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Novel Protein from *Labramia bojeri* A. DC. Seeds Homologue to Kunitz-Type Trypsin Inhibitor with Lectin-like Properties

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This study starts by isolating and characterizing the first protein from *Labramia bojeri* seeds, which belong to the *Sapotaceae* family. The purified lectin analyzed by SDS−PAGE with and without β-mercaptoethanol shows two protein bands (M_r = 19 and 20 kDa), which cannot be resolved. Protein bands have shown similar characteristics as molecular masses, determined by gel filtration and native gel; N-terminal sequences presented a difference in their isoelectric points. We have suggested that those protein bands might be variants of the protein named Labramin. The sequence database search has shown that the N-terminal sequence of Labramin presented a high degree of homology to Kunitz-type trypsin inhibitor (82−52%) despite no trypsin inhibition activity detection. The lectin-like form from Labramin was better inhibited by glycoproteins and has also presented growth inhibition of the fungus *Colletotrichum lindemuthianum* and the yeast *Saccharomyces cerevisiae*, but it has not presented an apparent effect on *Fusarium oxysporum*.

KEYWORDS: *Labramia bojeri*; lectin-like; trypsin inhibitor; *Colletotrichum lindemuthianum*; *Saccharomyces cerevisiae*

INTRODUCTION

An important cause of grain deterioration is infection by fungal diseases. Just like infestation by insect pests, fungal infection may start in fields, and it is carried to storage. High relative humidity is a crucial factor for encouraging fungal infestation (*1*). Fungal infestation results in a reduction of grain quality; change in color, taste, or smell; reduction in nutritional value; increase in free fatty acids; and reduction of germination ability (*2*).

Plants, to protect themselves from infection by pathogens, evolve a number of defense mechanisms. In the case of fungal infection, those mechanisms include synthesis of inhibitory compounds such as phenols, melanins, tannins, or phytoalexins and protein accumulation, which act directly on fungal growth inhibition (*3*). Thionins (*4*), plant defensins (*5*), proteinases

inhibitors (*6*), lectins and lectin-like proteins (*7*), thaumatin- and miraculin-like (*8*), and proteins belong to antimicrobial proteins classes.

Lectins, particulary the chitin-binding protein carrying the hevein domain, have been studied extensively. Lectins, such as hevein (4.7 kDa) and UDA (8.5 kDa), may be small enough to penetrate through the fungal cell wall and reach the plasma membrane, where they may have an effect on the active sites involved in cell-wall morphogenesis (*9*). On the other hand, WGA (21 kDa) binding to fungal cell walls of the *Fusarium* species was shown to affect the inner compartment and growth of the germ tubes as revealed by dramatic changes in fungal morphology (*10*).

Protein protease inhibitors (PIs) are proteins that inhibit proteolytic enzymes, specifically and competitively. The majority of proteinase inhibitors studied in plant kingdom originates from three main families, namely, *Leguminosae*, *Solanaceae*, and *Gramineae*. Protease inhibitors exhibit a very broad spectrum of activity including suppression of pathogenic nematodes and inhibition growth of many pathogens (*11*). The mechanism of antifungal activity of these proteins is not fully understood.

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Labramin, a Novel Protein Homologue to Kunitz Trypsin Inhibitor

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In this work, we describe the purification and physicochemical properties of the first protein belonging to the family *Sapotaceae* from *Labramia bojeri* seeds (Labramin), a protein that presents homology to Kunitz trypsin inhibitor, lacking inhibitory activity and at the same time presenting lectin-like activity. We also observed its biological activity on *C. lindemuthianum* and *S. cerevisiae*.

MATERIALS AND METHODS

Plant Material. *L. bojeri* seeds were collected in the State of Rio de Janeiro (Brazil). Sephacryl S-400, Superdex 75, DEAE-Sepharose, acrylamide, methylene bis-acrylamide, bovine serum albumin (BSA), 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), dithiothreitol (DTT), molecular weight markers for sodium dodecyl sulfate-polyacrylamide (SDS−PAGE) gel electrophoresis and other electrophoresis reagents, ampholine (pH range 3.5−9.5), and pI markers were from Amersham Biosciences (Uppsala, Sweden). *N*-benzoyl-DL-arginyl-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), *N*-succinyl-alanine-alanine-alanine *p*-nitroanilide (SA$_3$PNA), and sugars were from Sigma (St. Louis, MO). All other chemicals were reagent grade and obtained from local suppliers.

Fungi/Yeast. *S. cerevisiae* strain 1038 was obtained from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil. *Fusarium oxysporum* and *Colletotrichum lindemuthianum* were kindly supplied by CNPF/EMBRAPA, Goiânia, Goiás, Brazil.

Purification of the Protein. Dehulled *L. bojeri* seeds were ground finely and extracted with five volumes (w/v) of 150 mM NaCl for 24 h at 4 °C followed by centrifugation at 10 000*g*, for 30 min at 4 °C. The clear supernatant was dialyzed against distilled water for 48 h and lyophilized to provide a crude extract (CE). This extract (400 mg/5 mL) dissolved in 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl was applied to a column (2.5 × 80 cm) of Sephadex S-400 equilibrated with the same solvent. Hemagglutinating activity was measured in the resulting fractions, and the active fractions (peak 3) were pooled, dialyzed, and freeze-dried. This material (75 mg/mL) was further fractionated by ion-exchange chromatography on a DEAE-Sepharose column (2 × 20 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and eluted with a linear gradient of NaCl (0−1.0 M) in the same buffer. Twenty mg of DEAE-1 lyophilized was dissolved in 250 μL of 0.1% (v/v) trifluoroacetic (solvent A). The resulting solution was clarified by centrifugation at 10 000*g* for 3 min, and the supernatant was applied to a μ-Bondapack C18 column (0.78 × 30 cm) (Waters 991-PDA system). The column was eluted with a linear gradient (0−100%, v/v) of acetonitrile (solvent B) at a flow rate of 2.0 mL/min. The elution profile was monitored at 230 nm, and fractions were collected and lyophilized. The last peak was identified as being lectin based on their agglutinating activity. Rechromatography of LABOL with a shallower gradient also yielded only one peak (data not shown).

Hemagglutination Assay. Hemagglutinating activity was assayed in U-bottomed 96-well microtiter plates in a final volume of 100 μL containing 50 μL of a 2% suspension of erythrocytes of human and/or animals previously washed three times in saline solution and 50 μL of a 2-fold serial dilution of lectin solution. In most of the studies were used glutaraldehyde-stabilized erytrrhocytes. Agglutination was scored after 1 h at 37 °C as the reciprocal of the highest lectin dilution giving detectable agglutination (*12*).

Hapten Inhibition of Haemagglutination. To measure hapten inhibition of haemagglutination, 25 μL of a 2-fold dilution of glycoproteins, mono- and oligosaccharides, methyl monoglycosides, laminarin and agarose in 0.05 M PBS, pH 7.4, was mixed with an equal volume of Labramin solution corresponding to 2 haemagglutination units (2 HU). The plate was incubated at room temperature for 1 h. A volume of 25 μL of a 2% suspension of thrice-washed glutaraldehyde-stabilized human A Rh+ erythrocytes in the same buffer were added, and the inhibition of haemagglutination was read 1 h later at room temperature (*13*). Minimal inhibitory concentration was registered as the lowest glycoprotein or carbohydrate concentration capable of avoiding the 50% visible agglutination (IC$_{50}$).

Assay of Enzymes Inhibitory Activity. Trypsin and chymotrypsin inhibitory activity was determined by measuring the remaining activity of trypsin and chymotrypsin toward the substrate 1 mM BAPNA and 1 mM BTPNA, respectively, at pH 8.0 after preincubation with inhibitor. Elastase inhibitory activity was determined by measuring the remaining activity of elastase toward the substrate 1 mM SA$_3$PNA (*14*). In all cases, the reaction was stopped by addition of 30% acetic acid. The assays were monitored spectrophotometrically at 410 nm.

Protein Assay. Protein concentrations were determined as described by Bradford (*15*), using BSA as standard.

Polyacrylamide Gel Electrophoresis. SDS−PAGE was done in 15.0% polyacrylamide gel as described by Laemmli (*16*). Native PAGE was done, without SDS.

Detection of Sugars. The total neutral sugar content of protein was estimated colorimetrically by the phenol/H$_2$SO$_4$ method (*17*), using D-glucose as standard.

Molecular Mass Determination by Gel Filtration. The M_r of the native protein was determined by using a Superdex 75 column in a FPLC (fast protein liquid chromatography) system, equilibrated with 100 mM PBS, pH 7.4, containing 100 mM D-glucose. Used M_r standards were BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.5 kDa).

Two-Dimensional Gel Electrophoresis. Approximately 5 μg of protein was added to 340 μL of sample buffer containing 8 M urea, 4% CHAPS, 2% carrier ampholytes pH 3−10, 70 mM DTT, and 0.001% bromophenol blue. The samples were applied to IPG strips with a pH 3−10 nonlinear separation range (catalog number 17-1235-01, Amersham Pharmacia Biotech). After 10 h rehydration, isoelectric focusing was done, at 20 °C for 1 h at 500 V, 1 h at 1000 V, and 10 h at 8000 V in an IPGphor apparatus (Amersham Biosciences). The limiting current was 50 μA per strip. Strips have then been soaked for 10 min in a solution containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, and 2% DTT and followed by an additional 10 min in the same solvent containing 2.5% iodoacetamide instead of DTT. Second dimension electrophoresis (SDS−PAGE) was done in an SE-600 system connected to a Multitemp II refrigerating system (Amersham Pharmacia Biotech). After positioning the strip on the top of a 12.5% polyacrylamide gel and sealing it with agarose, gels were run for 1 h at 90 V, after which a constant amperage of 30 mA per gel was applied until the migration front reached the lower end of the gel. Proteins were detected by a standard silver nitrate staining protocol (*18*).

Protein Sequencing. The partial N-terminal sequence was analyzed on a Shimadzu PPSQ-10 automated protein sequencer performing Edman degradation. Phenylthiohydantoin amino acids (PTH-AA) were detected at 269 nm after separation on a reverse phase C18 Wakopack Wakosil HPLC column (4.6 × 25 mm) from Shimadzu, under isocratic conditions, by using 40% acetonitrile, 20 mM acetic acid, and 0.014% SDS as the mobile phase at a flow rate of 1.0 mL min^{-1} at 40 °C. The sequence was submitted to automatic alignment, which was performed by using the NCBI-Blast search system.

Effect of pH and Temperature. The effect of pH and temperature on the hemagglutinating activity of lectin-like was determined by incubating protein samples at various pH (2−12) for 1 h or at a defined temperature for 30 min. To determine the pH stability of Labramin, glutaraldehyde-stabilized human A Rh+ erythrocytes were used, while for the temperature assay, the treatment with glutaraldehyde was not used. The residual haemagglutinating activity was assayed after adjusting the mixtures to pH 7.0 or 37 °C. At least five replicates were done for each test to confirm the results. The means of those assays were compared by one-way analysis of variance test. Duncan's multiple range test (5% level) was used to determine the significance between means.

Effect of EDTA, Ca^{2+}, and Mn^{2+}. Partially purified Labramin (5 mg) was dissolved in 1 mL of 150 mM NaCl and dialyzed exhaustively (48 h) against 0.2 M EDTA to remove any metals associated to lectin. This was followed by dialysis (molecular weight cutoff of tubing; 12 000 Da) against 0.15 M NaCl for 24 h. At the end of this period, hemagglutinating activity was assayed in the absence and presence of added CaCl$_2$ and MnCl$_2$.

Affinity Chromatography. To examine the affinity of the purified protein to carbohydrate immobilized on inert matrix, the mannose—agarose (2 mL bed volume), glucose—agarose (2 mL bed volume), fetuin (2 mL bed volume), and chitin (20 mL bed volume) (*19*) columns were equilibrated with 50 mM phosphate buffer pH 7.6, containing 100 mM NaCl. After adsorption of the proteins, the columns were washed with the same buffer until the absorbance at 280 nm returned to 0, after which the adsorbed Labramin was eluted with 100 mM methyl α-mannosideo and 100 mM glucose for mannose—and glucose—agarose, respectively, followed by 100 mM HCl. On the chitin column, the protein was eluted with 100 mM HCl. Fractions from this column (2 mL) were collected, and proteins were estimated based on the absorbance at 280 nm after which the hemagglutinating activity was assayed.

Effect of Labramin on Fungal/Yeast Growth. To examine the effect of Labramin on the growth of fungi, 200 μL of cells (*S. cerevisiae*) and spores (*F. oxysporum* and *C. lindemuthianum*) (20 000 mL^{-1} of Sabouraud broth) was placed in multiwell plates at 28 °C followed by the addition of protein solution (120 μg mL^{-1}). Optical readings were taken at 660 nm at time zero and every 6 h during the following 60 h. A control without the addition of protein was also used. Readings were taken against a blank containing only culture medium. After 60 h, the cells were separated from the growth medium by centrifugation (3000*g*), washed once in 100 mM Tris-HCl, pH 8.0 to remove medium in excess, and processed for observation by light microscopy at magnification 400. All the experiments were run in triplicate, and averages of readings, standard errors, and coefficients of variation were calculated.

RESULTS

Isolation of the Labramin. Labramin was obtained in three chromatographic steps. Crude extract applied to a Sephacryl S-400 column presented three peaks (**Figure 1A**); the last one that showed hemagglutinating activity and no trypsin inhibitory activity was chosen for subsequent experiments. Ion exchange chromatography of this peak on DEAE-Sepharose (linear gradient of 0.0—1.0 M NaCl) yielded five peaks, where the second peak showed only hemagglutinating activity (**Figure 1B**). This peak was chromatographed on a C18 column with a 0—100% acetonitrile gradient. The elution profile obtained showed five main peaks; after gradient, the peak eluted with 50% acetonitrile was identified as Labramin, based on its agglutinating activity (**Figure 1C**). The yield of Labramin in this purification process was 6 mg/400 mg of crude extract.

Agglutination of Human and Animal Erythrocytes. Labramin agglutinates rat, hamster, mouse, rabbit, sheep, and cow erythrocytes the same way but produced weak agglutination of chicken erythrocytes. Human erythrocytes of all blood groups were agglutinated (**Table 1**). It was verified that Labramin hemagglutinated trypsin-treated and not treated human A Rh+ erythrocytes and rabbit have shown a hemagglutination titer of 64 (protein concentration 6 μg/mL). We have also observed that human A Rh+ erythrocytes (trypsin-treated or untreated) stabilized with glutaraldehyde required a higher concentration of lectin to be agglutinated (44 μg mL^{-1}) than did fresh erythrocytes.

Carbohydrate Specificity of LABOL. The carbohydrate-binding specificity of the Labramin was evaluated by the ability sugar has to inhibit glutaraldehyde-stabilized human A Rh+ erythrocytes. As shown in **Table 2**, glycoproteins such as fetuin, followed by casein, asocasein, ovalbumin, thyroglobulin, and orosomucoid better inhibited the hemagglutinating activity. Laminarin (up to 1 mg/mL) did not provoke inhibition. Simple sugars (D-mannose and D-glucose), sugar derivates (*N*-acetyl-D-mannosamine and *N*-acetyl-D-glucosamine), and agarose (1 mg/mL) only inhibited the hemagglutinating activity in high concentrations.
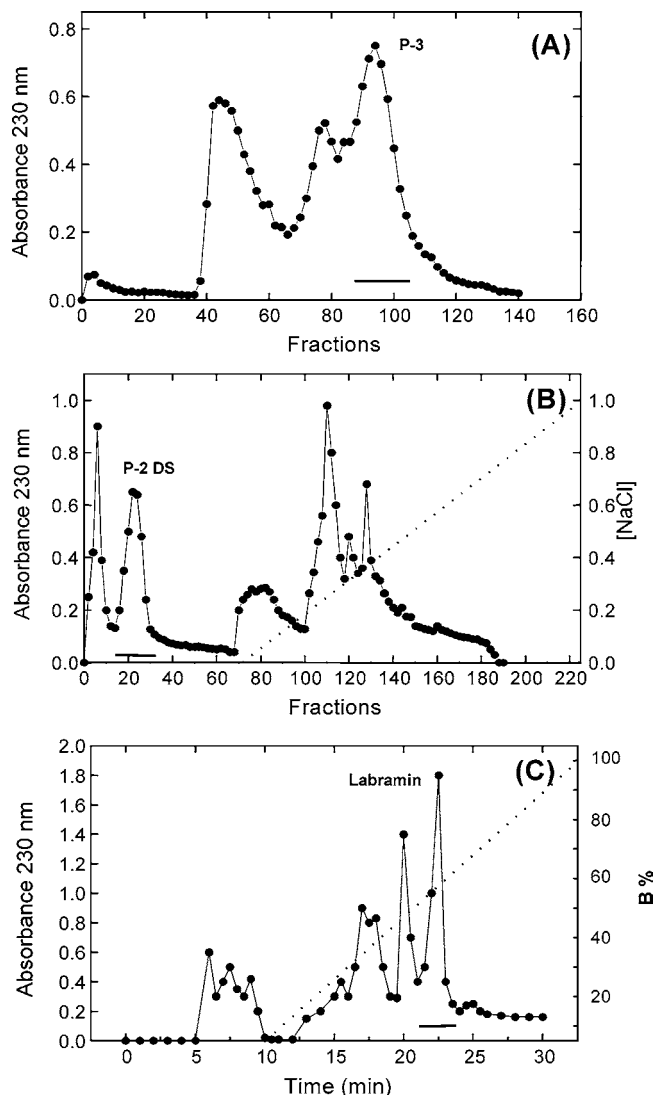

Figure 1. Purification of the Labramin: (**A**) Sephacryl S-400 chromatography of the crude extract of *L. bojeri* seeds [material eluted with 100 mM phosphate buffer (pH 7.6), containing 100 mM NaCl]; (**B**) fractions corresponding to peak P-3 further chromatographed in an ion exchange chromatography (DEAE-Sepharose); (**C**) reverse phase HPLC separation of the fraction P-2 DS from DEAE-Sepharose (C18 μ-Bondapack column); (−) hemagglutinating activity.

Chemical Characterization. Labramin purity was analyzed by SDS—PAGE with and without 2% β-mercaptoethanol. In both cases, the protein migrated as two bands with an apparent M_r of about 19 and 20 kDa (**Figure 2A**). This behavior indicates that Labramin has no disulfide-bonded subunits. The molecular mass of the native protein, determined by gel filtration in the presence of D-glucose, was 20 kDa (**Figure 2B**). Size-exclusion chromatography revealed that Labramin behaves as a monomeric protein. **Figure 2C** shows the silver-stained two-dimensional pattern of this protein, which showed components with distinct isoelectric points located at pI 5.1—5.25 at about 20 kDa and pI 5.2—5.4 at about 19 kDa.

Two ion exchange chromatographies (CM-Sepharose e DEAE-Sepharose) and different acetonitrile gradients on C$_{18}$ columns were assayed to separate these proteins (data not shown). None of these methods was successful in achieving separation. These results lead to the conclusion that the resulting material was composed of two polypeptide chains that may be separated only by SDS—PAGE and two-dimensional PAGE.

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Table 1. Hemagglutinating Activity of LABOL against Erythrocytes from Diverse Sources

erythrocyte	hemagglutination (titer[a])
human (type A)	6
human (type B)	5
human (type AB)	5
human (type O)	4
horse	5
cow	5
rabbit	6
sheep	7
mouse	6
rat	8
hamster	7
chicken	3

[a] Titer is defined as the reciprocal of the endpoint dilution that caused detectable agglutination of erythrocytes. The final lectin was 6 μg mL^{-1} in first well of the plate.

Table 2. Specificity of the Labramin

glycoprotein/sugar	μg mL^{-1}	IC$_{50}$[a] (mM)	relative potency
fetuin	155.0		1
thyroglobulin	530.4		3.4
ovalbumin	500.0		3.2
orosomucoid	581.2		3.7
casein	470.8		3.0
asocasein	500.0		3.2
heparin	NI[b]		
mannose		>100[c]	1.0
glucose		>200	0.5
N-acetyl-mannosamine		>100	1.0
N-acetyl-glucosamina		>100	1.0

[a] IC$_{50}$: concentration required to give a 50% inhibition of the agglutination of formaldehyde-stabilized trypsin-treated human A+ erythrocytes at a Labramin concentration of 44 μg/mL. [b] NI: no inhibition at 1 mg/mL. [c] The following monosaccharides and derivatives were not inhibitory at concentrations >100 mM: methyl α-glucopyranoside, methyl-α-galactopyranosideo, methyl-α-mannopyranosideo, glucosamine, mannosamine, galactosamine, N-acetylgalactosamine, fucose, fructose, sucrose, manitol, lactose, maltose, raffinose, rhamanose, ribose, xylose, sorbitol, rammose, cellobiose, arabinose, glucoronic acid, and trehalose.

To determine the N-terminal amino acid sequence, the 19 and 20 kDa spots obtained by two-dimensional PAGE electroblotted to PVDF membrane were sequenced. The partial NH$_2$-terminal sequence of both proteins (marked in **Figure 2C**) showed 82% identity. Thus, these proteins bands were called Labramin. This protein exhibited a high degree of homology (82%) with major trypsin isoinhibitor (DE5) isolated from seeds of the Brazilian Carolina tree (*Adenanthera pavonina* L.), soybean trypsin inhibitor (65%), Kunitz legume inhibitor (65%), and winged bean albumin-1 (WBA) with 52% (**Figure 3**). In both inhibitors, the conserved domains were detected (NCBI-Blast data bank). Labramin also presented high homology with other Kunitz trypsin inhibitor (82−52% of identity) despite no inhibitory activity having been detected (**Figure 3**).

Labramin priority was bound to chitin followed by fetuin column; the first column showed an absorption ca. 100% of the protein. However, when Labramim was applied to mannose and glucose columns, it was weakly eluted with its respective sugars but using 100 mM HCl the presence of a protein pick was verified, suggesting a probable interaction with agarose matrix (data not shown).

Periodic acid/Shiff staining indicated LABOL sugar content was 16.2% as determined by the phenol−sulfuric acid method. The hemagglutinating activity of purified labramin was constant, even when metal ions were removed (data not shown).
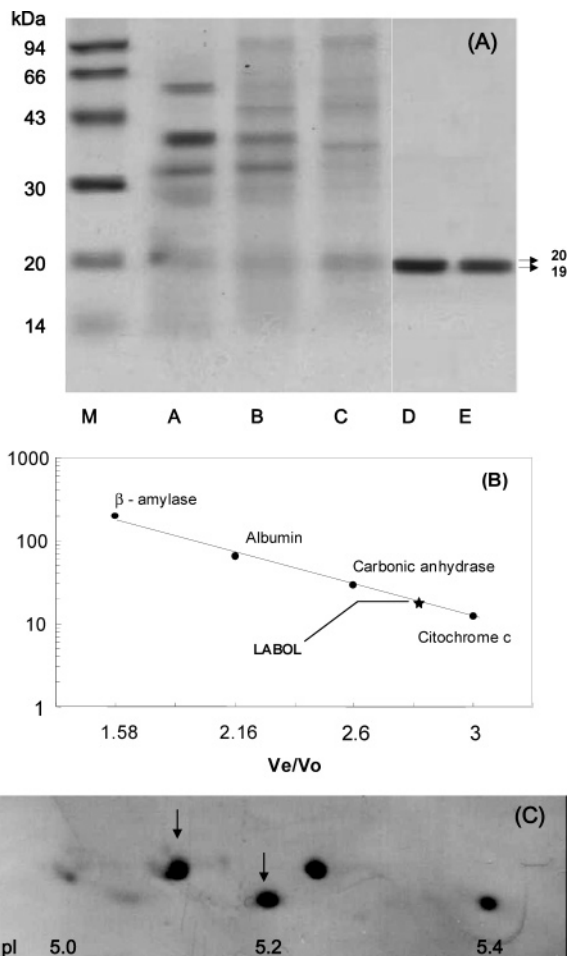

Figure 2. (**A**) SDS−PAGE analysis of purified Labramin: (lane M) molecular weight standards; (lane A) crude extract; (lane B) peak P-3; (lane C) P-2 DS; (lane D) Labramin; (lane E) Labraminin the presence of β-mercaptoethanol. (**B**) Molecular mass estimation from by gel filtration on Superdex 75. (**C**) Two-dimensional gel eletrophoresis of the Labramin was separated using a pH gradient from 3.0 to 10.0 in the first separation and then by SDS−PAGE (12.5%) in the second direction.

The protein showed hemagglutinating activity between a pH of 5 and 6. A further increase in the pH of the buffer (beyond pH 8) reduced the haemagglutining activity of Labramin by 55% (**Figure 4B**).

Assay of Enzymes' Inhibitory Activity. Because of the fact that Labramin exhibited homology with the trypsin inhibitor Kunitz family, it was particularly interesting to investigate the possible capacity of this protein to inhibit serinic proteinases. The inhibitory activity against bovine trypsin until 100 μg/mL Labramin and chymotrypsin or elastase inhibitory activity until 0.05 mg/mL Labramin was not observed. Labramin did not bind to the trypsin−Sepharose column (data not shown).

Antifungal Bioassay. Figure 5 shows the growth of *F. oxysporum*, *C. lindemuthianum*, and *S. cerevisiae* in the presence of Labramin (at the concentration of 120 μg mL^{-1}) and in control medium. Labramin exerted an inhibitory effect on the growth of *C. lindemuthianum* (35%) and *S. cerevisiae* (35%) after 62 h of growth. *F. oxysporum* growth was not affected by Labramin. Photomicrographs of C. *lindemuthianum* and *S. cerevisiae* taken after 60 h of growth showed normal development in control cultures (**Figure 6C,E**, respectively) but development inhibition in the presence of Labramin (**Figure 6D,F**, respectively). No apparent effect on *F. oxysporum* was

	Initial position	Sequence/Homology																					Identities (%)
20 kDa band	1	D	E	L	-	L	D	A	D	G	N	F	L	R	N	G	G	S	Y	Y	I	V	P	A	F	-
19 kDa band	1	K	-	L	F	-	D	V	D	G	N	F	L	K	N	G	G	S	Y	Y	I	V	P	A	F	82
DE5	1		R	E	L	L	D	V	D	G	N	F	L	R	N	G	G	S	Y	Y	I	V	P	A	F	82
STI	1		P	V	-	L	D	T	D	G	N	P	L	R	N	G	G	R	Y	Y	I	L	P	A	I	65
WBA-1	3	D	P	V	-	Y	D	A	E	G	N	K	L	V	N	R	G	K	Y	T	I	V	-	S	F	52
KUNITZ_LEG	1		P	V	-	L	D	T	D	G	N	E	L	R	N	G	G	T	Y	Y	I	L	P	A		65
PDTI	1		D	F	V	L	D	A	E	G	K	F	L	L	N	G	G	I	Y	Y	I	L				52
SBTI	1	D	F	V	-	L	D	N	E	G	N	P	L	S	N	G	G	T	Y	Y	I	L	S	D	I	52
MIRA	4	P	N	P	V	L	D	I	D	G	E	K	L	R	T	G	T	N	Y	Y	I	V	P	V	L	52
SP-B	8					L	D	I	N	G	D	E	V	R	A	G	E	N	Y	Y	I	V	S	A	I	43

Figure 3. Comparison of N-terminal amino acid sequences aligned with different regions of known plant protein and Labramin-1. DE5:*Adenanthera pavonina* trypsin inhibitor (*19*); STI: soybean trypsin inhibitor—gnl/CDD/14838; WBA: winged bean albumin from *Psophocarpus tetragonolobus* (*20*); KUNITZ_LEG: Kunitz trypsin inhibitor from legume—gnl/CDD/25437; PDTI: *Peltophorum dubium* trypsin inhibitor (*21*); SBTI: soybean trypsin inhibitor (*22*); MIRA: Miraculin (*23*); SP–B: Sporamin B (*24*). Letters in bold represent identified residues. Dots represent gaps introduced for maximal alignment.
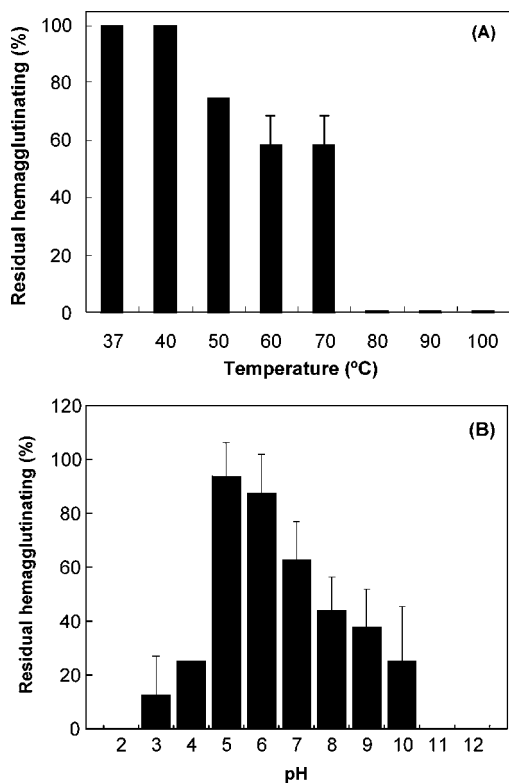

Figure 4. Thermal (**A**) and pH (**B**) stability of Labramin. Bars represent the average of five replicates. Full (100%) activity corresponded to a titer of 6.
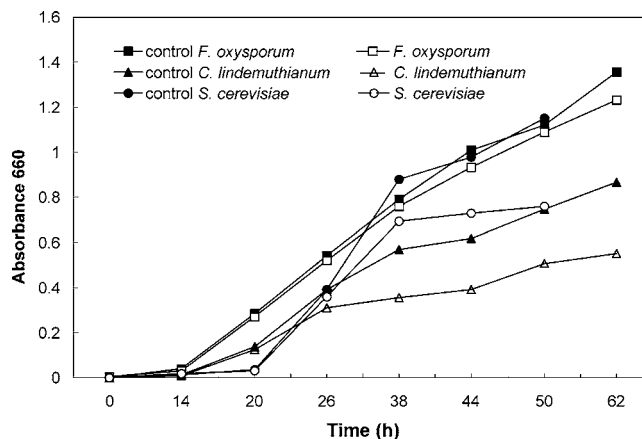

Figure 5. Effect of Labramin on fungal growth. The absorbance at 660 nm was used as a measure of fungal growth. The experiments were carried out in triplicate. The standard error bars (coefficients of variations <20%) have been omitted for clarity.

observed in the presence of Labramin (**Figure 5B**) when compared with this experiment control (**Figure 5A**).

DISCUSSION

This paper describes the isolation and partial characterization of a new protein from *L. bojeri*, a representative of the plant family *Sapotaceae*, first studied. Although Labramin presented NH₂ terminal sequence homology to Kunitz-type inhibitors, it did not show trypsin inhibitory activity but exhibited lectin-like activity.

The lectin-like properties of Labramin were evidenced by its hemagglutinating activity on various human and animals erythrocytes (**Table 1**). Labramin gave reproducible haemagglutinating titers of 64 (6 μg/mL) with human A Rh+ erythrocytes trypsin-treated or untreated. The hemagglutinating

activity of the protein was weak when compared with other typical lectins at least 100-fold, for example, PHA-E and Con A (*7*).

The study of Labramin lectin-like activity specificity by hemagglutination inhibition assays showed that the protein has affinity for fetuin, ovalbumin, tyroglobulin, rosomucoid, casein, and azocasein (**Table 2**). By contrast, there was no apparent inhibition by simple sugars and sugar derivatives. This affinity for glycoproteins was already verified in relationship Arcelin-1 (lectin-like) and with typical lectins such as PHA-E (erythro-agglutinin) and PHA-L (leucoagglutinating) (*7*) or PDTI, a trypsin inhibitor from *Peltophorum dubium* seeds that shows lectin-like properties (*21*). This specificity was also observed whether Labramin was applied to chitin, fetuin, or mannose and glucose columns showing a strongly adsorbed peak in chitin column. This lectin behavior bound to the chitin column was already observed in several species of plants (*25* and *26*). However, this bounding is not necessarily associated to *N*-acetyl-glucosamine affinity (*27*). Coelho and Silva (*28*) suggested that the inhibition of a lectin with carbohydrate, which is part of matrix structure, is not a guarantee of protein binding and desorption from the corresponding insoluble or cross-linked polysaccharide.

Taking into account all findings together with a single band obtained from the native PAGE, the native molecular mass by gel filtration, the high degree of identity on NH₂ terminal between 19 and 20 kDa (82%), and the results of two-
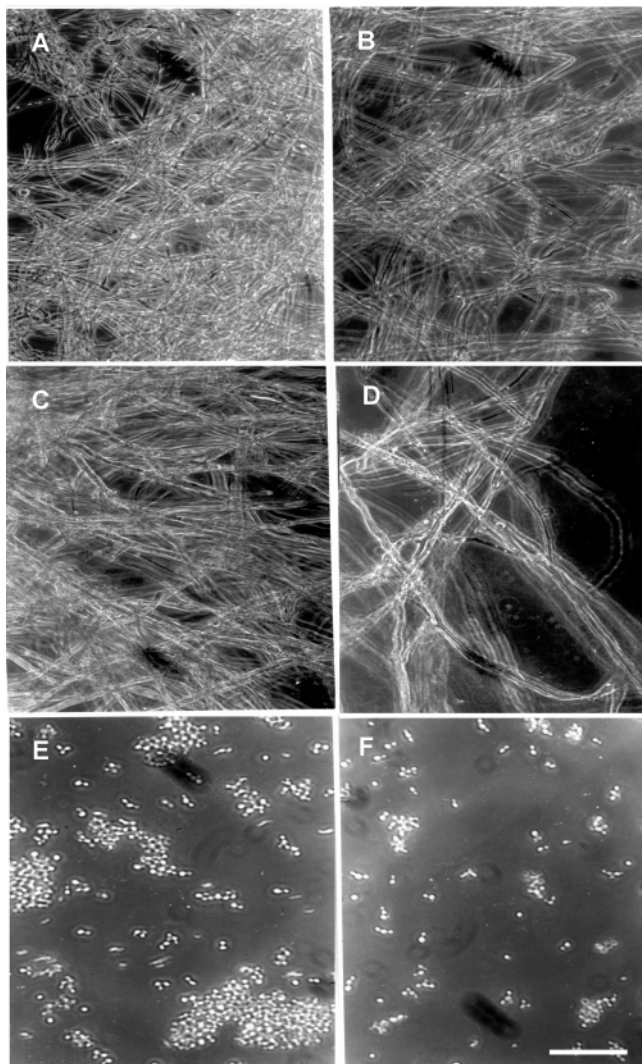
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Figure 6. Light micrographs of fungal mycelia after 60 h of growth: (**A**) *F. oxysporum* in control medium; (**B**) *F. oxysporum* in the presence of Labramin; (**C**) *C. lindemuthianum* in control medium; (**D**) *C. lindemuthianum* in the presence of Labramin; (**E**) *S. cerevisiae* in control medium; (**F**) *S. cerevisiae* in the presence of Labramin. Bar = 15.6 μm.

dimensional and SDS−PAGE, the protein bands can be assumed that, in fact, they are variants of the same protein or one of them is derived from the other. This described behavior was verified with *P. dubium* trypsin inhibitor (*21*).

Labramin showed conserved domains with two Kunitz inhibitors and any inhibitory activity. This Labramin behavior is similar to the major winged bean albumin from *Psophocarpus tetragonolobus*, which has a sequence similar to trypsin inhibitors Kunitz family, including the apparent conservation of the functional reactive site residue Lys64 at the position P_1 of the scissile bond in the trypsin inhibitors of Kunitz family. However, winged bean albumin did not inhibit trypsin. The authors explained the lack of inhibitory activity against trypsin in terms of conformation differences in the inhibitory loop (*29*). Another protein, obtained from *Pseudostellaria heterophylla* roots (*30*), with a molecular weight of 36 000 showed sequence simililarity to SBTI, but no substance was found to inhibit its hemagglutinating activity, and no trypsin inhibitory activity was reported for this protein.

Results described in the literature suggested that plant protein families such as lectins, protease inhibitors, and storage proteins could be encoded by a family of related genes. Some of these proteins have presented in common the similarity with the ancestral gene of the Kunitz family protease inhibitor (*31*).

Many proteins that bind to chitin were related to plant defense mechanisms against organisms that contained this polysaccharide as a constituent of their structures. This group included proteins such as lectins (*32*), lectin-like proteins (arcelin) (*7*), vicilins (*33*), zeatoxin (*34*), and some Kunitz family trypsin inhibitors that were toxic to insects and might affect the development of fungi (*35*).

Labramin inhibited the growth fungus *C. lindemuthianum* (35%) at a concentration of 120 μg mL^{-1} but manifested a negligible effect on *F. oxysporum*. Some lectins, such as *Urtica dioica* from stinging nettle rhizomes (*36*), inhibited *Botrytis cinerea*, *Septoria nodorum*, and *Trichoderma hamatum* by ca. 35% at concentrations of 90−600 and 35−45 μg mL^{-1}, respectively. TEL inhibited the growth of *F. oxysporum* and *C. lindemuthianum* ca. 50% at concentrations of 280 μg mL^{-1} (*32*). Mannan, chitin, and other saccharides were important components in most fungal cell walls. Labramin might inhibit fungal growth by interacting with these carbohydrates. Different inhibitory activities against various fungal strains might reflect differences in molecular structures of the fungal wall and/or the small lectins size, which allowed their penetration through the fungal cell wall (*36*).

The isolation and characterization of a new protein of a family never studied until now may be important both as a source of new plant protectants and to understand the evolutionary relationship of the plant proteins as the proteinase inhibitors and lectins.

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