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Purification and Characterization of an *N*-Acetylglucosamine-Binding Lectin from *Koelreuteria paniculata* Seeds and Its Effect on the Larval Development of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae)

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This study describes the purification of an *N*-acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effects on the larval development of *Callobruchus maculatus* and *Anagasta kuehniella.* The lectin (KpLec) was characterized and isolated by gel filtration, affinity column, and reverse phase chromatography. SDS-PAGE indicated that this lectin is a dimer composed of subunits of 22 and 44 kDa. The N terminus exhibited 40% similarity with *Urtiga dioica* agglutinin. KpLec was tested for anti-insect activity against *C. maculatus* and *A. kuehniella*. With regard to *C. maculatus*, an artificial diet containing 0.7 and 1% KpLec produced LD_{50} and ED_{50} value, respectively. However, for *A. kuenhiella*, an artificial diet containing 0.65% KpLec produced an LD_{50}, whereas 0.2% KpLec produced an ED_{50}. The transformation of genes coding for this lectin could be useful in the development of insect resistance in important agricultural crops.

KEYWORDS: ***Koelreuteria paniculata*; *N*-acetylglucosamine binding; lectin; *Callosobruchus maculatus*; *Anagasta kuehniella***

INTRODUCTION

Plant seeds contribute significantly to human and animal diets; cereal/legume seeds are a major part of the human diet. Legumes are major crops in many tropical countries and serve as important sources of carbohydrates and proteins to poor populations in these regions (*1*). The bean is a legume, and its seeds are an important source of protein in the tropics. Grains such as the cowpea [*Vigna unguiculata* (L.) Walp] are important sources of dietary proteins and carbohydrates in many countries, especially in sub-Saharan Africa and elsewhere in the tropics. The cowpea is also a source of animal fodder and income for many poor families (*2*). One of the most important insect pests of the cowpea is the bruchid weevil, *Callosobruchus maculatus* (F.) (Coleoptera), which attacks the seeds during storage and severely affects the quality and storability of the produce. In periods of severe infestation, postharvest seed losses caused by *C. maculatus* can reach 100% within a period of 6 months (*3*). The insect is a major pest of stored cowpeas in many of the developing nations of the world, including those of Africa, where losses may exceed $50 million annually (*4*). *Anagasta kuehniella* (Zeller), the Mediterranean flour moth (Lepidoptera), is found worldwide, particularly in stored grains, fruits, and nuts. Depending on temperature and humidity, a single female may lay nearly 600 eggs. *A. kuehniella* is of major economic importance as a flour and grain feeder; it is often a severe pest in flour mills. Few stored and dried vegetable products are safe from this voracious little moth: nuts, fruits, chocolates, biscuits, cakes, jellies, and candies have been consumed as well. It is believed that *A. kuehniella* is an indoor insect that requires a high temperature throughout the year to obtain maximum development (*5*).

Resistance in crop plants can be mediated by a wide range of metabolic products, including primary (e.g., proteinase, amylase inhibitors, and lectins) and secondary (e.g., alkaloids, tannins, and rotenoids) metabolites. Modified forms of the 7S storage proteins (vicilins) have been implicated in protection against *C. maculatus* in some cowpea cultivars (*6*, *7*). Canatoxin, a toxic protein isolated from *Canavalia ensiformis*, was also shown to be highly toxic when fed to *C. maculatus* (*8*). Zeatoxin, a glycoprotein isolated from *Zea mays* seeds, was toxic to *C.*

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maculatus when supplied as a diet of artificial seeds (*9*). TEL, a lectin isolated from *Talisia esculenta* seeds, produced ~90% mortality to *C. maculatus* and *Zabrotes subfasciatus* when it was incorporated in their diets at a level of 2% (*10*). To date, no investigation has been made in relation to protein insecticides to *A. kuehniella*.

Plant lectins are a large, heterogeneous group of proteins that reversibly bind specific mono- or oligosaccharides and possess at least one noncatalytic domain (*11*). The identification of an increasing number of plant lectins showing insecticidal activity toward economically important pest species (*10*, *12*−*18*) has fueled growing interest in their potential use in the field of transgenic crop protection. Lectins with specificity for *N*-acetylglucosamine residues appear to be insecticidal to many insects, including the cowpea weevil. Most cowpea weevil-active lectins and lectin-like proteins are members of the chitin-binding proteins (*19*, *20*).

Recently, our group purified and characterized from *T. esculenta* seeds (TEL) the first lectin belonging to the Sapindaceae family. This lectin inhibited the larval growth of *C. maculatus* and *Z. subfasciatus* (*10*). In this work, we describe the purification and physicochemical properties of a lectin isolated from *Koelreuteria paniculata* seeds belonging to the Sapindaceae family also, monitoring by an insect bioassay its insecticidal activity toward *C. maculatus* and *A. kuehniella* and determining the LD_{50} and ED_{50} values of the toxin via an insect bioassay with different concentrations of the above-mentioned lectin.

MATERIALS AND METHODS

Plant Material. The tree *K. paniculata* (Laxm), known as the Golden rain tree, belongs to the family Sapindaceae, is native to eastern Asia (China and Korea), and was introduced into Brazil as an ornamental species. The seeds used in this study were collected in the state of São Paulo, Brazil.

Insects. The colonies of *C. maculatus* used in this sutdy were reared in the Laboratório de Purificação de Proteínas e suas Funções Biológicas (LPPFB), Departamento de Ciências Naturais, Universidade Federal de Mato Grosso do Sul, Três Lagoas (MS), Brazil. The *A. kuehniella* colonies were supplied by the Departamento de Entomologia, ESALQ/USP, Piracicaba (SP), Brazil, and maintained in the LPPFB.

Isolation of Lectin. Lectin from *K. paniculata* seeds (KpLec) was extracted with 0.1 M phosphate buffer (pH 7.6) (1:10, w/v), and the mixture was allowed to stand overnight at 4 °C. After centrifugation (5000*g* for 30 min), the supernatant (crude extract) was dialyzed against distilled water, lyophilized, and diluted in 0.1 M phosphate buffer (pH 7.6), containing 0.1 M NaCl, and then applied to a Sephadex G-100 column that was eluted with the same buffer. The second peak (PII) containing lectin activity was collected, dialyzed, lyophilized, and chromatographed on an affinity column (trypsin-Sepharose) equilibrated with 0.1 M phosphate buffer (pH 7.6) containing 0.1 M NaCl. The protein was eluted with the same previous buffer, followed by 0.1 M HCl. Two peaks were obtained before the addition of HCl, the second of which showed hemagglutinating activity. This peak (Lec-ST) was dialyzed against distilled water, lyophilized, and chromatographed by reverse phase HPLC on a C18 μ-Bondapack column, obtaining a main peak with hemagglutinating activity (KpLec). KpLec was also applied to a chitin column equilibrated with 0.1 M sodium acetate (pH 4.5) and eluted with 0.1 M HCl.

Hemagglutination and Inhibition by Carbohydrate. Serial 2-fold dilutions of KpLec (50 μL) in saline solution (50 μL) were prepared in microtiter plates, and 50 μL of a 2% rat erythrocyte suspension was then added to each well. Agglutination was recorded after 1 h at room temperature. Hemagglutinating activity was expressed as the reciprocal of the highest dilution producing positive agglutination. The hemagglutinating activity of the KpLec was tested against mouse, rat, sheep, horse, cow, hamster, rabbit, and human erythrocytes.

The inhibitory activity of carbohydrates was studied by adding 50 μL of serial 2-fold dilutions of a sugar solution (100 mM) to an equal volume of KpLec. After 30 min of incubation, 50 μL of rat erythrocyte suspension was added, and the hemagglutinating activity was scored after 1 h. The lowest concentration of sugar that blocked hemagglutination was expressed as the concentration required to inhibit hemagglutinating activity by 50%. The sugars tested were D-mannose, d-raffinose, D-glucosamine, *N*-acetylglucosamine, D-galactose, D-maltose, D-glucose, D-fructose, d-sucrose, and D-lactose.

Neutral Sugar Content. The carbohydrate content of the KpLec was determined according to the phenol−sulfuric acid method (*21*), using D-glucose as the standard.

Protein Concentration. Protein concentration was determined as described by Bradford (*22*), using bovine serum albumin (BSA) as standard.

Amino Acid Composition. Amino acid analysis was done on a Pico-Tag amino acid analyzer (Waters system) as described by Henrikson and Meredith (*23*). One nanomole of KpLec was hydrolyzed in 6 M HCl/1% phenol at 106 °C for 24 h. The hydrolysates were reacted with 20 μL of fresh derivatization solution (methanol/triethylamine/water/phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature. After precolumn derivatization, PTC amino acids were identified on a reverse phase HPLC column by comparing their retention times to those of standard PTC amino acids (Pierce). Cysteine residues were quantified as cysteic acid.

Electrophoresis. SDS-PAGE of KpLec was done according to the method of Laemmli (*24*), using 5% stacking and 12.5% separating gels, under reducing and nonreducing conditions. Protein bands were detected by staining with Coomassie brilliant blue R-250. The molecular mass standards (Pharmacia) used were phosphorylase B (94 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Determination of Molecular Mass by Gel Filtration. The M_r of the native KpLec was determined by FPLC gel filtration on a Superdex 75 column (1 cm × 60 cm) equilibrated with 0.1 M ammonium bicarbonate (pH 8.0). The M_r standards (Pharmacia) used to calibrate the column were BSA (66 kDa), carbonic anhydrase (29 kDa), trypsin (24 kDa), and cytochrome *c* (12.4 kDa).

N-Terminal Amino Acid Sequence. KpLec was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was stained with Coomassie brilliant blue R-250, and the bands corresponding to the lectin were excised from the membrane. Automated Edman degradation was done using a Procise amino acid sequencer (Applied Biosystems). The PTH amino acids were identified in a 140 C microgradient PTH amino acid analyzer on the basis of their retention times.

Effect of Chelating Agents on Hemagglutinating Activity. To examine the metal cation requirement of the hemagglutinating activity, KpLec (1 mg mL^{-1}) was incubated with 25 mM EDTA or EGTA for 30 min at room temperature and the residual agglutinating activity was then determined.

Effect of Temperature and pH. The effect of thermal pretreatment of lectin on the hemagglutinating activity was determined by incubating KpLec samples at a defined temperature (37−90 °C) for 30 min. The heated solutions was rapidly cooled on ice and assayed for agglutinating activity. Control samples were incubated at 37 °C, and the results were expressed as a percentage of the activity of nonheated lectin.

KpLec was adjusted to different pH values (2−14). After incubation for 1 h at room temperature, the samples were adjusted back to pH 7.4 and assayed for the agglutinating activity. The results were expressed as a percentage of the hemagglutinating activity of KpLec kept in saline solution at pH 7.4 throughout the experiment.

Insect Bioassays. To examine the effects of KpLec on *C. maculatus* development, the artificial seed system previously developed by Macedo et al. (*10*). Artificial seeds (~400 mg each) were prepared from finely ground cowpea seeds (Epace 10 cultivar) using a cylindrical brass mal and hand press. Artificial seeds containing KpLec at concentrations of 0.1, 0.5, and 1% (w/w) were obtained by thoroughly mixing the lectin with cowpea seed meal and pressing as described above. Each treatment consisted of 10 artificial seeds for each of the above concentrations. After 48 h for adjustment in the growth chamber, the seeds were offered

to nine 2−3-day-old fertilized females. After 24 h had been allowed for oviposition, the number of eggs per seed was reduced to four (n = 40). Following incubation for 20 days at 28 °C and 70−75% relative humidity, the seeds were opened and the weight and number of larvae were determined. Control artificial seeds were prepared with Epace-10 cultivar meal containing no KpLec.

To examine the effects of KpLec on *A. kuehniella*, the moths were maintained in plastic boxes, with perforated plastic covers at a relative humidity of 65−75% and a temperature of 28 °C. An artificial diet was prepared by mixing wholee wheat flour, whole wheat husks, whole wheat, and yeast (8:2:1.9:0.1) with two concentrations (0.5 and 1.0%) of lectin. Control meal without lectin was also prepared. Each treatment was repeated eight times with five larvae (n = 40) for each of the above concentrations. After fourth instars, the relationship between lectin content and larval weight and number was determined.

Linear regression analysis and exponential decay (first order) were used to describe the response of *C. maculatus* and *A. kuehniella* to a series of doses of KpLec. Effective doses for 50% response (ED_{50}) are the concentration fractions that decrease the mass of the larvae to 50% of the control mass of the control. Lethal doses (LD_{50}) are the concentrations of the fraction that reduce the number of larvae to 50% of the number found in control seeds. X = dose and Y = mean weight or percentage mortality. Because of the range in mortality percentages (6.7−67 and 10.5−63% for *C. maculatus* and *A. kuehniella*, respectively) for the KpLec regression analysis, percentages were not transformed.

Statistical Analysis. The results were expressed as the mean ± SEM as appropriate. The data were compared using one-way analysis of variance (ANOVA) (general linear models of the GLM procedure) followed by the Student−Neuman−Keul test to identify the means that differed if ANOVA indicated significance. A p value of <0.05 indicated significance.

RESULTS

Purification of Lectin. Pure KpLec was obtained in three chromatographic steps. A crude extract was applied to a Sephadex G-100 column equilibrated with 0.1 M phosphate (pH 7.6) and eluted with the same buffer (**Figure 1A**). This profile resulted in three peaks (PI, PII, and PIII), the second showing hemagglutinating and antitryptic activities. Peak PII was chosen for subsequent experiments because of its lectin activity. After dialysis against water, centrifugation and lyophilization, peak PII was dissolved in 0.1 M phosphate (pH 7.6), and the protein solution then loaded onto a trypsin-Sepharose affinity column (**Figure 1B**), to separate the lectin from the trypsin inhibitor. Proteins were eluted with 0.1 M phosphate (pH 7.6), followed by 0.1 M HCl. Two peaks were obtained before washing with 0.1 M HCl, with the second of these showing only hemagglutinating activity. The peak with hemagglutinating activity (Lec-TS) was dialyzed, lyophilized, and chromatographed on a reverse phase C-18 μ-Bondapack column (HPLC system). The elution profile showed one major peak (KpLec) with hemagglutinating activity, eluted with ∼75% acetonitrile (**Figure 1C**). Lec-ST also was loaded onto a column of chitin. All of the material applied bound to the matrix and was eluted with 100 mM HCl (data not shown). The yield of KpLec in this purification process was of 130 mg/100 g of seeds.

Human and Animal Agglutination of Erythrocytes and Inhibition by Carbohydrate. The agglutination assays performed with KpLec demonstrated that the lectin showed no specificity in its ability to agglutinate human and animal blood cells (**Table 1**). The carbohydrate-binding specificity of KpLec was evaluated by the ability of sugars to inhibit the agglutination of rat erythrocytes. As shown in **Table 2**, the best inhibitor was *N*-acetylglucosamine.

Chemical Characterization. To determine the molecular structure of the lectin, the purified proteins were analyzed by SDS-PAGE and gel filtration. Two main protein bands with M_r of approximately 22 and 44 kDa were obtained in SDS-PAGE, although under reducing conditions (0.1 M DTT) only one band (22 kDa) was observed (**Figure 2A**), suggesting that KpLec is a disulfide-linked dimer. The apparent molecular mass of the lectin determined by FPLC-gel filtration on Superdex 75 was ∼22 kDa (**Figure 2B**).

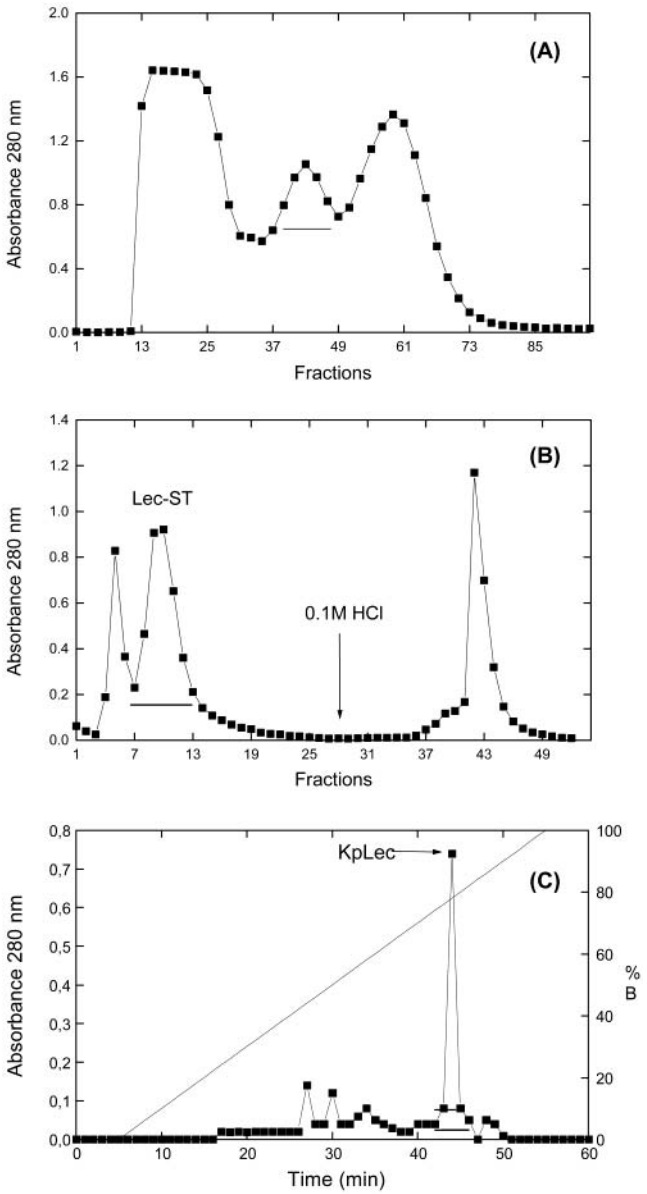

Figure 1. Purification of the lectin from *K. paniculata* (KpLec): (A) Sephadex G-100 chromatography of the crude extract of *K. paniculata* seeds [material eluted with 0.1 M phosphate buffer (pH 7.6), containing 0.1 M NaCl]; (B) fractions corresponding to peak P-2 further chromatographed in an affinity column (trypsin-Sepharose 4B); (C) reverse phase HPLC separation of the fraction Lec-ST from trypsin-Sepharose (C-18 μ-Bondapack column); (–) hemagglutinating activity.

Amino acid composition analysis (**Table 3**) revealed high contents of Asx (11.2%), Glx (9.5%), Gly (10.1%), and Leu (8.2%) and low levels of Met (0.9%), His (1.2%), and Cys (0.9%).

The neutral sugar content of KpLec was 14%, as determined by the phenol−sulfuric acid method.

The hemagglutinating activity of the purified lectin decreased appreciably after treatment with EDTA but not with EGTA.

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

erythrocyte	hemagglutination (titer[a])	erythrocyte	hemagglutination (titer[a])
human (type A)	8	sheep	6
human (type B)	6	horse	4
human (type AB)	5	cow	3
human (type O)	3	hamster	1
mouse	6	rabbit	0
rat	8		

[a] Titer is defined as the reciprocal of the endpoint dilution that caused detectable agglutination of erythrocytes. The final lectin concentration was 500 μg mL^{-1} in the first well of the plate.

Table 2. Inhibition of the Hemagglutinating Activity of KpLec by Carbohydrates

carbohydrate	IC_{50}[a] (mM)	carbohydrate	IC_{50}[a] (mM)
N-acetylglucosamine	0.09	D-lactose	ND[b]
D-fructose	ND	D-maltose	ND
D-galactose	6.24	D-mannose	3.12
D-galactosamine	1.56	D-raffinose	ND
D-glucosamine	0.19	D-sucrose	ND
D-glucose	0.78		

[a] IC_{50} = concentration required to inhibit hemagglutinating activity by 50%. The amount of KpLec used in these assays was 45 μg. [b] No inhibition detected at up to 100 mM.

Table 3. Amino Acid Composition of KpLec

amino acid	mol %	amino acid	mol %
Asx	11.19	Tyr	3.84
Glx	9.50	Val	6.86
Ser	5.26	Met	2.59
Gly	10.06	Cys[a]	0.89
His	1.17	Ile	5.95
Arg	3.76	Leu	8.21
Thr	5.19	Phe	5.87
Ala	5.14	Lys	6.78
Pro	7.72	Trp	ND[b]

[a] Cysteine was calculated as cysteic acid. [b] Not detected.

KpLec was heat stable up to 70 °C, but a sharp decrease in activity occurred between 70 and 90 °C (**Figure 3A**). Hemagglutination was markedly affected by pH between 2 and 4 and between 12 and 14, with maximum activity being retained at pH 5.0−10.0 (**Figure 3B**).

The partial NH_2-terminal sequence of the first 25 amino acids of KpLec (22 kDa subunit) showed 40% identity with *Urtica dioica* lectin when compared using the NCBI−Blast data bank (**Table 4**).

Insect Bioassay. The effect of KpLec on the development of *C. maculatus* and *A. kuehniella* was assessed by determining the number and mass of surviving larvae (fourth instars) fed a diet containing increasing amounts of KpLec. The dose−response effect KpLec on the growth and mortality of the insect larvae is shown in **Figures 4** and **5**. The effect of dietary KpLec on *C. maculatus* mortality and weight at day 20 is represented in **Figure 4**. The mortality and weight of cowpea weevil larvae feeding on control seeds (represented by *Y*-intercept value) were about 6.5% and 7.7 mg, respectively, whereas a seed containing about 0.7 and 1% KpLec produced an estimated 50% mortality (LD_{50}) and a decrease of 50% weight (ED_{50}), respectively. Regression analysis showed that for every 0.1% increase in KpLec dose, there was a 5.9% increase in mortality with an R^2 value of 0.98. For each increase in dose of 0.1% KpLec, there was a 0.42 mg decrease in weight with an R^2 value of 0.96.

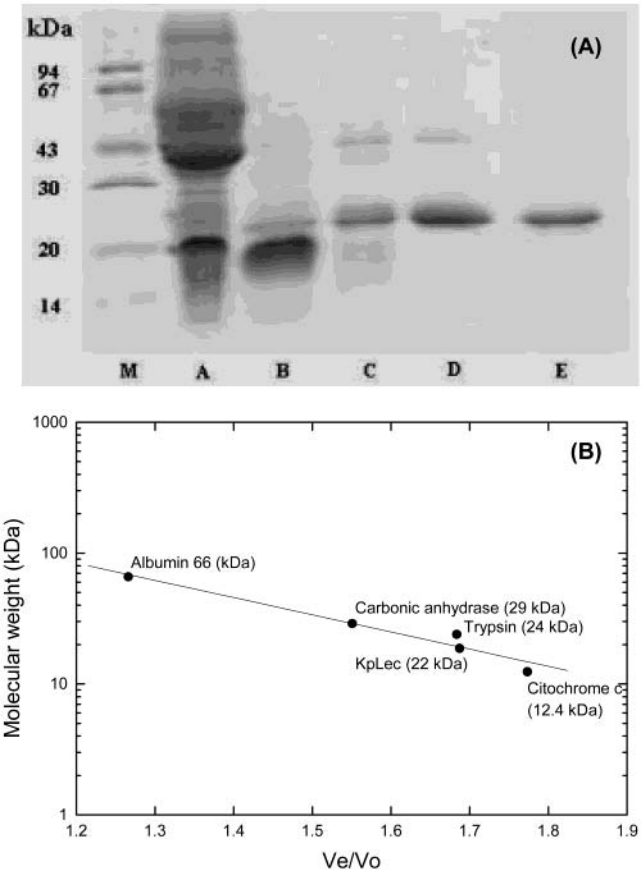

Figure 2. (A) SDS-PAGE analysis of purified KpLec: (lane M) molecular weight standards; (lane A) crude extract; (lane B) peak P-2; (lane C) Lec-ST; (lane D) KpLec; (lane E) KpLec in the presence of 0.1 M DTT. (B) Molecular mass estimation from KpLec by gel filtration on Superdex G-75. Standards used were cytochrome *c* (M_r 12.4 kDa), trypsin (M_r 24 kDa), carbonic anhydrase (M_r 29 kDa), and BSA (M_r 66 kDa).

Table 4. Comparison of the N-Terminal Amino Acid Sequences of KpLec and *U. dioica* Agglutinin (UDA)[a]

KpLec	**Q**KK**C**SQ**Q**C K I T I**C**Q**P** N-F **CCS** KI **-G**					
UDA	**Q** R- **C**GS**Q**GGGGT**C** – **P**ALW**CCS**IW**-G**					
	1	5	10	15	20	25

[a] Letters in bold represent completely conserved residues.

The effect of dietary KpLec on *A. kuehniella* mortality and weight of larvae of fourth instar is shown in **Figure 5**. Artificial diet containing 0.65% KpLec produced an estimated 50% mortality (LD_{50}), whereas a diet containing 0.2% KpLec produced a 50% decrease in weight (ED_{50}). Regression analysis showed that for each 0.1% increase in KpLec dose, there was a 5.3% increase in mortality with an R^2 value of 0.96. For each increase in dose of 0.1% KpLec, there was a 0.35 mg decrease in weight with an R^2 value of 0.9.

DISCUSSION

A lectin from *K. paniculata* seeds, commonly known as the Golden rain tree, was purified by gel filtration chromatography, affinity chromatography, and reverse phase HPLC on a C-18 μ-Bondapack column. In the latter, KpLec was eluted as the main peak with hemagglutinating activity at ~75% acetonitrile, indicating that the lectin bound strongly to the chromatographic

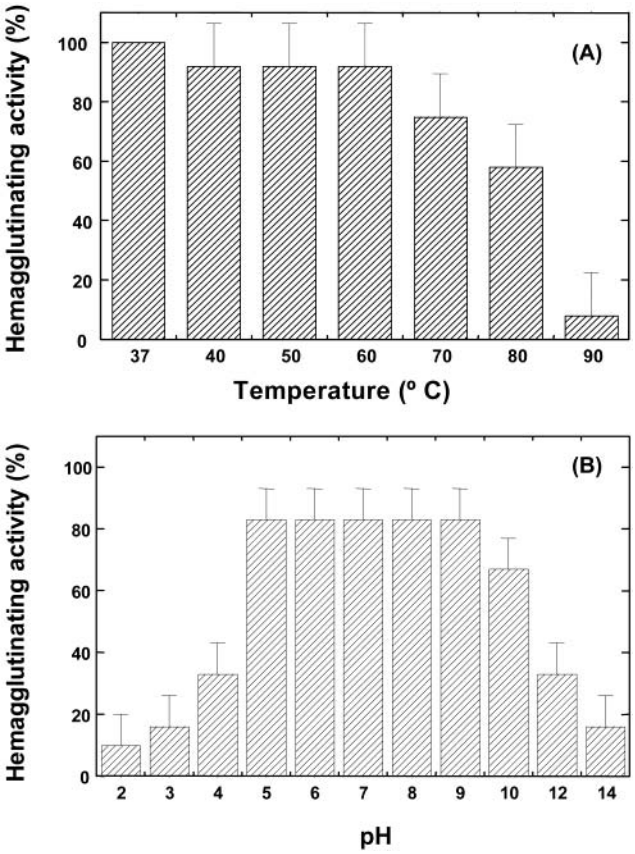

Figure 3. Thermal (A) and pH (B) stability of KpLec. Bars represent the average of five replicates. Full (100%) activity corresponded to a titer of 6.

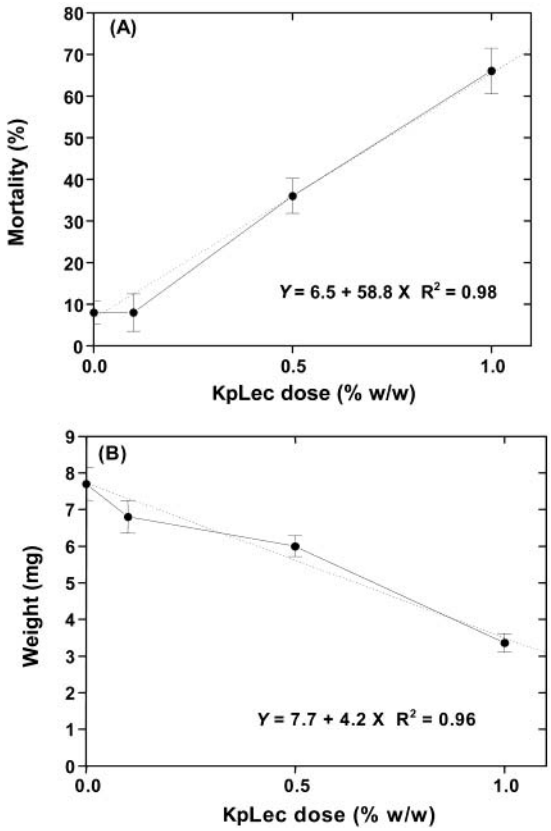

Figure 4. Effect of dietary KpLec on *C. maculatus* within-seed artificial larval: (A) mortality and (B) weight, using an artificial seed bioassay. *Y*-intercept in (A) is mortality, and (B) is weight of larvae in control seeds. Each point has an $n \geq 40$. Error bars indicate standard error of the mean.

matrix. KpLec also was loaded onto a column of chitin and the material applied bound to the matrix, confirming its affinity for *N*-acetylglucosamine. This same behavior was verified for the *T. esculenta* lectin (*10*). KpLec showed high contents of Gly, Val, Leu, Ile, Phe, and Pro, confirming its hydrophobicity, and low contents of His, Met, and Cys, a common finding in plant proteins (*25*). The methods used to extract and purify KpLec are commonly used for plant proteins and include extraction with saline solutions or acid, precipitation with organic solvents or salts, and fractionation by chromatographic methods. These methods were also used by Sampietro et al. (*26*) to isolate a lectin from the juice of ripe fruits of *Cyphomandra betacea* and by Freire et al. (*10*) to isolate a lectin from seeds of *T. esculenta*.

SDS-PAGE showed that KpLec consisted of two covalently bound subunits of 22 kDa, as shown by the altered electrophoretic profile in the presence of 0.1 M DTT (**Figure 2A**). The M_r of KpLec was also estimated by gel filtration on a Superdex 75 column. One major peak with a molecular mass of ~22 kDa was obtained (**Figure 2B**), suggesting that this value is probably due to protein−matrix interactions. Former assays with various gel supports (Sephadex, Superdex, Biogel P-100, and Sepharose) showed an abnormal retention of the protein; this behavior is attributed to the mildly hydrophobic nature of the lectin.

KpLec showed no specificity in its ability to agglutinate mouse, rat, sheep, horse, cow, hamster, and human erythrocytes. The activity of KpLec against hamster erythrocytes was low, and the lectin did not agglutinate rabbit erythrocytes (**Table 1**). The agglutinating activity of KpLec was best inhibited by sugars containing D-glucosamine residues (**Table 2**). Of these, *N*-acetylglucosamine was the most potent inhibitor (the minimum inhibitory concentration was 0.09 mM), followed by D-glucosamine and glucose (0.19 and 0.78 mM, respectively). The chromatography on an affinity column (chitin) confirmed its specificity (data not shown). This inhibitory profile was similar to that of lectins with high affinity for *N*-acetylglucosamine, such as the lectin from *Lentinus edodes* (*27*) and *Urtica dioica* agglutinin (*28*).

KpLec was a glycoprotein, with a carbohydrate content of 14% as confirmed using the method of Dubois et al. (*21*). This carbohydrate content of KpLec was higher than that of lectins from *Vatairea macrocarpa* (7.9%) (*29*) and *Araucaria brasiliensis* (6.3%) (*30*) and was similar to that of the lectin from *T. esculenta* which showed 16% of carbohydrate moiety (*10*).

KpLec was stable between 37 and 70 °C, but hemagglutinating activity decreased markedly at 90 °C (**Figure 3A**). These results are compatible with plant lectins, such as that from *Saraca indica*, which is stable up to 95 °C (*31*), and *Talisia esculenta* lectin (TEL), which is stable at 60 °C (*10*). KpLec was most stable at pH 5.0−9.0 (**Figure 3B**), again very similar to TEL, which was stable at pH 3.0−9.0 (*10*). Machuca et al. (*32*) reported that the lectin from *Sphenostyles stenocarpa* also was stable at pH 2.0−10.0.

Treating KpLec with EDTA (a divalent ion chelator) completely abolished the hemagglutinating activity. In contrast, EGTA (a calcium chelator) did not affect the hemagglutination. These results indicated that KpLec required divalent ions such as Mg^{2+} and Mn^{2+} for full hemagglutinating activity but did not require calcium.

The N-terminal sequence of the first 25 amino acids of the 22 kDa subunit of KpLec showed 40% identity with *Urtica dioica* agglutinin (UDA) (**Table 4**). UDA (8.5 kDa) from stinging nettle (*Urtica dioica*) rhizomes is a small chitin-binding

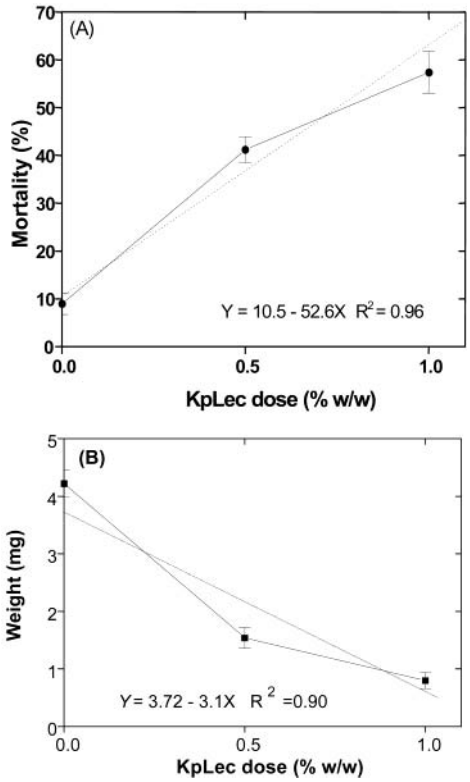

Figure 5. Effect of dietary KpLec on *A. kuehniella* within-diet artificial larval: (A) mortality and (B) weight, using an artificial diet bioassay. *Y*-intercept in (A) is mortality, and (B) is weight of larvae in control diet. Each point has an $n \geq 40$. Error bars indicate standard error of the mean.

protein, which, like KpLec, exhibits more affinity for *N*-acetylglucosamine and chitin-binding properties and has insecticidal activity that inhibits the development of the cowpea weevil *C. maculatus* and affects the survival of *Maruca vitrata* (*33*).

In the past few years, several lectins have been used by early workers to monitor the effect on cowpea wevill (*34*) and members of the Lepidoptera (*11*). However, no study had been accomplished with the lepidopteron *A. kuehniella*. In the study presented here, KpLec, with chitin-binding properties, was bioassayed on *C. maculatus* and *A. kuehniella* to judge its efficacy on the growth and developmental pattern.

KpLec was toxic to larvae and reduced the weight of *C. maculatus* (**Figure 4**). The LD_{50} of 0.7% for KpLec was comparable to the concentrations (0.1−1.0%) at which wheat germ agglutinin, rice, stinging nettle, and *T. esculenta* lectins caused 51.7, 58.5, 65, and 50% mortality, respectively, in *C. maculatus* (*10*, *35*). These lectins may provoke deleterious effects by binding to glycan receptors on the surface of cells lining the insect gut (*36*). Harper et al. (*37*) have recently demonstrated that lectins may interfere with the formation and integrity of the peritrophic membrane of the insect midgut. This membrane exists in most phytophagous insects and is composed primarily of chitin (containing *N*-acetylglucosamine residues) and proteins. The peritrophic membrane forms a barrier to protect the midgut epithelium from abrasive food particles. Although *C. maculatus* does not present the peritrophic membrane defined, Sales et al. (*38*) showed by chemical and immunocytochemical methods the presence of chitin in midgut structures of *C. maculatus* larvae. Macedo et al. (*10*) proposed that *T. esculenta* lectin would be acting like an insecticide protein against *C. maculatus* and *Z. subfasciatus* for two basic mechanisms: binding to the chitin component of the peritrophic membrane (or equivalent structures) in the weevil midgut and a strong resistance of TEL to digestion by midgut aspartic and cysteine proteinases in *C. maculatus* and *Z. subfasciatus*.

A. kuehniella larvae fed an artificial diet containing KpLec were affected more than *C. maculatus* larvae (**Figure 5**). KpLec administered at 1.0% (w/w) in the artificial diet caused an ∼84% reduction in larval weight against a reduction on the weight of *C. maculatus* larvae of ∼50% at the some concentration. In relation to an LD_{50} of KpLec the two insects presented similar patterns (0.5%, w/w). The literature mentions that lectin with *N*-acetylglucosamine specificity was the most effective against Coleoptera (*34*). However, another *N*-acetylglucosamine-binding lectin from *Triticum vulgaris* was very effective against *Ostrinia nubialis* (Lepidoptera: Pyralidae) larvae (*39*). This effect can be explained by the fact of *T. vulgaris* presents higher affinities for more complex carbohydrates, for example, the chitotriose (*39*).

The mechanism by which lectins exert their toxic effects in insects are poorly understood, but the molecules first have to bind to "receptors" on the midgut epithelium, resulting in subsequent systemic effects. Another possibility is that the lectins may bind to the peritrophic membrane in the midgut region and prevent or enhance movement between the endo- and exoperitrophic space or prevent the formation of the membrane itself. Lectins can also interfer with digestive enzymes and assimilatory proteins, thereby inhibiting food digestion and absorption. This action may contribute to the overall detrimental effect of lectins on nutrient absorption.

The action of KpLec on *C. maculatus* and *A. kuehniella* larvae may involve (1) binding to glycoconjugates on the surface of epithelial cells along the digestive tract, (2) binding to glycosylated digestive enzymes, thereby inhibiting their activity, and (3) binding to the chitin component of the peritrophic membrane (or equivalent structures) in the insect midgut.

Nevertheless, from the results presented above, KpLec may be considered to be a potent control component of integrated pest management due to its ability to act on the larval development of *C. maculatus* and *A. kuehniella*.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HPLC, high-performance liquid chromatography; M_r, relative molecular mass; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PTC, phenylthiocyanate; PTH, phenylthiohydantoine; Tris, tris(hydroxymethyl)aminomethane.

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