Effects of a Chitin Binding Vicilin from *Erythrina velutina* Seeds on Bean Bruchid Pests (*Callosobruchus maculatus* and *Zabrotes subfasciatus*)

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**Abstract:** *Erythrina velutina* vicilin, EvV, is a dimeric glycoprotein with Mr of 124.6 kDa. EvV was tested for anti-insect activity against bean bruchid larvae. EvV had LD₅₀ of 0.10% and ED₅₀ of 0.14% for *Z. subfasciatus* and LD₅₀ of 0.26% and ED₅₀ of 0.19% for *C. maculatus*. EvV was not digested by bean larvae enzymes until 12 h of incubation, and at 24 h EvV was more resistant to *Z. subfasciatus* enzymes.

**Keywords:** Chitin binding vicilin, *Erythrina velutina*, *Callosobruchus maculatus*, *Zabrotes subfasciatus*.

**INTRODUCTION**

A major portion of human diet throughout the world consists of cereals and legumes. According to FAO estimates, 70% of human food comprises cereals and legumes and the remaining 30% comes from animals that feed on these seed meals [1]. Legume seeds represent an important food source for low income populations in subtropical and tropical areas where bean crops such as cowpeas (*Vigna unguiculata*) and common beans (*Phaseolus vulgaris*) are rich sources of protein (20-25%), carbohydrates (50-60%) and lipids (1-2%) [2]. However, losses in bean production caused by pests and pathogens have been estimated at more than 37% [3]. One of the most important insect pests of the cowpea (*Vigna unguiculata*) is the bruchid weevil *Callosobruchus maculatus* (F.), which attacks seeds during storage [4]. The Mexican bean weevil, *Zabrotes subfasciatus* (Boh.) is the major pest of legume seeds such as *Phaseolus vulgaris, Phaseolus lunatus* and *V. unguiculata* [5]. Both insects severely affect grain quality and storability.

The ever-increasing demands on yield and the intensification of farming practices have increased the problem of pest and pathogen damage and hence control. Bruchid and fungi controls are expensive and performed by treating stored seeds with fungicides, methyl bromide, carbon disulfide, and several other chemicals [6]. These are considered environmentally undesirable and are also expensive for subsistence farmers. In order to increase the resistance of cultivated varieties, plant breeders are interested in understanding resistance mechanisms involving defence proteins and other metabolites that operate in wild varieties.

Storage proteins of the 7S vicilin type from cultivar IT81D-1045 of *Vigna unguiculata* and others from leguminous seeds have been included as some of the most recent defensive protein plant groups [7-12]. The involvement of vicilins in the resistance of cowpea seeds to *C. maculatus* was first suggested during investigations into the possible role of trypsin inhibitors in this process. Analysis of protein fractions from the seeds indicated that the detrimental effects observed were associated with the globulin fraction [7]. Purification of vicilins from both *C. maculatus*-resistant and susceptible cowpea seeds and their incorporation into artificial seeds showed that the purified 7S globulins were responsible for at least some of the detrimental effects caused by resistant seeds [13].

In this paper, we describe the purification and characterization of a chitin binding vicilin from *Erythrina velutina* plant seeds and report on its action against two species of bean bruchid pests (*Callosobruchus maculatus* and *Zabrotes subfasciatus*).

**MATERIAL AND METHODS**

**Isolation of *E. velutina* Globulin**

*Erythrina velutina* plant seeds were obtained from the IBAMA (Brazilian Environmental Institute) seed bank of Natal/RN-Brazil. Globulin fraction was isolated by the procedure developed by Macedo et al. [14] with modifications.

**Preparation of the Larvae Midgut Homogenates**

Bean bruchids, *C. maculatus* and *Z. subfasciatus* were supplied from Departamento de Bioquímica, UFRN, Natal, Brazil. Permanent insect colonies were established on cowpea and common bean seeds and reared at 28-30 °C and 55-60 % relative humidity. The guts were surgically removed from the larvae and placed into an iso-osmotic saline [0.15 M NaCl] solution. Midgut tissue was stirred and centrifuged for 10 min at 10,000 x g at 4°C. The supernatants were then removed and used for *in vitro* assays.
Detection of Proteinase Inhibitory Activities in *E. velutina* Globulin

Globulin inhibitory assays against proteinase extracts from both Larvae were measured using 1% azocasein solution at pH 7.5 (serine proteinase activities), 1% azocasein solution at pH 5.6 (cysteine proteinase activities) and 1% hemoglobin solution at pH 3.5 (acid proteinase activities) as substrates [15].

Detection of Amylase Inhibitory Activities in *E. velutina* Globulin

Globulin inhibitory assays against midgut amylase from both larvae were measured by the Bernfeld method [17].

Detection of Haemagglutinating Activities in *E. velutina* Globulin

The haemagglutinating activity was assayed in microtiter V plates (Nunc Brand products, Denmark) according to a twofold serial diluting procedure [18].

Purification of *E. velutina* Vicilin

Globulin (10 mg.ml⁻¹) (enzyme inhibitor and lectin free) was applied to a size exclusion column Sephacryl 300-SH (84 cm x 2.5 cm column), equilibrated with 0.05 M borax buffer, pH 7.5. Fractions of 1.5 ml were collected using a flow rate of 30 ml.h⁻¹. The first peak PI (5 mg.ml⁻¹) was then applied to another gel filtration Sephacryl 200-SH column (72 cm x 2.5 cm) calibrated with protein markers: Cytocrom C (12 kDa), SBTI (20 kDa), bovine serum albumin (66 kDa), Alcohol dehydrogenase (150 kDa) and β-Amylase (200 kDa). Fractions of 1.5 ml were collected using a flow rate of 30 ml.h⁻¹. All chromatographies were monitored at 280 nm. The peak obtained, denominated EvV, was pooled, and freezer dried and subjected to further analysis.

Protein and Carbohydrate Determination

Protein content was measured by Bradford's [19]. Carbohydrate content was measured by Dubois et al’s procedure [20].

Characterization of EvV by Polyacrylamide Gel Electrophoresis

The presence of sub-units in EvV was determined by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature, as described by Laemmli [21]. EvV Homogeneity was observed by native polyacrylamide gel electrophoresis (PAGE) also as described by Laemmli [21], without SDS. Bromophenol blue was used as the tracking dye.

Binding of EvV to Chitin-Matrix

To examine the interaction of EvV with chitin matrix, the vicilin was chromatographed on a chitin column (5 ml bed volume) equilibrated with 50 mM Borax, pH 7.5. Controls were chromatographed on the same chitin column and under the same conditions described above. Positive protein controls were: IT81D-1045 *Vigna unguiculata* vicilin, *Canavalia ensiformis* vicilin, *Phaseolus lunatus* vicilin, WGA (wheat germ agglutinin), *Vigna unguiculata* chitinase and negative protein controls: Chymotrypsin, SBTI (soybean trypsin inhibitor), BSA (bovine seric albumin), Ovalbumin, PHA (*Phaseolus* phytohaemagglutinin) and Rabbit Gamma Globulin.

Insect Bioassay

The developmental performance of *C. maculatus* and *Z. subfasciatus* in artificial seeds system was assessed following the previously described method by Macedo et al. [7]. The experiments were run in four replicates and the mean (±SEM) was determined. Control artificial seeds were made with cowpea and common bean seed meal.

Detection of Chitin in the Midgut of Bruchid Larvae

Larvae were dissected and midguts were perforated and luminal contents were aspirated and reserved. Midguts were then thoroughly washed to remove remaining luminal contents. The presence of chitin in both larval midguts was ascertained by the von Wisselingh color test [22].

**In Vitro Digestibility of EvV to Midgut Homogenate of Larvae**

Vicilin was dissolved in 0.01 M phosphate buffer, pH 6.0 at 0.8 μg.μl⁻¹ concentration. Vicilin (aliquot of 240 μl) was incubated with 240 μl homogenate midgut from both larvae (0.2 midgut. μl⁻¹) at 37°C for periods of 0, 6, 12 and 24 hours. The ratio of enzyme to substrate was 4:1. Adding a 10% SDS solution stopped the digestion. Protein hydrolysis was observed by SDS-PAGE [21].

**Statistical Analysis**

The data were examined using one-way analysis of variance (ANOVA). The Student *t*-test was used to identify the means that differed if ANOVA indicated significance.

**RESULTS AND DISCUSSION**

Vicilins or 7S globulins consist of multi-subunit combinations with molecular masses between 20.1 and 94 kDa [23]. Combinations of multiple structural genes and extensive post-translational processing results in a high degree of polymorphism for these proteins [24]. In legume seeds, vicilins exhibit a considerable amount of sequence homology and microheterogeneity and may contribute to plant defense mechanisms [25]. In this study vicilins from *E. velutina* seeds were purified by precipitation with ammonium sulphate at 70-90% saturation and protein fraction F70-90 (globulins) obtained was assayed to detect contaminants such as midgut proteinase inhibitors, midgut amylase inhibitors and lectin activities. This fraction showed no inhibitory and lectin activities (data not shown), then was applied on Sephacryl 300-SH gel filtration and the peak PI was then applied to previously calibrated Sephacryl 200-SH gel filtration. The EvV peak had a molecular mass of 124.6 kDa (Fig. 1A). EvV is a glycoprotein, composed of 1.02% carbohydrates and when applied to a SDS-PAGE, proved to be a multimeric protein with 2 relative molecular mass subunits of 55 kDa (Fig. 1B) in agreement with similar data previously reported by several authors [8,14,26-27] for other leg-
ume vicilins. EvV homogeneity was confirmed in a PAGE where it is a unique protein band with slow mobility in this native gel, similar to acid protein (Fig. 1C).

To test the affinity of EvV to chitin, this protein was applied to a chitin matrix column. The EvV affinity was similar to those chitin binding protein such as WGA, cowpea chitinase and chitin binding vicilins (IT81D-1045 V. unguiculata vicilin, C. ensiformis vicilin and P. lunatus vicilin) (Table 1).

![Figure 1.](image)

**Figure 1.** (A) Elution profile on Sephacryl 200-SH calibrated column of PI (10mg) Protein markers: (a) β-Amylase (200 kDa); (b) EvV (151 kDa); (c) Alcohol dehydrogenase (150 kDa); (d) bovine serum albumin (66 kDa) and (e) SBTI (20kDa); (B) SDS–PAGE analysis of purified EvV stained with Coomassie Blue. (M) β-galactosidase (116.0 kDa); Bovine Serum albumin (66.2 kDa); Ovalbumin (45.0 kDa); Lactate dehydrogenase (35.0 kDa); Restriction enzyme Bsp98 (25.0 kDa); β-lactoalginin (18.4 kDa). (EvV) E. velutina vicilin; (C) PAGE analysis of purified EvV stained with Coomassie Blue. (EvV) E. velutina vicilin.

The observation that legume vicilins bind to a chitin matrix led to the discovery that vicilins from cowpeas [28] and other legumes such as adzuki beans [Vigna angularis], jack beans [Canavalia ensiformis], soybeans [Glycine max], and lima beans [Phaseolus lunatus] strongly bind to chitin [8]. Vicilins from these distantly related species showed a highly detrimental effect on the larval development of *C. maculatus* [8]. EvV had an affinity to chitin matrix similar to that of chitin binding proteins such as WGA [29], Hevein [30], α-amylase inhibitor/endochitinase [31], class I chitinase [32] and cowpea chitinase [33]. Through bioassays it was observed that EvV, like other chitin binding proteins such as vicilins, lectins and chitinases, had lethal and detrimental effects on organisms that contain chitin in cell walls (fungi) [34] and in the peritrophic membrane of some insects, which forms a barrier to protect the midgut epithelium from abrasive food particles [35]. Fig. (2A,B,C and D) show the influence of EvV on the weight and number of survivors of *C. maculatus* and *Z. subfasciatus* during larval development when larvae were fed with a diet containing different EvV concentrations. EvV was very lethal to *C. maculatus* with LD50 (0.26%) and ED50 (0.19%) ten and/or twenty lower than those observed in IT81D-1045 resistant V. unguiculata vicilin (LD50 of 2.0% and ED50 of 1.07%) [7] and among other legume vicilins such as *G. max* vicilin (ED50 of 1.66%), *P. lunatus* vicilin (ED50 of 1.74%), *C. ensiformis* vicilin (ED50 of 2.15%), which are non-host legume seeds of *C. maculatus* [8]. The lethal EvV dose was comparable to the concentrations (0.1-1.0%) of WGA, rice lectins, natté lectins and TEL (Talisia esculenta lectin) tested for *C. maculatus* [36-37]. Differently from IT81D-1045 resistant V. unguiculata vicilin, which was neither lethal nor detrimental to *Z. subfasciatus* [7], EvV was highly lethal (LD50 of 0.1%) and detrimental (ED50 of 0.14%) to this insect. In comparison to TEL, EvV was ten time more lethal than TEL (LD50 of 1.0%) to *Z. subfasciatus* [37]. The EvV action mechanism is probably due to the binding with chitin or chitinous structures.

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### Table 1. Affinity of Proteins from Diverse Origins to Chitin Matrix at pH 7.6

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Binding to Chitin</th>
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<tbody>
<tr>
<td>Vicilins</td>
<td>+</td>
</tr>
<tr>
<td>IT81D-1045 - Resistant V. unguiculata</td>
<td>+</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>+</td>
</tr>
<tr>
<td>Phaseolus lunatus</td>
<td>+</td>
</tr>
<tr>
<td>Erythrina velutina (EvV)</td>
<td>-</td>
</tr>
<tr>
<td>Lectins</td>
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</tr>
<tr>
<td>WGA (Triticum aestivum)</td>
<td>+</td>
</tr>
<tr>
<td>PHA (Phaseolus vulgaris)</td>
<td>-</td>
</tr>
<tr>
<td>Vigna unguiculata Chitinase</td>
<td>+</td>
</tr>
<tr>
<td>Bovine Chymotrypsin</td>
<td>-</td>
</tr>
<tr>
<td>Soybean Trypsin Inhibitor (SBTI)</td>
<td>-</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>-</td>
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<tr>
<td>Ovoalbumin</td>
<td>-</td>
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<td>Rabbit Gammaglobulin</td>
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* (+) plus sign means binding of the protein to the matrix and (-) minus sign means that the proteins did not bind to the matrix under the conditions employed. 

Figure 2. Effects of EvV (%. w/w) on C. maculatus and Z. subfasciatus larvae development in an artificial diet system. (A) Mass C. maculatus larvae and (B) number C. maculatus larvae. (C) Mass Z. subfasciatus larvae and (D) number of Z. subfasciatus larvae. Y-intercept in (a) is the number of larvae and (b) is the mass of larvae in control seeds. Each mean represents 4 replicates (±S.E.).

Figure 3. SDS-PAGE patterns of EvV digested by midgut homogenate of (A) C. maculatus and (B) Z. subfasciatus stained with Coomassie Blue. (M) molecular mass markers (M): β-galactosidase (116.0 kDa); Bovine Serum albumin (66.2 kDa); Ovalbumin (45.0 kDa); Lactate dehydrogenase (35.0 kDa); Restriction enzyme Bsp98 (25.0 kDa); β-lactoalbumin (18.4 kDa); (GUT): midgut homogenate; Incubation time (hours): 0, 6, 12 and 24h.

present in the midgut of these bruchids, as proved here by von Wisselingh’s color test for chitin (data not shown). This effect on the midgut of both C. maculatus and Z. subfasciatus suggests that the binding of vicilins to the peritrophic membrane of insect guts either causes interference with nutrient absorption [38], prevents or enhances movement between the endo- and exoperitrophic space, or prevents the formation of the membrane itself [39]. EvV was also resistant to digestion by enzymes from the midgut of C. maculatus and highly resistant to those of Z. subfasciatus. Larval
proteases were unable to digest EvV until 12 hours of digestion (Fig. 3A and 3B). EvV was completely digested by C. maculatus larval proteases after a 12-hour assay. EvV was more resistant to Z. subfasciatus larval proteases and at 12 hours of digestion, the presence of protein bands of high molecular mass was still observed. Similar resistance was found to TEL, which was hydrolyzed with a mixture of pepsin and papain after 16 hours of incubation [37]. This fact, along with chitin binding, suggests that at least part of the effect on insects can be accounted for by these lower rates of EvV hydrolysis.

The strong negative effects of EvV on bruchids observed in this study suggest that EvV might be able to provide a viable alternative in designing transgenic crops to control insect pests or be used as a pesticide.

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REFERENCES


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