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Potential of the lectin/inhibitor isolated from *Crataeva tapia* bark (CrataBL) for controlling *Callosobruchus maculatus* larvae development

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Abstract

Callosobruchus maculatus is an important predator of cowpeas. Due to infestation during storage, this insect affects the quality of seed and crop yield. This study aimed to investigate the effects of CrataBL, a multifunction protein isolated from *Crataeva tapia* bark, on *C. maculatus* larvae development. The protein, which is stable even in extreme pH conditions, showed toxic activity reducing the larval mass 45% and 70% at concentrations of 0.25% and 1.0% (w/w), respectively. Acting as inhibitor, CrataBL decreased by 39% the activity of cysteine proteinases from larval gut. Conversely, the activity of serine proteinases was increased about 8-fold. The toxic properties of CrataBL may also be attributed to its capacity of binding to glycoproteins or glycosaminoglycans. Such binding interferes with larval metabolism, since CrataBL-FITC was found in the fat body, Malpighian tubules, and in the feces of larvae. These results demonstrate the potential of this protein for controlling larvae development.

Graphical Abstract

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Keywords

bioinsecticide; *C. maculatus*; *Crataeva tapia*; glycosaminoglycan; inhibitor; lectin

INTRODUCTION

The legumes (seeds of beans) are an important component of nutrition in the tropics. For example, the cowpea (*Vigna unguiculata* (L.) Walp) is an important source of carbohydrates and proteins in dietary needs of various countries. The bean beetle *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchinae) is among the most important insect pests of cowpea, severely affecting the quality and storability of the produce, by attacking seeds during storage. Due to the extensive adaptability of these storage pests, the destruction of seeds is often extremely high, making the beans useless for human consumption or for replanting.¹

Plants produce various metabolic products that may be responsible for pest resistance. These compounds can be classified as primary (proteinase inhibitors and lectins) and secondary (alkaloids and tannins) metabolites.² Examples of toxic primary metabolites of plants are canatoxin isolated from *Canavalia ensiformis*,³ zeatoxin glycoprotein isolated from *Zea mays*,⁴ and the proteinase inhibitor isolated from *Bauhinia rufa* seeds.⁵

Lectins are proteins not belonging to the immune system that are able to reversibly bind to carbohydrates by recognizing specific sites on the target molecules, without altering the covalent structure of the glycosides.^{6–10} Thus lectins may bind to glyco components on the cell surface of viruses, bacteria, and on tissues from plant and animals source.^{9,11} Such interactions can subsequently trigger other responses, including antimicrobial, anti-tumor, mitogenic, and insecticidal activity.^{12–14} It has been reported that some plant lectins are toxic to insects belonging to the orders of Coleoptera, Diptera, Lepidoptera, and Isoptera.^{15–20}

Crataeva tapia (Capparidaceae), also known as *Crateva tapia*, is a tree found in northeastern Brazil, where its wood is used in construction and for building canoes, because of its great resistance to putrefaction.²¹ CrataBL (*Crataeva tapia* Bark Lectin) is a bifunctional protein (lectin and protease inhibitor of trypsin and factor Xa) that was isolated from the *Crataeva*

tapia bark. The primary and tertiary structure of this protein was characterized previously.²² CrataBL exhibits binding specificity to N-acetyl glucosamine,²³ glucose, and galactose.¹⁴ It was already demonstrated that this protein has several important biological functions including changes in the intrinsic pathway of the coagulation cascade proteins leading to the delay time of formation of the blood clot,²⁴ as well as thrombus formation.²⁵ In addition, CrataBL was shown to exhibit analgesic and anti-inflammatory,^{26–27} anti-tumoral,^{22,26} and insecticidal (against *Nasutitermes corniger*¹⁴) properties.

In this report, we present the results of monitoring the insecticidal effects of CrataBL on *C. maculatus* utilizing an insect bioassay.

MATERIALS AND METHODS

Purification of CrataBL.

The purification the CrataBL was accomplished following the previously described methodology.^{14,22} Briefly, protein from the fine powder of *Crataeva tapia* bark was extracted with saline (0.15 M NaCl) (10% w/v). The saline extract was subjected to protein fractionation with ammonium sulfate (0–30% and 30–60% (w/v)). The 30–60% fraction, containing lectin activity, was dialyzed against 0.01M phosphate citrate buffer pH 5.5 and applied to a CM-cellulose column that was previously equilibrated with the dialysis buffer. Elution of CrataBL was performed by adding the equilibration buffer plus 0.5 M NaCl. This eluate was applied to a Superdex 75 column attached to Äkta Purifier (GE Healthcare) and equilibrated with 0.15 M NaCl. The homogeneity of CrataBL preparation was followed by reverse phase chromatography in a C₁₈ protein/peptide column (15 cm X 4.6 mm; Vydac) in a linear gradient of acetonitrile in trifluoroacetic acid (TFA) (0.1%, v/v) at a flow rate of 0.7 mL/min and monitored at 280 nm.

Circular Dichroism (CD) analysis.

CD spectra were obtained with a J-810 JASCO spectropolarimeter. Measurements were carried out at 25 °C at a CrataBL concentration of 10 µM in a 1 mm pathlength cuvette and were recorded in the 190–250 nm range as an average of eight scans. The results were expressed as the mean residue ellipticity, $[\theta]$, defined as $[\theta] = \theta_{\text{obs}} / (10 \cdot C \cdot l \cdot n)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the pathlength of the cuvette (cm), and n is the number of amino acid residues (165, as described by Ferreira et al. (2013)²²). The CDPro software was used to estimate the fractions of the secondary structure²⁸ and the Cluster program was used to determine the tertiary structure class.²⁹

The effect of pH on the CrataBL conformation was determined by CD. CrataBL, at an initial concentration of 2.0 mg/mL, was diluted with 10 mM acetate/phosphate/borate (PBA buffer), pH 2.0; 4.0; 5.5; 6.0; 7.4; 8.0; 10.0; and 12.0 to a final concentration of 0.2 mg/mL. The protein was incubated for six hours at room temperature.

Insects.

C. maculatus colony was maintained at the Laboratório de Química e Função de Proteínas, Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, SP, Brazil.

The bruchids were reared on *V. unguiculata* host seeds (cv. Fradinho), purchased at supermarkets in the city, in glass bottles at 28 °C and 60–80% relative humidity inside a B.O.D. incubator.

The coats of *V. unguiculata* seeds were separated from cotyledons by manual peeling and a fine flour of *V. unguiculata* cotyledons was made. The flour was placed into a cylindrical brass mold containing variable concentrations (w/w) of CrataBL. These artificial seeds have a solid consistency, are 8 mm in diameter and 5 mm in height, with the final mass of 400 mg.³⁰ After the removal of a seed from the mold it was exposed to three *C. maculatus* females (2 days old) over 24 h, under conditions described above. Subsequently the females were removed, and only four eggs were left on each seed, with the excess eggs removed. The control artificial seeds consisted of only *V. unguiculata* flour, without addition of CrataBL; they were maintained in the same conditions, including the number of eggs. Both the control and CrataBL-containing seeds were incubated for a period of 18 days (28 °C and relative humidity 60–80%). Upon completion of the incubation the seeds were opened and the larval mass, as well as the number of emerging larvae, were determined.

Conjugation of FITC (isothiocyanate fluoresceine) to CrataBL.

A solution of the compound FITC was prepared by dissolving 50 mg in 1 mL of anhydrous DMSO. In order to covalently couple it to CrataBL, this solution was immediately diluted in 0.75 M bicarbonate buffer pH 9.5 and then added to the solution of CrataBL, to yield the final concentration of 1 mg FITC for 1 mg of protein. The solution was kept in the dark and constantly rotated at room temperature for 1 h. After this period the solution was dialyzed through 14 kDa membrane against Milli-Q water to remove FITC which was not conjugated. The solution of the FITC-CrataBL complex was subsequently lyophilized and was used later for making artificial seeds.

Artificial seeds containing CrataBL coupled to FITC.

Artificial seeds which contained 1% CrataBL coupled to FITC (w/w) were made as described above. Artificial seeds used as controls included only *V. unguiculata* flour. After 18 days the larvae were removed and placed on glass plates for 30 min to collect feces. They were subsequently dissected in 0.15 M NaCl for collection of midgut, malpighian tubules, and fat body and were further analyzed through Leica confocal microscopy.

Enzyme activity assays.

Cysteine proteinase activity.—For extraction of enzymes from midgut, five larvae, after 18 days of maintenance on artificial seeds incorporating 1% (w/w) CrataBL, were dissected in 0.15 M NaCl and their midgut macerated with 250 µL of 0.1 M sodium phosphate buffer pH 6.3 with 0.01 M EDTA, 0.4 M NaCl and 0.005 M DTT. The extract was maintained in constant rotation at 5 °C for 1 h, centrifuged at 4000g for 5 min at 4 °C, and the supernatant was used for enzymatic assay.

Proteinase activity of the extract was measured using the $4 \cdot 10^{-4}$ M peptide Z-Phe-Arg-pNan (Calbiochem Ltda, Darmstadt, Germany) as a substrate. Twenty microliters of the extract diluted 10x in 0.1 M sodium phosphate buffer pH 6.3 with 0.01 M EDTA, 0.4 M NaCl and

0.005 M DTT were incubated at 37 °C in microplate assay, in a 250 µL final volume of 0.1 M sodium phosphate buffer, pH 6.3 with 0.01 M EDTA, 0.4 M NaCl and 0.005 M DTT. The reaction was monitored for 30 min. Substrate hydrolysis was monitored by measuring the absorbance of the released p-nitroaniline at 405 nm in a spectrophotometer (Spectra max plus 384, Molecular Devices).

Serine proteinase activity.—The midgut enzymes were extracted as described above. However, in this case the maceration was performed in 150 µL of 0.05 M Tris/HCl buffer pH 8.0 with 0.02% CaCl₂. The extract was maintained in constant rotation at 5 °C for 1 h and was subsequently centrifuged at 4000g for 5 min at 4 °C, with the supernatant used for enzymatic assays.

Proteinase activity of the extract was measured using α-benzoyl-DL-arginine p-nitroanilide (BAPA) (Bachem, Bubendorf, Switzerland) as a substrate. Forty microliter of the extract were incubated with 20 µL 0.01 M BAPA for in microplate assay in a 250 µL final volume at 37 °C for 120 min. Substrate hydrolysis was monitored by measuring the absorbance of released p-nitroaniline at 405 nm in a spectrophotometer (Spectra max plus 384, Molecular Devices).

RESULTS AND DISCUSSION

Circular Dichroism (CD) analysis.

The CD spectrum of CrataBL in pH 7.4 is characterized by two negative bands, one at around 198 nm and the other one around 210 nm (Figure 1). This indicates that this protein possesses a large fraction of β-sheet structure and a low content (or complete lack) of α-helix structure, because no peaks were observed around 208 nm and 222 nm.³¹ Additionally, the CD spectrum of CrataBL shows a positive contribution below 190 nm and a positive band around 228 nm that are due to contribution of aromatic residues and disulfide bridges.³² (CrataBL contains four tyrosine residues, three tryptophan residues, seven phenylalanine residues and two disulfide bridges.²²) The contents of secondary structure estimated using the CDPro software were 2% α-helix, 46% β-sheet, 21% β-turn, 31% irregular structures and an RMSD (root mean square deviation) was lower than 0.1%. Cluster analysis showed CrataBL to be a β-class protein (proteins containing mainly β structure), and this result was corroborated by CDPro analysis (46% all-β structures) and by crystallographic data which showed that this lectin belongs to the β-trefoil superfamily.²² Proteinase inhibitors from the Kunitz family^{33,34} have secondary structure content similar to that of CrataBL. CD spectra of other Kunitz family inhibitors are generally similar, although, for example, the soybean trypsin inhibitor (STI), a model protein of this family, displays only one negative band around 200 nm in his CD spectrum. However, the papaya Kunitz-type trypsin inhibitor³³ has a very similar CD spectrum that includes positive and negative CD bands, as observed in the CD spectrum of the CrataBL.

The secondary structure of CrataBL was not affected by the changes in pH ranging from 2.0 to 10.0, as shown by the CD spectra (Figure 2). Furthermore, an increase in pH to 12.0 resulted in the loss of CD band intensity centered at 199.5 nm and the reduction of the light intensity of the band with a maximum at 228.5 (Figure 2B). However, the profile of protein

CD spectrum at this highly basic pH is not characteristic of disordered structure, suggesting that the molecule still retains considerable native-like order. These results demonstrate the stability of the secondary structure of the protein at both the high and low extremes of pH. The β -trefoil structure of CrataBL, also common in many other lectins and protease inhibitors,^{33,35} leads to increased stability of these proteins required in the performance of their biological functions.

The conformational stability of the CrataBL at different pH values makes this protein a good target for use in a bioassay with *C. maculatus* larvae, considering that the midgut of such insects has pH value around 5.6 to 6.2.³⁶

Insect assay.

Previous studies have shown that plant lectins interfere with the development of insect larvae, in particular those of *C. maculatus*.^{37–41} The mass of the larvae was significantly lower in all CrataBL-fed groups compared to controls, demonstrating the efficiency of this protein as a deleterious component for *C. maculatus*. A mass reduction of about 45% and 70% was observed when the seeds used as the feed incorporated 0.25% or 1% (w/w) of CrataBL, respectively (Figure 3). No mortality among the larvae was found at the tested doses. The effect was similar to the effects of some other lectins. For example, the BmoLL lectin, purified from *Bauhinia monandra* leaves,⁴² reduced the larval mass of *C. maculatus* by 50% when incorporated at a concentration of 0.4% in artificial seeds,¹⁸ whereas the TEL lectin purified from *Talassia esculenta* seeds reduced the larval mass by 50% when applied at a concentration of 1% (w/w).⁴³

Investigation of CrataBL accumulation in *C. maculatus*.

In order to verify biodistribution, clearance, or specific retention of CrataBL in the *C. maculatus* larvae we used CrataBL-FITC conjugated with FITC. Trafficking of FITC-labeled CrataBL was investigated using a Leica confocal microscope (488 nm excitation, emission detected at 490–550 nm). CrataBL was found in the midgut (Figure 4 A-B), Malpighian tubules (Figure 4 C-D), fat body (Figure 4 E-F), and feces (Figure 4 G-H). Retention of CrataBL in these tissues may be attributed to its lectin properties, primarily to its capacity for binding to *N*-acetylglucosamine (GlcNAc). Toxic activity of CrataBL on the caste of soldiers and workers of *N. corniger* insect (termite) was previously reported.¹⁴

Whereas millimolar levels of GlcNAc binding have been shown to inhibit hemagglutinating activity of CrataBL, the structural basis of this phenomenon has not yet been established, since the crystal structure of CrataBL has been determined only for the free lectin,²² while attempts to cocrystallize it with bound carbohydrates have not been successful as yet.

Lectins capable of specific binding of GlcNAc residues appear to be frequently insecticidal, including targets such as *C. maculatus*.^{44,45} The mechanism by which insecticidal lectins exert their function is not well elucidated, but may involve the binding to glycoproteins present in midgut epithelial cells, thereby disrupting cellular function.

Glycoproteins are major constituents of insect digestive tract membranes, i.e., the gut of these insects contains specific binding molecules that are targets of plant lectins.^{18,46–48} The

lectin wheat germ agglutinin was demonstrated to interfere with the formation and integrity of the peritrophic membrane of the insect midgut.⁴⁹ The peritrophic membrane is a barrier that protects the midgut epithelium from abrasive food particles. This membrane is present in most phytophagous insects and is composed primarily of chitin (containing GlcNAc residues). It is noteworthy that *C. maculatus* does not have a defined peritrophic membrane;⁵⁰ however, the presence of chitin located in the apical part of microvilli in the midgut epithelium was demonstrated by chemical and immunocytochemical methods.⁵¹

Other proteins, for example vicilin, act by a mechanism similar to that of lectins, by binding to the midgut structures which are major components of the insect peritrophic membranes rich in GlcNAc.^{52–55} It was demonstrated that vicilin is concentrated in microvilli of enterocytes, interfering with the digestive physiology of larvae.⁵¹ Moreover, these proteins also cross the intestinal epithelium and are found in the cells of the fat body,^{56,57} destabilizing the internal organs of the larvae.⁵⁸ Based on the results of this one and previous studies we may suggest that, similarly to the case of vicilin, broad biodistribution of the glycoprotein/lectin CrataBL might be related to its high affinity to GlcNAc.

Enzyme activity assay.

It has been shown that lectins, in addition to binding to glycoconjugates, can also bind to glycosylated digestive enzymes, interfering with their activity. Such binding results in a decrease of chitin hydrolysis in peritrophic membrane of the insect midgut, interfering with uptake of proteins and thereby inhibiting the digestion and absorption of food.^{25,43}

To investigate if CrataBL affects the activity of digestive enzymes we measured the specific activity of two classes of enzymes from the midgut of *C. maculatus* larvae. The use of inhibitors of cysteine proteinases for Coleoptera plague control has also being attributed to the inhibition of exogenous activity of this class of proteinases.^{56,58,59} It should be noted, however, that CrataBL does not inhibit papain, the enzyme used most commonly as the model of cysteine proteinases.²² Our kinetic results indicate the activity of cysteine proteases in *C. maculatus* larvae, since the midgut extract cleaves better a more specific substrate for this class of enzymes, and the conditions used for the hydrolysis, such 0.1 M sodium phosphate buffer, pH 6.3 with 0.01 M EDTA, 0.4 M NaCl and 0.005 M DTT, are very suitable for cysteine proteinases. This activity decreased by 39% in the larvae fed for 18 days with artificial seeds containing CrataBL (1% w/w) when compared with control (Figure 5A). Conversely, specific activity of serine proteinases (Figure 5B) increased approximately 8-fold when compared with control. Previous studies suggested that, by binding to a sugar moiety on the enzyme surface, lectins decrease proteolysis, thus impairing larval development.⁶⁰ A significant increase of the serine proteinase-like activity can be explained by the fact that, in order to overcome the deleterious effects of the lectin, the larvae upregulate other classes of proteolytic enzymes; however, this effect was not sufficient to overcome the toxicity of the protein. We can suggest that CrataBL, functionally active as both a lectin and/or as an inhibitor, interferes directly in production in the *C. maculatus* midgut of proteolytic enzymes necessary for larval development.

In conclusion, our results indicate that the biological activity of CrataBL involves its binding to specific proteins in the intestinal epithelium, resulting in anti-nutritional effects that may be considered in the development of biopesticides.

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Figure 1.

Measurements of the circular dichroism of CrataBL. A CD spectrum of CrataBL was obtained in 0.01 M PBA pH 7.4, at 25 °C. Measurements are the averages of eight scans using a solution containing 10 μM of protein. CD spectrum deconvolution using CDPro software calculated 2% α-helix, 46% β-sheet, 21% β-turn, 31% irregular structures.

Figure 2.

Circular dichroism spectra of CrataBL at different pH values. CD spectra of CrataBL (0.2 mg/ml) were obtained after incubation for 6 hours in phosphate-borate-acetate buffer at pH (A) 2.0; 4.0; 6.0 and 7.4, (B) 7.4; 8.0; 10.0 and 12.0.

Figure 3.

Effects of CrataBL on the development of *C. maculatus* larvae in artificial seeds. **(A)** Mass of surviving larvae at 18 days. Values represent mean ($\pm$ SEM) and the statistical treatments were performed using One-way ANOVA (Tukey test), p-value $\leq$ 0.001(*), n=20. **(B)** Photographs of *C. maculatus* larvae developed in artificial seeds with the incorporation of 0.25%, 0.5%, 1% (w/w) CrataBL, and without this protein (control).

Figure 4. Detection of CrataBL in *C. maculatus* larvae. Magnification 10x. **A-B** midgut, **C-D** Malpighian tubules, **E-F** faty body, **G-H** feces of larvae. Bar = 145μm. Images obtained with a confocal microscope Leica TCS SP8.

Figure 5.

Specific activity of the midgut of larvae of *C. maculatus* treated with CrataBL. The larvae were kept for 18 days in artificial seeds containing CrataBL (1% w/w). **(A)** Specific activity of cysteine proteinases. **(B)** Specific activity of serine proteinases. The values represents means ($\pm$ SEM) and the statistical treatments were performed using t-test, p-value ≤ 0.05 (*). Specific activity (U/mg), U – unit of enzyme activity (U=1 μ mol/min).