chromatographed on 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 M1, M2 and M3. M1 represents the purified thaumatin-like protein (TLP). N-terminal sequencing of TLP was conducted using a Hewlett-Packard (HP) G-1000A Edman degradation unit and an HP 1000 HPLC System. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of TLP was performed according to the method of Laemmli and Favre (7).

The assay for antifungal activity was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. Around the central disk (0.625 cm in diameter) and at a distance of 1 cm away from it were placed sterile blank paper disks of the same size. An aliquot (6 μl) of the test sample in 10 mM sodium acetate buffer (pH 5.5) containing 130 mM NaCl was added to a disk. The plates were incubated at 23°C for 72 hours until mycelial growth from the central disk had enveloped peripheral disks containing the control (phosphate buffered saline) and had formed crescents of inhibition around disks containing samples with antifungal activity. The fungal species used included *Fusarium oxysporum, Rhizoctonia solani, Pleurotus ostreatus,* and *Coprinus comatus.*

RESULTS

The French bean TLP was adsorbed on Affi-gel Blue Gel and could be eluted by a NaCl gradient (Fig. 1). Further purification was achieved by ion exchange on CM-Sepharose; French bean TLP appeared as a major peak, M1 (Fig. 2). Table 1 summarizes the purification of French bean TLP. In SDS–PAGE the protein exhibited a molecular weight of 20 kDa (Fig. 3). Table 2 presents a comparison of N-terminal amino acid sequence of French bean TLP with those of other TLPs, pathogenesis-related proteins, antifungal proteins, osmotin-like proteins, ripening-associated proteins, and thaumatins I and II. It can be seen that there is a pronounced similarity. French bean TLP exerted a sig-

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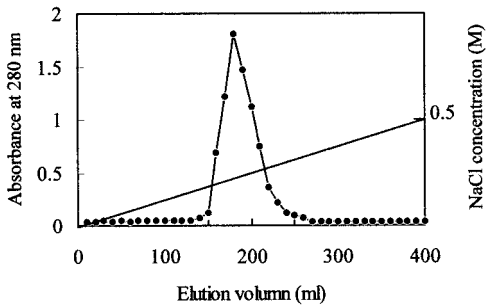

FIG. 1. Fractionation of the crude extract of French bean on Affi-Gel Blue Gel column equilibrated with the binding buffer (10 mM Tris-HCl, pH 7.2). The gel was washed with the binding buffer and eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer.

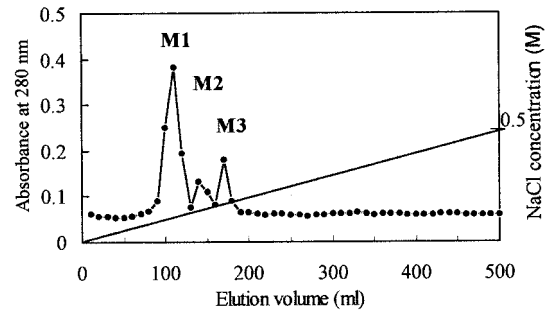

FIG. 2. Elution profile from the CM-Sepharose column. After chromatography on Affi-Gel Blue Gel, the adsorbed fraction was dialyzed and then applied to CM-Sepharose column in 10 mM Tris-HCl buffer (pH 7.2). The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear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

nificant inhibitory effect on the growth of *Fusarium oxysporum*, *Pleurotus ostreatus* and *Coprinus comatus* (Figs. 4–6). No effect was observed on *Rhizoctonia solani*, however (Fig. 7). By comparison thaumatin (Sigma) had only weak or indiscernible effects on the same fungi.

DISCUSSION

A variety of sweet proteins with different structures have been discovered in Nature. They include monellin from *Dioscoreophyllum cumminsii* (8, 9), mabinlin from *Capparis masaikai* (10), brazzein and pentadin from *Pentadiplandra brazzeana* (11, 12), miraculin from *Richadella dulcifera* (13), curculin from *Curculigo latifolia* (14) and thaumatin from *Thaumatococcus danielli* (15). Engineering of plant proteins including mabinlin has been reviewed (16). Thaumatin, as a sweetener, flavor enhancer and masking agent, has been employed in a wide range of beverages, confectionery and bread, dairy products, desserts, agricultural, marine and livestock processed foodstuffs, flavorings, medicines, animal feeds and pet foods (17, 18). The economic importance of thaumatin is immense.

TLPs are proteins with 40% to 70% sequence identity to thaumatin. They have been isolated from a variety of species including grape berries (19), tomato fruits (20), *Diospyros texana* fruits (21), maize seeds (22), tobacco leaves (23), wheat, sorghum, oat and barley (24–25). With regard to leguminous plants, a thaumatin-like basic protein was demonstrated to be induced in the bean *Phaseolus vulgaris* in the abscission zones after ethylene treatment (26). A protein with marked structural resemblance to TLPs was isolated from mature soybean (*Glycine max*) leaves (27).

Del Campillo and Lewis (26) were the first to report ethylene-dependent accumulation of a thaumatin-like protein in plants and more specifically in bean tissues. In their study the seeds of *Phaseolus vulgaris* cv *greensleeves* were germinated into plants. The proteins that accumulated in proximal abscission zones (2 mm proximal pulvinus plus 2 mm stem) after ethylene treatment were analyzed with a two-dimensional gel electrophoretic procedure which combined a cationic polyacrylamide gel electrophoresis at near neutral pH with SDS-polyacrylamide gel electrophoresis. The proteins which appeared to be homogeneous were sequenced. One of them, protein no. 6, was sequenced up to the 20th residue. It was identical in sequence to the French bean TLP isolated in the present study. Antibodies against acidic TLP from tobacco cross-reacted with protein no. 6. Graham *et al.* (27) isolated soybean TLP from leaves. However, the proteins from *P. vulgaris* (26) and soybean (27) have not been tested for antifungal activity.

The present investigation represents the first reported endeavor to chromatographically isolate the TLP from French bean legumes and demonstrate that it exhibited antifungal activity. The French bean protein resembled its counterpart in tomato fruits (20) in that they were adsorbed on CM-Sepharose and S-Sepharose respectively. It differed from the TLP in *Diosphyros texana* fruits in that the latter was adsorbed on DEAE-Sepharose (21). The Affi-gel Blue Gel utilized in the purification scheme of the French bean TLP was useful because it could remove some extrane-

TABLE I

Summary of Purification of Thaumatin-like Protein from French Bean

Fraction	Protein (mg[a])
Crude extract	1818
Affi-gel Blue gel	42.0
CM-Sepharose	5.6

[a] Protein obtained from 225 g starting material.

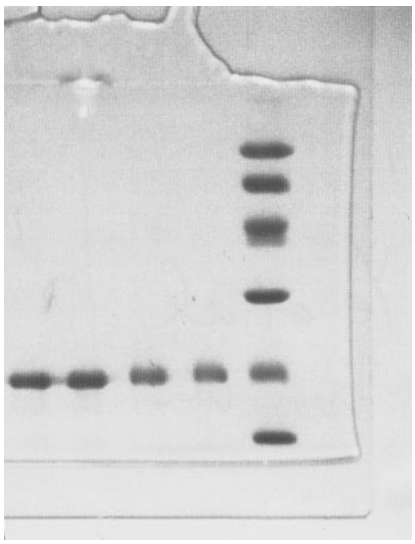

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of French bean thaumatin-like protein (TLP). From left to right: lanes 1 and 2, 25 μg TLP; lanes 3 and 4, 15 μg TLP; lane 5, Pharmacia molecular weight markers. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14 kDa).

ous materials. The isolation protocol for French bean TLP was different from those previously used for isolating other TLPs. For instance, $(NH_4)_2SO_4$ precipitation, chitin extraction and Mono S column chromatography were used for maize TLP (22); $(NH_4)_2SO_4$ precipitation, followed by chromatographic separations on S-Sepharose, hydroxyapatite and butyl Sepharose for tobacco TLP (26); chromatography on S-Sepharose, DEAE-Sephadex and Mono S for tomato TLP (20); $(NH_4)_4SO_4$ precipitation, DEAE-Sepharose chromatography and reversed phase HPLC on C18 column for *Diospyros texana* TLP (21); and Q-Sepharose and Superdex 200 chromatography for grape TLP (19); and chromatography on P-60 and phenyl-Sepharose followed by HPLC using a polypore phenyl column for soybean TLP (27). Nevertheless, the purification procedure for French bean TLP was a simple and efficient one and served its purpose. The molecular weight of French bean TLP was close to those reported for grape TLP (24 kDa), *Diospyros texana* RIP (27 kDa), maize TLP (22 kDa), and tomato TLP (23 kDa) (19–22). The N-terminal amino acid sequence of French bean TLP was identical to that of *Cicer arietinum* (28, Blast research results) deduced from gene sequence.

Maize TLP inhibited the growth of the agronomically important pathogens of potato wilt (*Fusarium oxysporum*) and tomato early blight (*Alternaria solani*) (22). Tobacco TLP retarded the growth of the nonphytopathogenic fungi *Neurospora crassa* and *Trichoderma reesei* and the phytopathogenic fungi *F. oxysporum, A. solani* and rice brown spot *Cochiliobolus miyabeanus* (26) Tomato TLP was very potent against *Phomo betae* and *Verticillium dahliae* (22). *Diospyros texana* and tobacco TLPs were effective against *Phytophthora infestans,* the causative agent of potato late blight (21, 29). In the present investigation French

TABLE II

Comparison of N-Terminal Sequence of French Bean Thaumatin-like Protein (TLP) with Those of Other TLPs, Pathogenesis-Related Proteins (PRP), Antifungal Proteins (AFP), Osmotin-like Proteins (OLP), Ripening-Associated Protein (RAP), and Thaumatins I and II

	Residue		Residue	% Identity
French bean TLP	1	ANFEIVNNCPYTVWAAASP-GGGRRLDRGQT	30	100
Chick pea TLP	22	ANFEIVNNCPYTVWAAASP-GGGRRLDRGQT	51	100
Soybean TLP	1	ARFEITNRCTYTVWAASVPVGGGVQLNPGQS	31	60
Tomato PRP P23	9	ATFEVRNNCPYTVWAASTPIGGGRRLDRGQT	44	80
Tobacco, Samsun NN, osmotin II	1	ATIEVRNNCPYTVWAASTPIGGGRRLDRGQT	31	77
Arabidopisis thaliana osmotin	23	ATFEILNQCSYTVWAAASP-GGGRRLDAGQS	52	80
Grape TLP	25	ATFDILNKCTYTVWAAASP-GGGRRLDSGQS	54	76
Commerson's wild potato OLP	22	ATIEVRNNCPYTVWAASTPIGGGRRLDRGQT	52	77
Wheat TLP	26	ATFNIKNNCPYTVWPAATPIGGGRQLNTGET	56	67
Barley TLP	1	ATFTVINKCQYTVWAAAVPAGGGQKLDAGQT	31	64
Maize TLP	1	AVFTVVNQCPFTVWAASVPVGGGRQLNRGES	31	61
Rice TLP	32	ATFAITNRCQYTVWPAAVPSGGGTKLDPGQT	62	64
Flax seed AFP	1	ARFDIQNKCPYTVWAASVPVGGGRQLNSGQT	31	67
Avena sativa TLP	22	ATFTITNNCGYTVWPAAIPVGGGQQLDQGQT	52	67
Pseudotsuga menziesii TLP	36	VKNQCSYTVWAAGSP-GGGKQLGQGET	61	61
Musa acuminata RAP	27	ATFXIVNRCSYTVWAAAVP-GGGRQLNQGQS	56	73
Thaumatin I	1	ATFEIVNRCSYTVWAAASK-G*GGRQLNSGET	36	58
Thaumatin II	23	ATFEIVNRCSYTVWAAASK-G*GGRQLNSGET	58	58

* The sequence DAALDA in both thaumatins I and II.

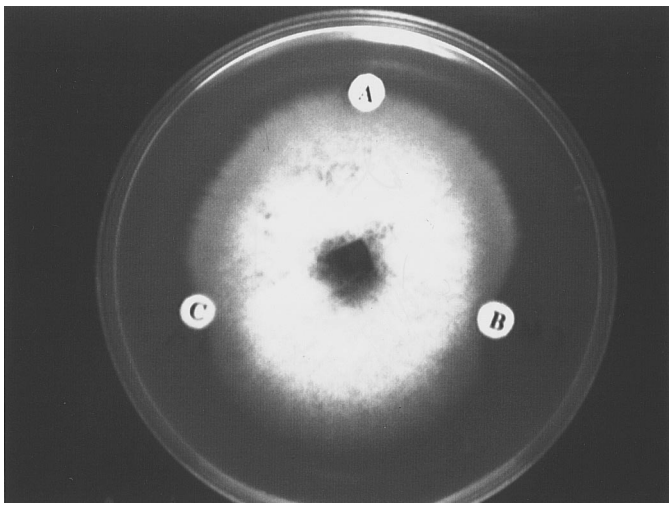

FIG. 4. Inhibitory activity of French bean thaumatin-like protein (TLP) toward *Fusarium oxysporum*. (A) 10 mM NaOAC buffer, pH 5.5, (B) 300 μg TLP, and (C) 60 μg TLP.

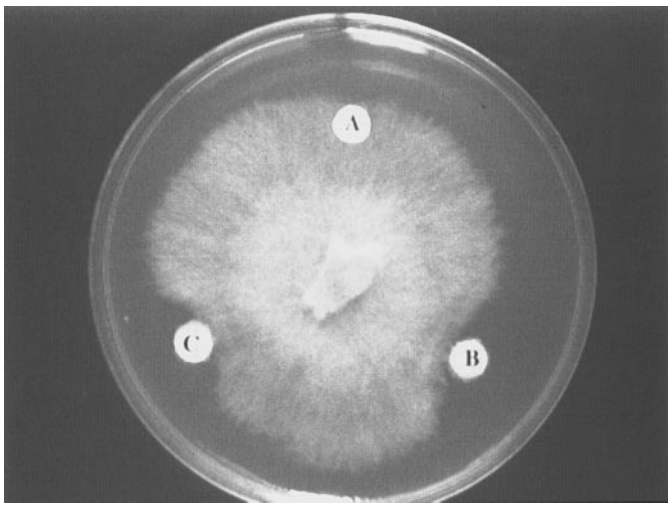

FIG. 6. Inhibitory activity of French bean thaumatin-like protein (TLP) toward *Coprinus comatus*. (A) 10 mM NaOAC buffer, pH 5.5, (B) 300 μg TLP, and (C) 60 μg TLP.

bean TLP exhibited growth-retarding effects on one pathogenic fungus, *Fusarium oxysporum*, failed to do so on another pathogenic fungus, *Rhizoctonia solani* and inhibited the growth of two edible mushrooms, *Pleurotus ostreatus* and *Coprinus comatus*. The antifungal activity of French bean TLP was very conspicious compared with thaumatin. Thaumatin alone has not been reported to be antifungal (30) although possibility of an antifungal action of thaumatin has been raised (24).

From the BLAST search results it is interesting to note that the first 30 amino acid residues of French bean TLP correspond to the first 30 or so residues of thaumatin I and TLPs from soybean, barley, maize and flax seeds and osmotin II from tobacco *Nicotiana tabacum* cv *Samsun NN*. Other TLPs, osmotins and osmotin-like proteins contain an N-terminal sequence of 20 or more residues which is absent in French bean TLPs, and the resemblance between these proteins and French bean TLPs begins at the end of this sequence. The similarity of French-bean TLP to *Cicer arietinum* TLP is most remarkable. Interestingly, the resemblance between French bean TLP and another legume TLP, the soybean TLP, is less extensive and not much greater than that existing between French bean TLP and thaumatin.

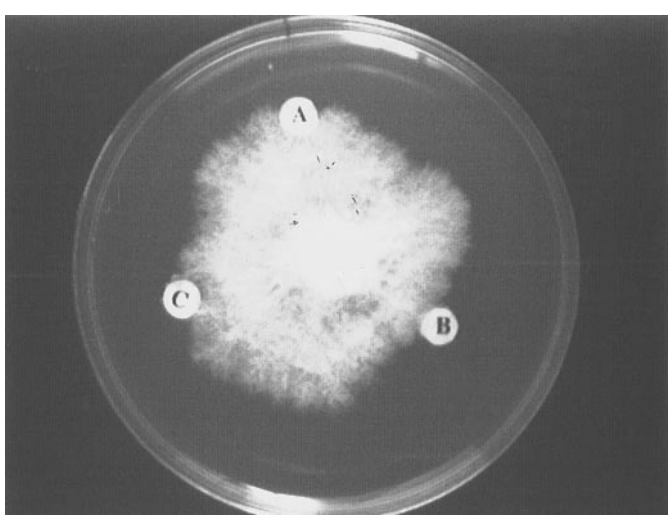

FIG. 5. Inhibitory activity of French bean thaumatin-like protein (TLP) toward *Pleurotus ostreatus*. (A) 10 mM NaOAC buffer, pH 5.5, (B) 300 μg TLP, and (C) 60 μg TLP.

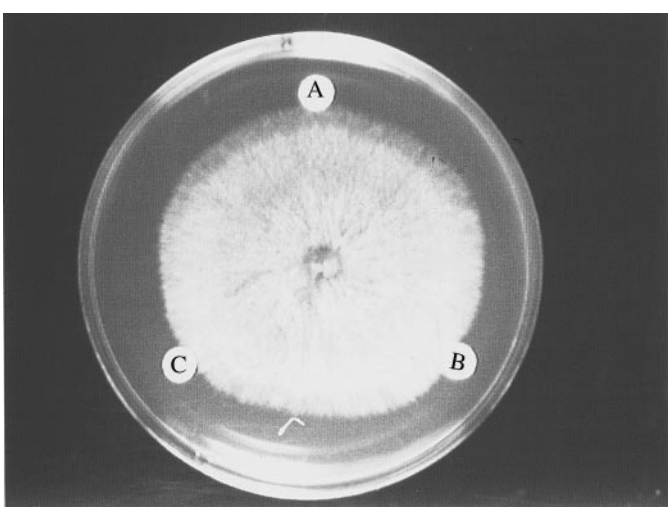

FIG. 7. Lack of inhibitory activity of French bean thaumatin-like protein (TLP) toward *Rhizotonia solani*. (A) 10 mM NaOAC buffer, pH 5.5, (B) 300 μg TLP, and (C) 60 μg TLP.

In summary, a relatively simple chromatographic procedure is described herein for purifying a TLP from the pod and seeds of a leguminous species. It furnishes evidence for the existence of TLP in legumes in addition to previously demonstrated TLPs in the leaf and stem tissues of these plants. Antifungal activity was first established as an attribute of bean TLP.

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