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Insecticidal and Antifungal Activity of a Protein from *Pouteria torta* Seeds with Lectin-like Properties

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This paper describes the purification and characterization of a novel protein from the seeds of *Pouteria torta* (family Sapotaceae). The protein was purified by a combination of gel filtration, ion-exchange, and reverse phase chromatographies. SDS-PAGE of the purified protein resulted in a single protein band of 14 kDa in the presence and absence of DTT. The lectin-like activity of pouterin was best inhibited by glycoproteins such as fetuin, asialofetuin, heparin, orosomucoid, and ovoalbumin. Pouterin inhibited the growth of the fungi *Fusarium oxysporum* and *Colletotrichum musae* and of the yeast *Saccharomyces cerevisiae*. The incorporation of pouterin into an artificial diet (final concentration = 0.12%, w/w) caused 50% mortality in larvae of the insect *Callosobruchus maculatus*, whereas 0.08% pouterin produced an ED_{50}.

KEYWORDS: ***Pouteria torta*; seed; lectin-like; antifungal protein; *Callosobruchus maculatus***

INTRODUCTION

Lectins are proteins or glycoproteins that interact specifically with saccharide residues and are common in the seeds of several plants. The hemagglutinating activity of lectins has generally been the basis for their detection and purification and has led to their use as probes for studying various biological functions (*1*, *2*). In contrast to classic plant lectins that reversibly bind to specific mono- or oligosaccharides, plant lectin-like proteins show affinity for complex oligosaccharides, and their hemagglutinating activity is generally weaker than that of classic lectins such as phytohemagglutinin E (PHA-E) and concanavalin A (Con A) (*3*). Lectin-like proteins that have been isolated from plant seeds include arcelin-1, an insecticidal protein purified from kidney beans (*Phaseolus vulgaris*) (*3*), labramin, from *Labramia bojerii* seeds (*4*), and PDTI, a trypsin inhibitor from *Peltophorum dubium* seeds (*5*).

One of the most important insect pests of cowpeas (*Vigna unguiculata*) is the bruchid weevil *Callosobruchus maculatus* F. (Coleoptera), which attacks the seeds during storage and severely affects the quality and storability of the product. In periods of severe infestation, postharvest seed losses caused by *C. maculatus* can reach 100% within 6 months (*6*). In Brazil, cowpeas are cultivated mainly in the northeastern part of the country and are the main source of protein for poor populations (*7*). Cowpea plants have a low resistance to insects and microorganisms and, consequently, produce very low crop yields (*8*). A grain potential of over 1500 kg/ha is never obtained without the use of synthetic insecticides (*9*). However, controlling insect pests through the use of synthetic insecticides is costly and environmentally hazardous. The search for naturally occurring, cheaper, and more environmentally friendly biocontrol agents is currently an area of intense research (*10*). In this regard, there is evidence that lectins and lectin-like proteins can protect against insects and other herbivores. Arcelin-1 is insecticidal toward the larvae of various bruchids, for example, the Mexican bean weevil (*Zabrotes subfasciatus*) (*11*). TEL, a lectin isolated from *Talisia esculenta* seeds, produced ∼90% mortality in *C. maculatus* and *Z. subfasciatus* larvae when incorporated into the diet at a concentration of 2% (*12*).

Lectins, particularly chitin-binding proteins, apparently help to defend plants against fungi and insects. Indeed, some of these proteins, such as hevein (4.7 kDa) and *Urtica dioica* agglutinin

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(UDA; 8.5 kDa), may be small enough to penetrate the fungal cell wall and reach the plasma membrane, where they can block the active sites of enzymes involved in cell-wall morphogenesis (*13*). Lectin-like proteins from *L. bojerii* seeds inhibit the growth of *Colletotrichum lindemuthianum* and *Saccharomyces cerevisiae* (*4*), and Freire et al. (*14*) reported that TEL inhibited the growth of the fungi *Fusarium oxysporum*, *C. lindemuthianum*, and *S. cerevisiae*.

We recently reported the purification and characterization of labramin from the seeds of *L. bojerii*. This protein, the first to be isolated from the family Sapotaceae, is structurally homologous to Kunitz trypsin inhibitors but lacks inhibitory activity, although it has lectin-like activity. In this paper, we describe the purification and physicochemical properties of pouterin, a protein with lectin-like activity isolated from the seeds of *Pouteria torta* (family Sapotaceae). We also examined the antifungal and insecticidal activities of this protein.

MATERIALS AND METHODS

Plant Material. *P. torta* seeds were collected in the state of Mato Grosso do Sul, Brazil. Acrylamide, bovine serum albumin (BSA), DEAE-Sepharose, dithiothreitol (DTT), methylene bis(acrylamide), molecular mass markers, and other reagents for Tricine gel electrophoresis and Sephacryl S-100 were from Amersham Biosciences (Uppsala, Sweden). Ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and sugars were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were obtained from local suppliers.

Fungi/Yeast. *S. cerevisiae* strain 1038 was obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil. *F. oxysporum* and *Colletotricum musae* were kindly supplied by CNPF/EMBRAPA, Goiânia, G, Brazil.

Insects. The colonies of *C. maculatus* used were originally supplied by Dr. J. H. R. Santos (Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, CE, Brazil). The insects were housed at 28 ± 1 °C and a relative humidity of 65−75% and were fed seeds of the susceptible cultivar Epace-10.

Purification of the Lectin-like Protein. Dehulled *P. torta* seeds were finely ground and extracted with 5 volumes (w/v) of 150 mM NaCl for 24 h at 4 °C followed by centrifugation at 10000*g* for 30 min at 4 °C. The supernatant was dialyzed against distilled water for 48 h and lyophilized to provide a crude extract. This extract was dissolved (300 mg/5 mL) in 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl and then applied to a column (2.5 cm × 40 cm) of Sephacryl S-100 equilibrated with the same buffer. Proteins were eluted with phosphate buffer at a flow rate of 45 mL/h, and 3 mL fractions were collected, with the elution profile being monitored at 230 nm. Hemagglutinating activity (see below) was measured in the resulting fractions, and the active fractions (peak 3) were pooled, dialyzed, and lyophilized. This material (50 mg/mL) was further fractionated by ion-exchange chromatography on a column (2 cm × 20 cm) of DEAE-Sepharose 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. Fractions containing hemagglutinating activity were pooled, dialyzed, and lyophilized. Ten milligrams of lyophilized material from the DEAE step was dissolved in 250 μL of 0.1% (v/v) trifluoroacetic acid (solvent A) and clarified by centrifugation at 10000*g* for 5 min. The resulting supernatant was applied to a μ-Bondapack C_{18} column (0.78 cm × 30 cm) (Waters 991-PDA system), and the proteins were eluted with a linear gradient (0−100%, v/v) of acetonitrile (solvent B) at a flow rate of 2 mL/min. The elution profile was monitored at 230 nm, and fractions were collected manually and lyophilized. The second peak of the elution profile showed hemagglutinating activity and contained a lectin-like protein that was named pouterin.

Hemagglutination Assay. The hemagglutinating activity of pouterin was assayed using U-bottom microwell plates as previously described (*15*). The final assay volume of 100 μL contained 50 μL of a 2% suspension of human or animal erythrocytes previously washed three times in saline solution and 50 μL of a 2-fold serial dilution of protein solution. In most experiments, untreated or trypsin-treated erythrocytes were used in buffer containing 10 mM $CaCl_2$, $MnCl_2$, or $CaCl_2$ plus $MnCl_2$. Hemagglutination was determined after incubation for 1 h at room temperature, and the hemagglutination titer was defined as the highest dilution that produced complete hemagglutination.

Hapten Inhibition of Hemagglutination. To assess hapten inhibition of hemagglutination, 25 μL of 2-fold dilutions of glycoprotein, mono- and oligosaccharides, methyl monoglycosides, laminarin, and agarose in 0.05 M PBS, pH 7.4, was mixed with an equal volume of pouterin corresponding to two hemagglutination units (2 HU), and the microwell plate was incubated at room temperature for 1 h. Subsequently, 25 μL of a 2% suspension of glutaraldehyde-stabilized human A Rh+ erythrocytes was added in the same buffer, and the inhibition of hemagglutination was determined 1 and 24 h later at room temperature (*16*). The minimal inhibitory concentration was defined as the lowest glycoprotein or carbohydrate concentration able to reduce the extent of hemagglutination by 50% (IC_{50}).

Protein Assay. Protein concentrations were determined as described by Bradford (*17*) using BSA as standard.

Polyacrylamide Gel Electrophoresis. SDS-PAGE was done in 17.0% polyacrylamide gel as described by Laemmli (*18*).

Detection of Sugars. The total neutral sugar content of the protein was estimated colorimetrically according to the phenol/H_2SO_4 method (*19*), using D-glucose as the standard.

Amino Acid Composition. Amino acid analysis of pouterin was done on a Pico-Tag amino acid analyzer (Waters system), as described by Henrikson and Meredith (*20*).

Effect of pH and Temperature. The effect of pH and temperature on the hemagglutinating activity of pouterin was determined by incubating protein samples at various pH values (3−11) for 1 h or at a defined temperature for 30 min. The residual hemagglutinating activity was assayed after the mixtures had been adjusted to pH 7.0 or 37 °C. At least three replicates were done for each test to confirm the results.

Effect of Pouterin on Fungal/Yeast Growth. The assay was conducted as described by Freire et al. (*14*) using cells (*S. cerevisae*) and spores (*F. oxysporum* and *C. musae*).

Insect Bioassays. The artificial seed system previously developed by Macedo et al. (*21*) was used to examine the effects of pouterin on the development of *C. maculatus* larvae.

Statistical Analysis. The results were expressed as the mean ± SEM, when appropriate. Statistical comparisons were done using one-way analysis of variance (ANOVA) (as part of the General Linear Models procedure) followed by Tukey's multiple-comparisons test to compare the differences between means. A p value of <0.05 indicated significance.

RESULTS

Isolation of Pouterin. Pouterin was purified in three chromatographic steps. Fractionation of the crude extract on a Sephacryl S-100 column resulted in three peaks, the last of which (P-3) showed hemagglutinating activity (**Figure 1A**). Ion-exchange chromatography of this peak on DEAE-Sepharose in which proteins were eluted with a linear gradient (0−1.0 M) of NaCl yielded two peaks, the first of which (P-1DS) had hemagglutinating activity (**Figure 1B**). This peak was chromatographed on a C_{18} column, and the proteins were eluted with a gradient (0−100%) of acetonitrile. The elution profile contained three peaks, with the peak that eluted with 47% acetonitrile being identified as pouterin, on the basis of its hemagglutinating activity (**Figure 1C**). The yield of pouterin using this purification process was 0.33% (1 mg/300 mg of crude extract).

Agglutination of Human and Animal Erythrocytes. Pouterin agglutinated rabbit, cow, and rat erythrocytes, as well as human erythrocytes of all blood groups (**Table 1**). Pouterin also

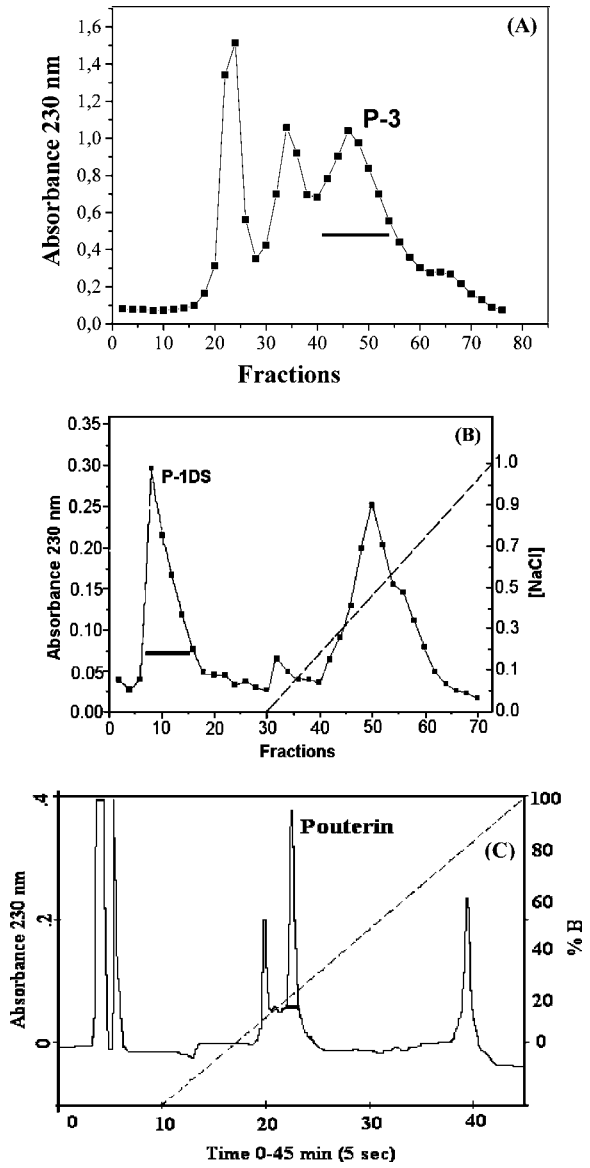

Figure 1. Purification of pouterin: (**A**) Sephacryl S-100 chromatography of a crude extract of *P. torta* seeds. Proteins were eluted with 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl. (**B**) Fractions corresponding to peak P-3 were pooled and fractionated by ion-exchange chromatography on DEAE-Sepharose. (**C**) Reverse phase HPLC of fraction P-1DS from DEAE-Sepharose on a μ-Bondapack C_{18} column. (–) hemagglutinating activity.

hemagglutinated trypsin-treated human A Rh+ and rabbit erythrocytes. Pouterin showed a reproducible hemagglutination titer of 16 (protein concentration = 3.6 μg/mL) for non-trypsinized human A Rh+ and rabbit erythrocytes and a titer of 256 (protein concentration = 0.2 μg/mL) for the corresponding trypsinized cells. The hemagglutination of human A Rh+ erythrocytes was enhanced by treatment with Ca^{2+} plus Mn^{2+} (hemagglutination titer of 64, corresponding to a protein concentration of 1.1 μg/mL), but not by other divalent ions.

Carbohydrate Specificity of Pouterin. The carbohydrate-binding specificity of pouterin was determined on the basis of the ability of different sugars to inhibit the hemagglutination induced by glutaraldehyde-fixed human A Rh+ erythrocytes. As shown in **Table 2**, glycoproteins such as fetuin, asialofetuin, heparin, orosomucoid, and ovoalbumin provided the greatest inhibition of hemagglutinating activity. Casein (up to 1 mg/mL) was not inhibitory.

Table 1. Pouterin-Induced Hemagglutination of Erythrocytes from Several Species

erythrocyte	hemagglutination (titer[a])
human (type A)	4
human (type B)	3
human (type AB)	4
human (type O)	3
rat	5
cow	5
rabbit	4
human A, trypsin-treated	8
rabbit, trypsin-treated	8
human A, Ca^{2+}	5
human A, Mn^{2+}	4
human A, Ca^{2+} + Mn^{2+}	6

[a] Titer is defined as the reciprocal of the endpoint dilution that caused detectable agglutination of erythrocytes. The final lectin was 3.6 μg mL^{-1} in the first well of the plate.

Table 2. Sugar Specificity of Pouterin[a]

glycoprotein	μg mL^{-1}	glycoprotein	μg mL^{-1}
fetuin	77	orosomucoid	145
asialofetuin	121	ovoalbumin	590
heparin	31	casein	NI[b]

[a] The minimal concentration required to give a 50% inhibition of the agglutination of glutaraldehyde-stabilized human A+ erythrocytes at a pouterin concentration of 172 μg mL^{-1}. [b] NI, no inhibition at 1 mg/mL. The following monosaccharide and derivates were not inhibitory at concentration >100 mM: methyl α-glucopyranoside, methyl α-galactopyranoside, methyl α-mannopyranoside, glucosamine, mannosamine, galactosamine, *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, fructose, sucrose, lactose, maltose, glucose, mannose, maltose, and raffinose.

Chemical Characterization. Electrophoresis of pouterin by SDS-PAGE in the absence and presence of 1 M DTT resulted in a single protein band with an M_r of ~14 kDa (**Figure 2**). This behavior indicates that Pouterin has no disulfide-bonded subunits. Amino acid analysis (**Table 3**) revealed high contents of Asx (8.4%), Glx (33%), Arg (9.6%), Cys (6.0%), and Leu (12%) and low levels of Phe (1.2%), Ile (1.2%), Tyr (1.2%), and Thr (1.2%). Neutral sugars accounted for 22% of the mass of pouterin, as determined by the phenol−sulfuric acid method. Pouterin was heat stable up to 60 °C, but there was a sharp decrease in activity at higher temperatures (**Figure 3A**). Hemagglutination was maximal between pH 5.0 and 10.0 and decreased markedly at pH <5 and >10 (**Figure 3B**).

Antifungal Activity. Figure 4 shows the growth of *F. oxysporum*, *C. musae*, and *S. cerevisiae* in the presence of pouterin (280 μg mL^{-1}) and in control medium. Pouterin inhibited the growth of *F. oxysporum*, *C. musae*, and *S. cerevisiae* by 85, 54, and 100%, respectively, after 50 h. Photomicrographs of *F. oxysporum* and *C. musae* taken after 60 h of growth confirmed the inhibition by pouterin (data not show).

Insecticidal Activity. The effect of pouterin on the development of *C. maculatus* larvae was assessed by determining the number and mass of surviving larvae (fourth instars) fed a diet containing increasing amounts of this protein. **Figure 5** shows the effect of pouterin on the mortality and weight of *C. maculatus* larvae on day 20. The mortality and weight of larvae that were fed control seeds (represented by the *Y*-intercept value) were about 7.5% and 7.8 mg, respectively, whereas seeds containing 0.3% pouterin caused 100% mortality (data not shown). At a concentration of up to 0.12%, pouterin killed 50% of the larvae (LD_{50}), whereas at 0.08% it caused a 50% reduction

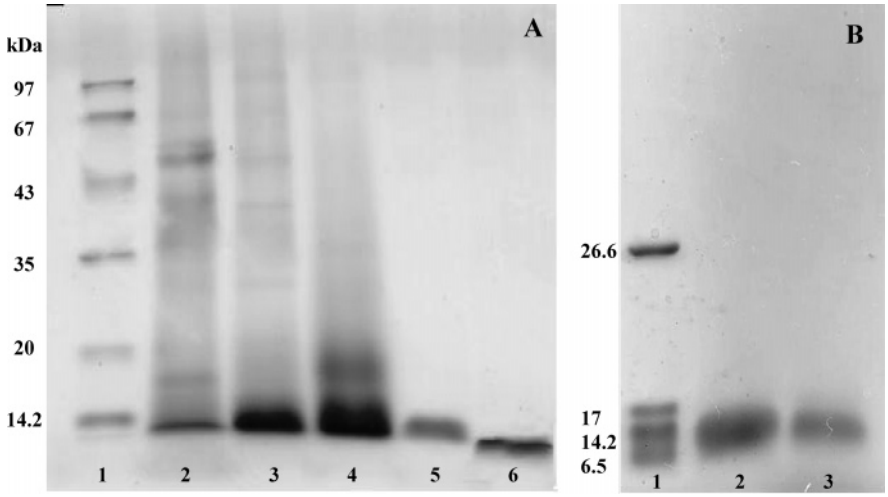

Figure 2. SDS-PAGE analysis of purified pouterin: (**A**) Lane 1, molecular mass standards; lane 2, crude extract; lane 3, peak P-3; lane 4, P-1DS; lane 5, pouterin; lane 6, pouterin in the presence of DTT. (**B**) Lane 1, molecular mass standards; lane 2, pouterin; lane 3, pouterin in the presence of DTT.

Table 3. Amino Acid Composition of Pouterin

amino acid	mol %	amino acid	mol %
Asx	7	Tyr	1
Glx	28	Val	2
Ser	3	Met	2
Gly	3	Cys	5
His	3	Ile	1
Arg	8	Leu	10
Thr	1	Phe	1
Ala	2	Lys	3
Pro	3	Trp	ND[a]

[a] ND, not detected.

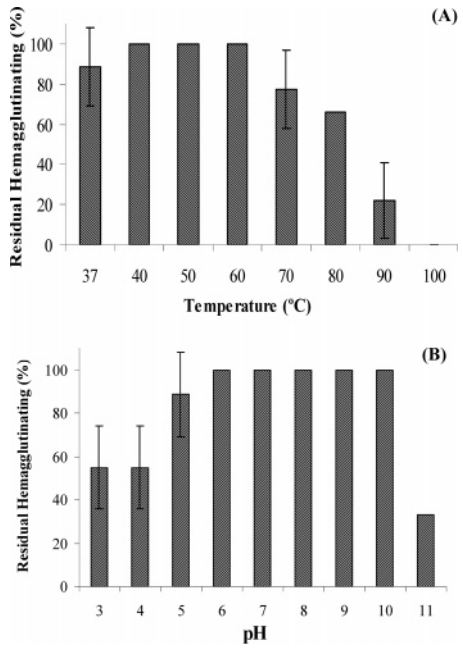

Figure 3. Thermal (**A**) and pH (**B**) stability of pouterin. The bars represent the mean of five replicates. Full activity (100%) corresponded to a titer of 4.

in the average weight of the larvae (ED_{50}) (**Figure 5**). Regression analysis showed that for every 0.01% increase in the concentration of pouterin, there was a 4.98% increase in mortality ($r^2 = 0.95$) and a 0.61 mg decrease in weight ($r^2 = 0.98$).

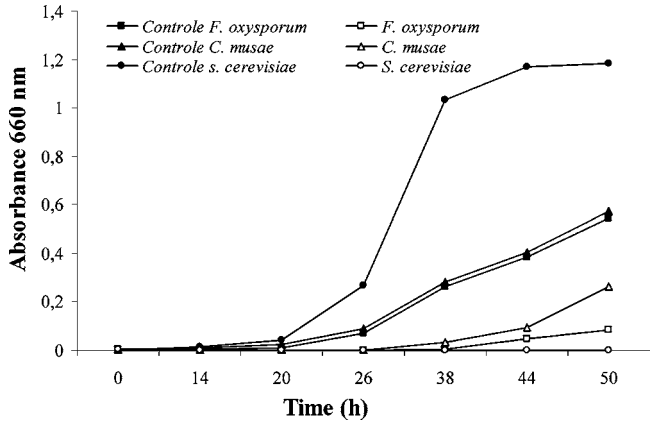

Figure 4. Effect of pouterin on fungal growth. The increase in absorbance at 660 nm was used as a measure of fungal growth. The experiments were done in triplicate. Standard error bars (coefficients of variation < 20%) have been omitted for clarity.

DISCUSSION

Lectin-like proteins have been detected in plant seeds (*3*−*5*), and the isolation and characterization of pouterin, a lectin-like protein from *P. torta* seeds, agrees with these studies. Pouterin was purified by a combination of gel filtration, ion-exchange chromatography, and reverse phase HPLC. In the presence and absence of 1 M DTT, the purified protein showed a single protein band of ∼14 kDa in SDS-PAGE (**Figure 2B**, lanes 1 and 2). Pouterin had a relatively large content of hydrophilic amino acids such as Asx, Glx, and Leu, and there was also a high number of Cys residues (**Table 3**). Pouterin was a glycoprotein, with neutral sugars accounting for ∼22% of the molecular mass. This carbohydrate content was similar to that of other lectins and lectin-like proteins such as labramin (*4*), TEL (*14*), and KpLec (*22*), in which carbohydrates account for 16.2, 18.8, and 14% of the protein, respectively.

The lectin-like properties of pouterin were confirmed by the hemagglutination of erythrocytes in the presence of Ca^{2+} and Mn^{2+} (**Table 1**). Pouterin gave a reproducible hemagglutination titer of 16 (3.6 μg/mL) with human A Rh+ and rabbit erythrocytes; a higher titer of 256 (0.2 μg/mL) was obtained with the corresponding trypsin-treated cells. The inability of pouterin to agglutinate human erythrocytes was probably related to a requirement for Ca^{2+} and Mn^{2+} in the medium (data not

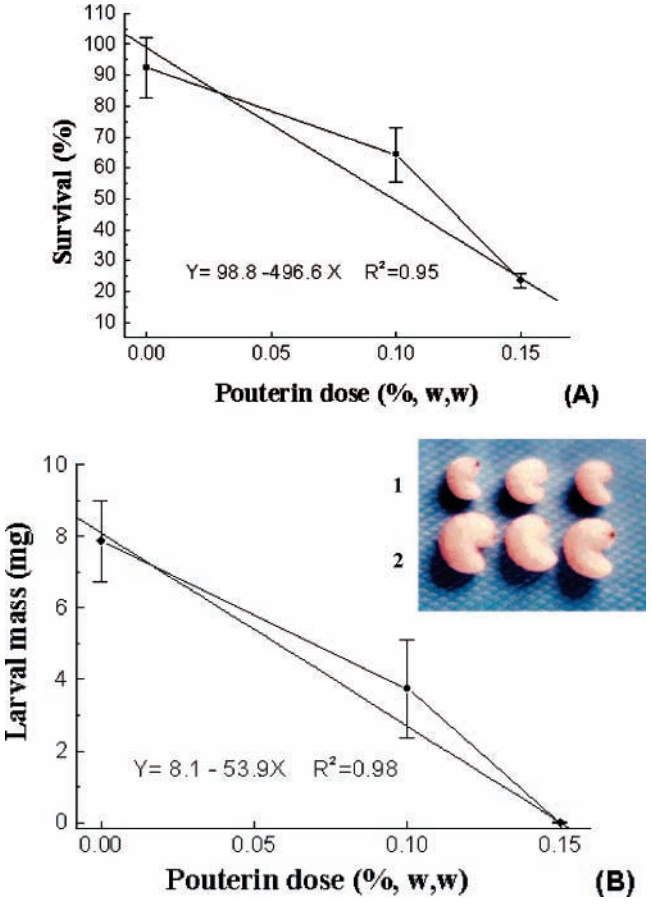

Figure 5. Effect of pouterin on *C. maculatus* larvae fed an artificial diet containing this protein: (**A**) mortality; (**B**) weight. The *Y*-intercept in (**A**) is mortality, and (**B**) is the weight of larvae fed the control diet. (Inset) (1) Larvae fed 0.1% pouterin and (2) larvae fed the control diet. Each point represents $n \geq 100$, and the error bars indicate the SEM.

shown). The hemagglutinating activity of pouterin was similar to that of labramin, which had a titer of 64 (6 μg/mL) with trypsinized or nontrypsinized human A Rh+ erythrocytes (*4*). Fabre et al. (*3*) reported that the lectin-like protein arcelin-1 had titers of 128 (30 μg/mL) and 32 (125 μg/mL) with trypsinzed human O Rh+ and rabbit erythrocytes, respectively. Pouterin showed affinity for glycoproteins such as fetuin, asialofetuin, ovoalbumin, and orosomucoid, as well as heparin (**Table 2**). In contrast, there was no apparent inhibition by simple sugars and sugar derivatives. A similar affinity for glycoproteins has also been described for labramin (*4*), arcelin-1 (*3*), and PDTI (*5*).

The hemagglutinating activity of pouterin was maintained over the pH range of 5.0−10; at pH 3−4 there was a 55% loss of activity, whereas at pH 11 there was a 67% loss of activity. These results are similar to those for the plant lectins KpLec, which retained maximum activity at pH 5.0−10.0 (*22*), and ACLEC (lectin from *Annona coriacea*), which was stable at pH 3.0−9.0 (*23*). A general property of most proteins involved in plant defense mechanisms is their marked heat stability (*24*). In agreement with this, pouterin was stable at up to 60 °C, with a progressive loss of activity at higher temperatures: 22% loss at 70 °C, 34% at 80 °C, and 78% at 90 °C; TEL is also stable at 60 °C (*14*).

Many proteins contribute to plant defense systems by providing resistance to insects, bacteria, and fungi. In particular, chitin-binding lectins apparently have a role in defending plants against fungi and insects. The ability of lectins to inhibit fungal growth varies among fungal species. Freire et al. (*14*) showed that TEL inhibited the growth of *F. oxysporum*, *C. lindemuthianum*, and *S. cerevisiae* by ∼50% at a concentration of 280 μg/mL^{-1}. Macedo et al. (*4*) showed that labramin inhibited the growth of *C. lindemuthianum* by 35% at a concentration of 120 μg/mL^{-1} but had a negligible effect on *F. oxysporum*.

Pouterin bound to chitin (data not shown) and inhibited the growth of *F. oxysporum* (85%), *C. musae* (54%), and *S. cerevisiae* (100%). The concentration of pouterin required to inhibit fungal growth was 280 μg/mL^{-1}. Mannan, chitin, and other saccharides are important components of most fungal cell walls, and pouterin may inhibit fungal growth by interacting with these carbohydrates. Variation in the susceptibility of fungal strains to inhibition by lectins and related proteins may reflect differences in the molecular composition and organization of the fungal wall and/or be related to the small size of many lectins that allows them to penetrate through the fungal cell wall (*25*).

Several plant lectins are detrimental to insect growth, and most bioassays of this activity have used an artificial diet and *C. maculatus* as the test species (*26*−*28*). The mechanisms by which lectins exert their toxic effects in insects are poorly understood, but because glycoproteins are major constituents of insect digestive tract membranes, it is plausible that the insect gut contains specific ligand-binding molecules that are targets for plant lectins (*29*).

Pouterin was toxic to *C. maculatus* larvae and caused a loss of weight. The LD_{50} of 0.12% estimated for pouterin was comparable to the concentration range (0.1−1%) at which TEL, KpLec, and *Bauhinia monanbra* lectin caused 50%, 50, and 50% mortality, respectively (*12*, *22*, *27*). These lectins may provoke deleterious effects by binding to glycan receptors on the surface of cells lining the insect gut (*30*). Macedo et al. (*30*) proposed that TEL was insecticidal to *C. maculatus* larvae, possibly (1) by binding to chitin components (or equivalent structures) in the insect gut, (2) by interacting with glycoconjugates on the surface of epithelial cells along the digestive tract, (3) by binding to the sugar moiety of any of a variety of glycosylated digestive enzymes and/or assimilatory proteins in midgut extracts, and (*4*) by resisting enzymatic digestion by cysteine-like proteinases.

In conclusion, we have identified a new lectin-like protein with antifungal and insecticidal activities in *P. torta* seeds. This protein could be a potentially useful tool for the management of pest insects and fungi.

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