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Bioinsecticidal activity of *Talisia esculenta* reserve protein on growth and serine digestive enzymes during larval development of *Anticarsia gemmatalis*

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ABSTRACT

Plants synthesize a variety of molecules to defend themselves against an attack by insects. Talisin is a reserve protein from *Talisia esculenta* seeds, the first to be characterized from the family Sapindaceae. In this study, the insecticidal activity of Talisin was tested by incorporating the reserve protein into an artificial diet fed to the velvetbean caterpillar *Anticarsia gemmatalis*, the major pest of soybean crops in Brazil. At 1.5% (w/w) of the dietary protein, Talisin affected larval growth, pupal weight, development and mortality, adult fertility and longevity, and produced malformations in pupae and adult insects. Talisin inhibited the trypsin-like activity of larval midgut homogenates. The trypsin activity in Talisin-fed larvae was sensitive to Talisin, indicating that no novel protease-resistant to Talisin was induced in Talisin-fed larvae. Affinity chromatography showed that Talisin bound to midgut proteinases of the insect *A. gemmatalis*, but was resistant to enzymatic digestion by these larval proteinases. The transformation of genes coding for this reserve protein could be useful for developing insect resistant crops.

1. Introduction

Soybean (*Glycine max*) is the second most cultivated agricultural crop in Brazil and is grown extensively in southern and central-western regions of the country. Attack by pests can severely damage the quality of the grains or seeds and result in important economic losses in productivity.

Anticarsia gemmatalis (Hübner) (Lepidoptera: Noctuidae), the velvetbean caterpillar, is native to tropical and subtropical areas of the western hemisphere and was first found in Florida in 1903 (Hinds, 1931). This species is widespread in tropical America and considered the major pest of soybean crops in Brazil.

Problems associated with widespread pesticide usage have lead to greater interest in the potential for exploiting plant defensive proteins to help combat insect-related crop damage. Some proteins have characteristics which suggest that they have a primary, if not an exclusive role, in nutrient reserve storage. The best-studied examples are the storage proteins that accumulate specifically in developing seeds (Shewry, 2003).

Plants store proteins in embryo and vegetative cells to provide carbon, nitrogen, and sulfur resources for subsequent growth and development. The storage and mobilization cycles of amino acids that compose these proteins are critical to the life cycle of plants. Mechanisms for protein storage and mobilization serve many different developmental and physiological functions. In agriculture, proteins stored in seeds and vegetative tissues account for much of the protein consumed directly as food by humans and livestock (Herman and Larkins, 1999). These proteins can accumulate to a high abundance, up to 50% of the total soluble proteins, in various vegetative storage organs.

Many, but not all, storage proteins exhibit a well-defined biological activity. For example, patatin exhibits acyl hydrolase and esterase activity (Racusen and Weller, 1984), sporamin acts as a trypsin inhibitor (Hattori et al., 1989; Yeh et al., 1994) and dioscorin has antioxidative properties (Hou et al., 2001). It is therefore probable that storage proteins play a dual role in storage and defense (Van Damme et al., 2002; Gaidamashvili et al., 2004).

Several reports have shown reserve proteins acting as plant defenses against insects and other herbivores. A toxic vicilin (EcV) isolated from *Enterolobium contortisiliquum* and *Vigna unguiculata* seeds was highly toxic to *Callosobruchus maculatus* (Macedo et al., 1993; Moura et al., 2007), as were globulins and albumins isolated from *Luetzelburgia auriculata* seeds (Soares et al., 2007). The glycoprotein zeatoxin (globulin) isolated from Zeamays seeds was toxic to *C. maculatus* when supplied in an artificial diet (Macedo et al., 2000). In coffee, Legumin-like proteins from the seeds of *Coffea arabica* and *Coffea racemosa* were toxic to *C. maculatus* (Coelho et al., 2010).

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For some of the biologically active storage protein it is believed that, through their carbohydrate-binding, ribosome-inactivating, or trypsin-inhibiting activity, they acquired in addition to their storage function a role in plant defense (Peumans and Van Damme, 1995; Yeh et al., 1997; Freire et al., 2009).

In our previous study (Freire et al., 2009), we presented the biochemical characterization and cloning of the major protein from the seeds of *Talisia esculenta* (Talisin), a member of the Sapindaceae family. The sequence analysis of the cloned cDNA, demonstrated a 756 bp sequence encoding a peptide of 198 amino acids. The deduced peptide presented high similarity to a typical VSP, the 22-kDa protein in lychee (73%) and 50.0% identity to *Theobroma bicolor* reserve protein. There were identities of 52.0% and 44.0% from *Treobroma mammosum* and *Populus tremula* trypsin inhibitors, respectively. Talisin was defined as a seed storage protein with affinity properties, i.e. it interacts with chitin and trypsin.

In the present study, we investigated the effects of Talisin on the biology of larval development, pupation, adult emergence and fertility in *A. gemmatalis*. We also examined the effects of the Talisin on two enzymes involved in protein digestion, the soluble endoproteases trypsin and chymotrypsin, and assessed the binding of Talisin to larval intestinal fluid in order to determine the mechanism of action in *A. gemmatalis*.

2. Materials and methods

2.1. Materials

T. esculenta seeds were collected in the State of Ceará (Brazil), during the months from January to March in coastal regions. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) or Amersham Biosciences (Uppsala, Sweden).

2.2. Insects

A. gemmatalis Hübner (Lepidoptera: Noctuidae) larvae were from a laboratory colony and provided by Dr. J.R.P. Parra (Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil). The photoperiod used for the colony was L14:D10, and the larvae were maintained in an incubator at 25 ± 1 °C and a relative humidity of 60 ± 10% during feeding trials.

2.3. Extraction and purification of Talisin

Talisin was prepared according to Freire et al. (2009). Dehulled *T. esculenta* seeds were finely ground and extracted (meal to buffer ratio of 1:5) with 150 mM NaCl for 24 h at 4 °C and then centrifuged at 10,000 *g* for 30 min at the same temperature. The clear supernatant (crude extract or CE) was used to determine the protein content. The CE (350 mg) was diluted in 150 mM NaCl and applied to a Sephadex G-100 column (2.5 cm × 80 cm) equilibrated with the same solution. The talisin-rich (60 μg) fraction was recovered and applied to a chitin column (20 mL) equilibrated with 50 mM phosphate buffer, pH 7.6, and eluted with 100 mM HCl. The purified talisin was dialyzed and lyophilized.

2.4. Enzyme assays

Bovine pancreatic trypsin bovine (EC 3.4.21.4) and pancreatic chymotrypsin (EC 3.4.21.1) and midgut extracts were used for the enzymatic assays. Tryspin-like acitivities were assayed using BApNA (Nα-benzoyl-DL-arginine *p*-nitroanilide) as substrate (Erlanger et al., 1961). Chymotrypsin-like acitivities were assayed using BTpNA (N-benzoyl-L-tyrosine *p*-nitroanilide) and SAAPFpNA (N-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide) as substrates (Christeller et al., 1989). All substrates were used at a final concentration of 1 mM in 1% (v/v) DMSO in a final volume of 1000 μL and pH 8 (0.1 M Tris–HCl buffer). Buffer and enzyme were warmed at 37 °C for 10 min before adding substrate to start the reaction, which was allowed to proceed for 20 min and then stopped with 30% (v/v) acetic acid. The resulting absorbance was read at 405 nm. The linearity of the relationship between change in absorbance with the time was checked to ensure substrate concentrations were not rate limiting. Substrate and controls were run to ensure the validity of the sample absorbance readings. *p*-Nitroanilide standards were used to obtain a calibration of moles of product formed vs absorbance for the trypsin and chymotrypsin assays.

2.5. Polyacrylamide gel electrophoresis

SDS polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) was done as described by Laemmli (1970). The proteins used as molecular mass standards were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

Native PAGE was done as described earlier, but without SDS. Protein bands were detected with Coomassie brilliant blue R-250.

2.6. Insect biossays

To examine the effects of talisin on *A. gemmatalis* development, the artificial diet system previously developed by Greene et al. (1976) was used. Talisin was dissolved in water and incorporated into an artificial diet. Each bioassay ($n = 240$) included four treatments (diets 1, 2, 3 and 4 with 0%, 1%, 1.5% and 2% of talisin, respectively). For the bioassays, neonate *A. gemmatalis* were placed in glass containers (8.5 cm long × 2.5 cm in diameter) containing the test diet ad libitum and the larval weights were recorded 16 days after hatching.

Linear regression analysis was used to describe the response of *A. gemmatalis* to various doses of talisin. The effective dose for a 50% response (ED_{50}) was defined as the concentration of talisin that decreased the mass of the insect to 50% of that of control larvae. The lethal dose (LD_{50}) corresponded to the concentration of talisin that reduced the number of insects to 50% of those fed the control diet. Because of the range in the mortality percentages for talisin regression analysis, these values were not transformed. The ED_{50} determined for 16-day larvae was used to analyze other biological parameters such as duration of the larval and pupal period, larval and pupal mortality, pupal weight (24 h after pupation), sex ratio, larval and pupal viability, pupal and adult malformation, and fertility.

An equal number of males and females (6 pairs), randomly selected from larvae reared on the different diets, were isolated after emergence and placed in a glass box (35 × 25 × 20 cm) for 3 days to ensure mating. Each female was subsequently introduced into a plastic vial (24 × 10 cm) for a further 3 days. In both experiments, the insects were provided with a small plug of cotton wool soaked in honey solution (10%) (Hoffman-Campo et al., 1985). The eggs produced were removed and counted.

Fifteen female and male insects emerging from diets 1 and 4 each were placed in plastic vials (8 × 5 cm) and monitored daily for mortality.

Larval consumption and faecal output were analyzed on a dry weight basis. The protein content and tryptic and chymotryptic activities of the faecal samples were also determined.

2.7. Midgut preparation

Proteinases were obtained from the midguts of fourth instar larvae, according to Macedo et al. (1995). Fourth instar larvae were cold-immobilized and the midgut, along with its contents, was removed in cold 150 mM NaCl and stored frozen (−20 °C) until

needed. Guts from larvae of *A. gemmatalis* were subsequently homogenized in 150 mM NaCl, centrifuged at 6000×*g* for 5 min, and the supernatants pooled and kept on ice for enzymatic assays. The protein concentration of the extracts was determined according to Bradford (1976), using BSA as the standard.

2.8. Assay of talisin activity against serine and endogenous proteinases

The inhibition of trypsin (20 μg) and chymotrypsin (2 0 μg) was determined by measuring the remaining activity towards 1 mM BApNA and BTpNA, respectively, at pH 8.0, after incubation with talisin at 37 °C for 20 min. The effect of talisin (1 μg) on the proteolytic activity of midgut extracts was measured using 1 mM BApNA and SAAPFpNA at pH 8.0 after incubation with talisin at 37 °C for 20 min. The residual enzymatic activity was assayed as described earlier. The assays were run in triplicate with appropriate blanks.

When used, the protease-inhibitor TLCK (N-p-tosyl-lysine chloroketone, 1 mM) was preincubated at 37 °C with the midgut extracts or the commercial proteases for 20 min, prior to addition of substrate.

2.9. Effect of pH on inhibition

The effect of pH on *A. gemmatalis* midgut proteinases and on the inhibition of *A. gemmatalis* proteinases by talisin was determined by

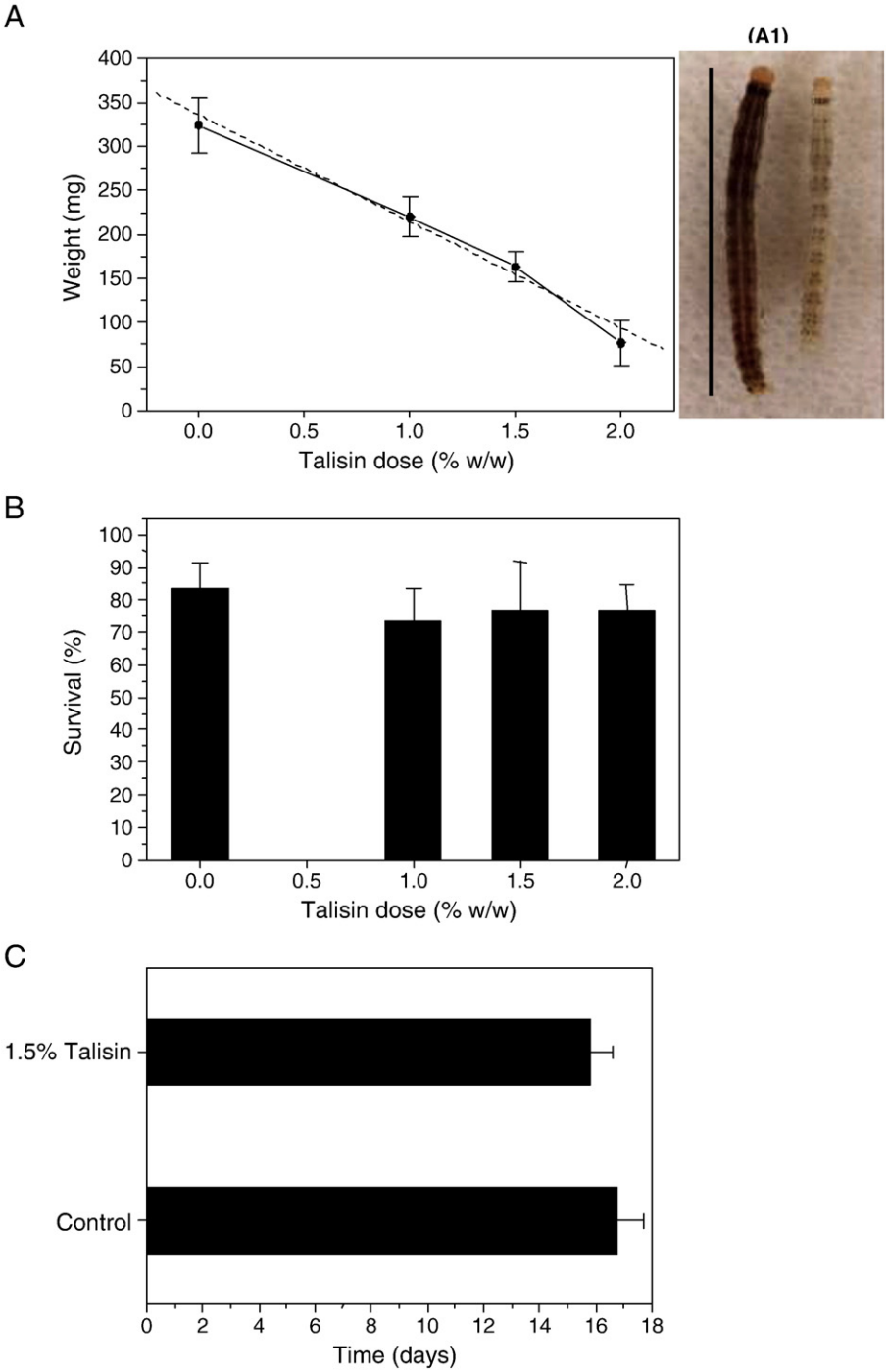

Fig. 1. Effect of dietary Talisin on *A. gemmatalis* larval mass (A) and survival (B). Effects of 1.5% Talisin on larval development time (C). A1: Variation in the size of fourth instar *A. gemmatalis* larvae fed 1.5% Talisin (right) and control (left) diets. Bar = 3 cm.

preincubating midgut extract (10 μg of protein) (with or without talisin, 10 μg) in 200 μL of 0.1 M phosphate (pH 6.0–7.0), Tris (pH 8.0–9.0) or glycine (pH 10.0–11.0) at 37 °C for 20 min. The residual trypsin activity was determined by adding BApNA to a final concentration of 1 mM in a final volume of 1000 μL in 0.1 M Tris pH 8.0. The reaction was allowed to proceed for 20 min and then stopped with 30% (v/v) acetic acid. The resulting absorbance was read at 405 nm.

2.10. Proteinase activity of midgut and faecal extracts in polyacrylamide gels containing 0.1% gelatin

Proteins extracted from the midguts and faecal extracts of larvae fed diets with or without talisin, and without prior boiling or reduction, were run on SDS-PAGE (10% gels), as described earlier, with some alterations. Midgut and faecal extract proteins from the larvae fed with an artificial diet and fed with talisin at 1.5% were incubated with TLCK (100 μM) for 10 min at 30 °C. These mixtures were then run on SDS-PAGE in gels containing 0.1% gelatin. Following electrophoresis at 5 °C, the gels were washed with 2.5% Triton X-100 solution for 2 h with shaking to remove the SDS, after which the gels were incubated with 0.1 M Tris–HCl, pH 8.0, for 2–3 h. The gels were subsequently stained with Coomassie brilliant blue R-250.

2.11. Talisin-Sepharose column chromatography

An affinity matrix was prepared by cross-linking purified talisin with CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences). The midgut extracts of *A. gemmatalis* larvae (10 mg of protein in 1 mL) were applied to a 5 mL Talisin-Sepharose column. The column was washed with 100 mM potassium phosphate buffer containing 100 mM NaCl (pH 7.6) until the absorbance at 280 nm was zero, after which protein was eluted with 100 mM HCl. Two milliliter fractions were collected and the elution profile was monitored based on the absorbance at 280 nm. Trypsin activity was determined in each fraction using BApNA as the substrate as described above.

2.12. Digestion of talisin and gel electrophoresis

The digestion of talisin by midgut extract and bovine trypsin was done as described by Macedo et al. (2003). The midguts of fourth instar larvae were dissected, extracted in 1 mL of 0.1 M Tris, pH 8.0, and processed as described earlier. Talisin was incubated with this homogenate in Tris buffer (final concentration, 2 mg/mL). The talisin: midgut protein ratio was 1:1. Digestion was done for 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h at 30 °C and was stopped by immersing the tubes in boiling water for 2 min. Talisin was digested with bovine trypsin at a substrate:proteinase ratio of 20:1 for 15 min and 0.5, 1, 2, 4, 8 and 16 h at 30 °C in 0.1 M Tris, pH 8.0. The digestion was stopped as described earlier. The relative molecular masses of the digestion products were estimated by SDS-PAGE using protein markers of known molecular mass.

2.13. Statistical analysis

The results were expressed as the mean ± S.D., where appropriate. The data on initial mortality, duration of the larval and pupal period, larval and pupal average weight, and nutritional parameters were analyzed using analysis of variance (ANOVA) (general linear moldels or GLM procedure). When a difference was found between treatments, the Tukey test was used to determine the level of significance ($P<0.05$).

3. Results

3.1. Effects of talisin on different stages of insect development

To examine the effects of talisin on *A. gemmatalis* development, neonate larvae up to the fourth instar were fed with an artificial diet containing talisin.

The effect of talisin on the development of fourth instar *A. gemmatalis* larvae was assessed by determining the number and mass of surviving larvae fed diets containing increasing amounts of talisin. The dose–response effect of talisin on larval growth and mortality is shown in Fig. 1A and B. Larvae on fed the control diet weighed ca. 324 mg, while a diet containing 1.5% talisin produced an approximate 50% decrease in weight (ED_{50}) (Fig. 1A). Regression analysis indicated that for each increase of 0.13% in talisin content there was a 15.3 mg decrease in larval mass ($R^2 = 0.98$). Talisin up to 2.0% caused ca. 76% decrease in larval weight (Fig. 1A). However, the talisin did not significantly decrease larval survival (Fig. 1B) or larval development time (Fig. 1C).

The growth and development of *A. gemmatalis* pupae were affected when 1.5% talisin was supplemented in their diet (Table 1). The pupal viability was significantly ($P<0.05$) reduced by ca. 50% for larvae which fed a diet supplemented with 1.5% talisin. Although there was no difference in the duration of pupation, 24 h after pupation the weights of larvae which fed a talisin-containing diet were significantly lower (ca. 20%) than those of larvae which fed the control diet. Pupal malformation was also observed, with the pupae of the larvae that were fed with talisin being 90% smaller in size than those of larvae which fed the control diet ($P<0.05$). In addition, 50% of the pupae showed abdominal and thoracic deformations ($P<0.05$) (Fig. 2A). The pupae of the larvae that were fed with the control diet showed no such deformations, with only 6% being smaller than the normal size ($P>0.05$).

The viability of adult moths was evaluated by counting the number of malformed moths at emergence and by determining their longevity and fertility. Adult insects from larvae fed with talisin were significantly smaller in size ($P<0.05$) than those from larvae fed with the control diet. In addition, 20% of the insects had wing defects (Table 1; Fig. 2B and C). The longevity of adult insects from the larvae fed with the control diet was about 1.4 day greater than that of adults from the larvae fed with talisin ($P<0.05$; Table 1). The fertility of larvae fed with the different diets was assessed by determining the sex of each pupa. There was no difference in the sex ratio (data not shown). Table 1 shows that adult females fed with the control diet

Table 1
Effect of Talisin on survival and growth of *A. gemmatalis*. Sample sizes are indicated in parentheses under the mean.

Diet type	Pupal				Adult		
	Mass	Duration	Mortality	Malformation	Mean egg no.	Longevity	Malformation
	(mg ± SE)	(days ± SE)	(%)	(%)	(no./female ± SE)	(days ± SE)	(%)
Control	267.9 ± 15.1a (75)	10.5 ± 0.3a (66)	10.1 ± 0.5 a (75)	5.0 ± 0.3a (66)	69.3 ± 3.1a (6)	6.1 ± 0.5a (55)	0.0 ± 0.8a (60)
1.5% Talisin	205.2 ± 19.2b (100)	11.4 ± 0.5a (46)	53.7 ± 5.0b (100)	90.5 ± 1.7b (46)	0.0 ± 0.0b (6)	4.7 ± 0.3b (40)	100.0 ± 0.0b (44)

Means followed by the same letter within each parameter are not significantly different ($P>0.05$; Tukey test).

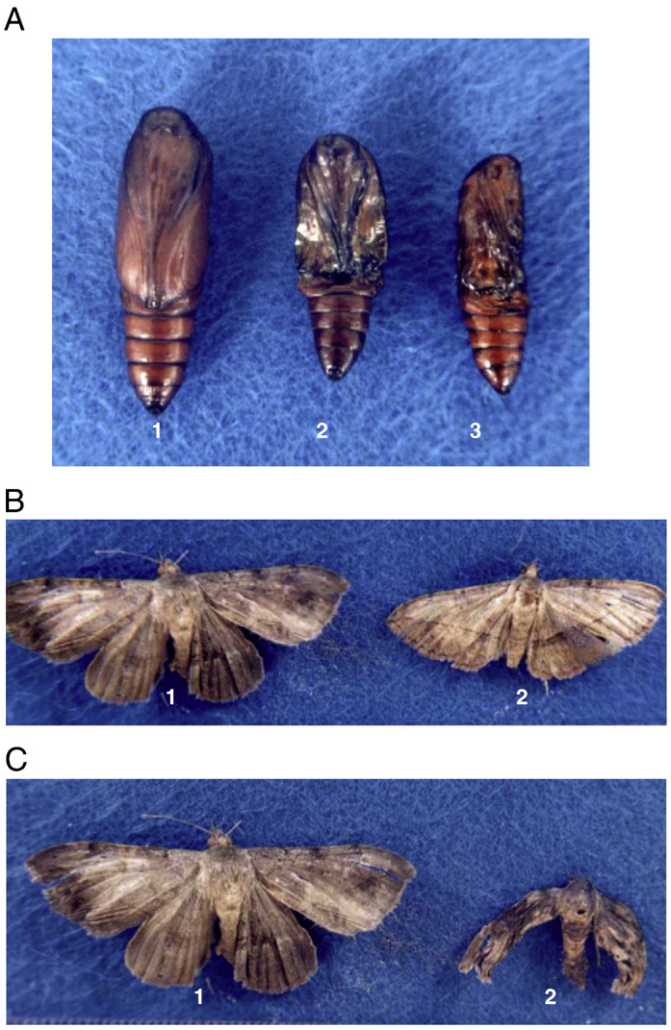

Fig. 2. Effect of Talisin on the formation of pupae and adult insects. (A) Pupa: lane 1, control-fed larva; lanes 2 and 3, Talisin-fed larvae. (B) and (C) Adult insects: lane 1, control-fed larva; lane 2, Talisin-fed larva.

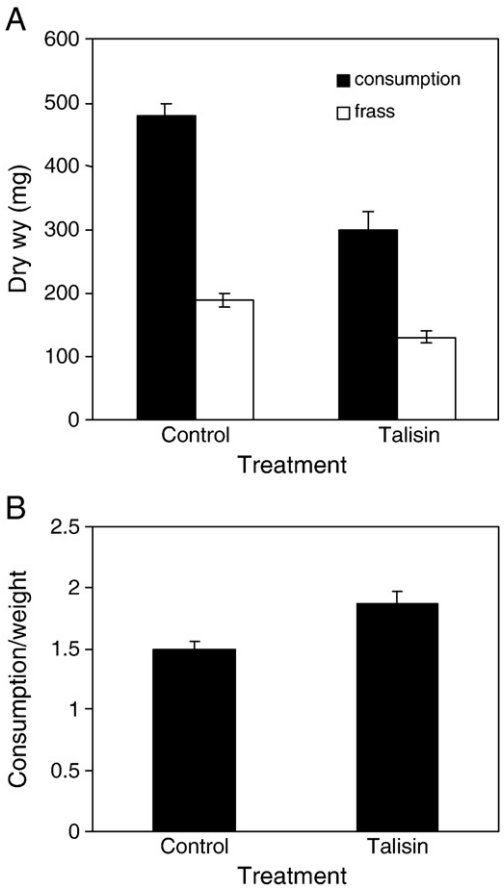

Fig. 3. Physiological parameters measured for *A. gemmatalis* larvae. The larvae were fed with a control diet or diets containing talisin for 16 days. (A) Diet consumption and faecal production by larvae (in mg, based on dry mass). (B) Mean food consumption as a ratio of the mean larval weight. Bars show the mean ± SE. There was a significant difference between talisin and control larvae in all treatments (ANOVA, $n = 40$, $P < 0.05$).

produced ca. 69 eggs/female while the corresponding females fed with talisin were unable to produce eggs ($P < 0.05$), indicating that talisin affects *A. gemmatalis* fertility.

3.2. Nutritional data

All experimental diets contained a constant level of 1.5% talisin ($n = 60$) and its effect against fourth instar larvae was analyzed. Overall, the larvae reared on a talisin-containing diet consumed 40% less food and produced 30% less frass than the controls (Fig. 3A). However, when food consumption was expressed as a ratio of body weight, the larvae fed with talisin consumed 25% more than the controls (Fig. 3B).

3.3. Digestive enzyme activity of larvae reared on talisin-containing diets

Extracts of soluble proteins prepared from *A. gemmatalis* midguts contained enzymes capable of hydrolysing the synthetic substrates BApNA and SAAPFpNA, but unable to hydrolyse BTpNA (data not shown). Therefore, all subsequent assays to detect chymotrypsin-like activity were done with SAAPFpNA.

The differences in the trypsin and chymotrypsin activities from midgut between the control and talisin-fed larvae are shown in Fig. 4A and C, respectively. Talisin-fed larvae showed a significant reduction in trypsin activity (approx. 55%) when compared with control larvae. In contrast to the trypsin activity, there was no significant change in the midgut chymotrypsin activity of talisin-fed larvae (Fig. 4C). Typsin and chymotrypsin activities were also detected in faecal material (Fig. 4B and D), with talisin-fed larvae having ca. 30% less trypsin activity than the controls whereas there was no alteration in the chymotrypsin activity of these larvae.

3.4. Effect of talisin on endogenous proteinases of A. gemmatalis and bovine trypsin

Since talisin-fed larvae showed a 55% decrease in trypsin-like activity, we examined the ability of talisin to inhibit endogenous proteinase of *A. gemmatalis* larvae and bovine trypsin.

The chymotrypsin-like activity of midgut larval extracts and of bovine chymotrypsin assayed using SAAPFpNA was not inhibited by up to 60 μg of talisin (data not shown). However, Fig. 5A shows that talisin decreased the hydrolysis of BApNA by the larval midgut extract. The inhibitory activity of talisin (ca. 10 μg) against insect proteases was ca. 80%.

In a separate experiment, aliquots of midgut larval extract from *A. gemmatalis* were preincubated with TLCK (inhibitor of trypsin-like proteases) and talisin prior to assaying for trypsin-like activity with BApNA as substrate (Fig. 5B). These assays showed that TLCK and talisin inhibited the trypsin-like activity by 87% and 67%, respectively.

To examine the effect of talisin on the enzyme activity of midgut extracts from 1.5% talisin-fed larvae, trypsin-like activity was measured using BApNA as substrate. Talisin-fed larvae showed reduced trypsin activity (ca. 66%) after incubation with talisin (Fig. 5C).

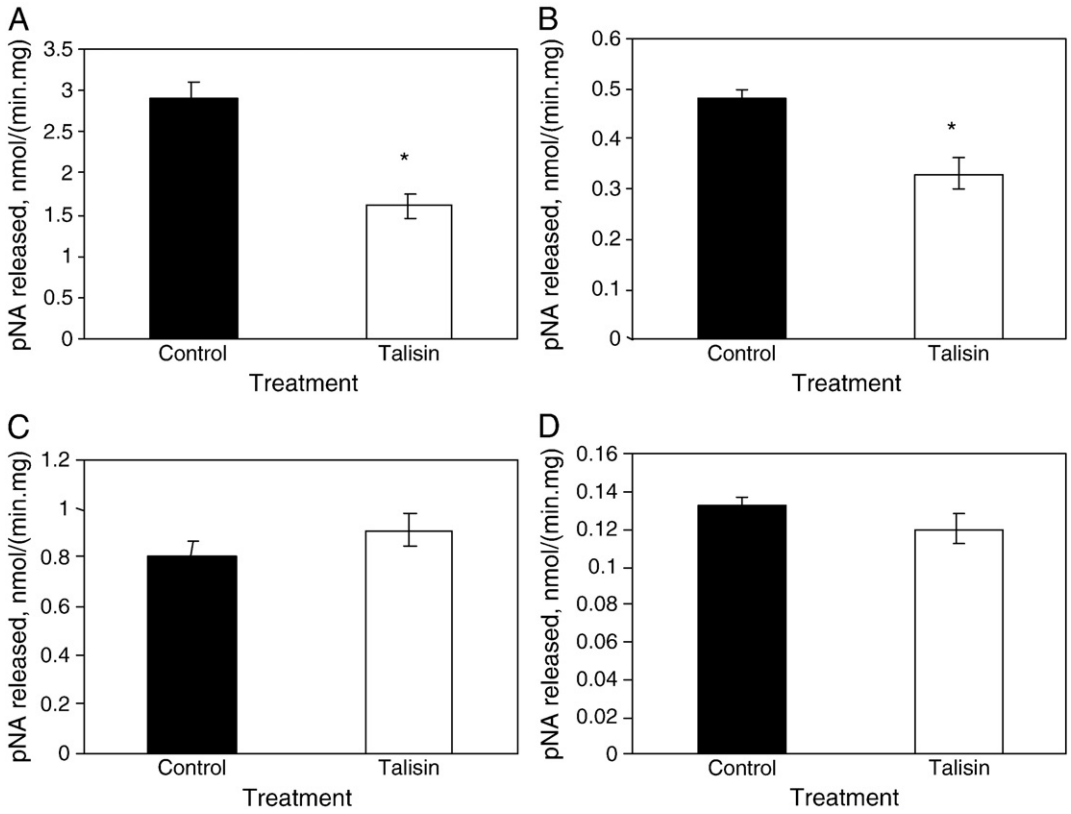

Fig. 4. Digestive activities of *A. gemmatalis* larvae after feeding a control diet or diets containing talisin for 16 days. Trypsin and chymotrypsin activities in midguts (A and C, respectively) and faecal material (B and D, respectively). Bars show the mean ± SE; *$P<0.05$ compared to the control (ANOVA, $n=40$).

3.5. Effect of pH on inhibition

Soluble trypsin was more active towards BApNA at pH 8–9 (Fig. 6). Talisin inhibited trypsin activity throughout the entire pH range. The inhibition, expressed as a percentage of the control values, was highest (ca. 80%) at pH 8 and decreased steadily with increasing pH, i.e. inhibition was 67% at pH 9, 53% at pH 10, and 36% at pH 11.

3.6. Proteinase activity of midgut and faecal extracts in polyacrylamide gels containing 0.1% gelatin

Polyacrylamide gels containing 0.1% gelatin were used to examine the action of talisin on trypsin activity and to analyze the profile of these enzymes. Proteolytic activity appeared as a clear zone against a dark blue background. Fig. 7 shows the trypsin activities of midgut extracts from control and talisin-fed larvae in lanes 1 and 2, respectively. The trypsin activity of midguts from control and talisin-fed larvae were inhibited by TLCK (Fig. 7, lanes 1A and 2A). There was a decrease in the trypsin activity of midgut extracts from talisin-fed larvae when compared with that of the control larvae.

The trypsin activities of faecal extracts from control and talisin-fed larvae were visualized in Fig. 7 (lanes 3 and 4), respectively. The trypsin activity of faecal extracts from control and talisin-fed larvae were inhibited by TLCK (Fig. 7, lanes 3 A and 4A). There was a decrease in the trypsin activity of faecal extracts from talisin-fed larvae when compared with that of the control larvae.

3.7. Talisin-Sepharose column chromatography

When midgut extracts (3 mg of protein) of *A. gemmatalis* larvae were chromatographed on a Talisin-Sepharose column, two peaks with proteolytic activity were obtained, one eluting in the wash buffer (100 mM phosphate, pH 7.6) and the others with 100 mM HCl (Fig. 8). Trypsin-like activity towards BApNA was detected in the non-retained and one retained fraction. The zymogram (insert) revealed what enzymes bind to Talsin-Sepharose column.

3.8. Digestion of talisin and gel electrophoresis

The susceptibility of talisin to the action of basic proteolytic enzymes from *A. gemmatalis* midgut homogenates was assayed by incubating these enzymes with the talisin followed by SDS-PAGE. After 30 min of incubation with trypsin, talisin was hydrolyzed (data no shown). In contrast, when incubated with *A. gemmatalis* enzymes talisin was not hydrolyzed after up to 72 h of incubation (Fig. 9).

4. Discussion

The present study provides a detailed analysis of the effects of talisin on the survival, development, and food consumption of the fourth instar *A. gemmatalis* larvae. Novel data are provided on the effects of this lectin and its insecticidal action on the larvae of this lepidopteran. This protein may act as a storage protein, presenting lectin-like activities i.e. caracterized by a weakly hemagglutination inhibition in high concentrations (starting from 500 μg mL^{-1}) (Freire et al., 2002), already as verified for Arcelin-1 (lectin-like) in contrast to typical lectins (Fabre et al., 1998), as well the capacity of interaction with carbohydrates. In addition talisin also displays protease-inhibitor properties. The first property was characterized by a strong interaction with chitin immobilized on an affinity column; with carbohydrates on neutrophil or mononuclear cells (Freire et al., 2003) and binding to the chitin component of the peritrophic membrane (or equivalent structures) in the weevil midgut. The sequence analysis of the cloned cDNA of talisin, demonstrated a 756 bp sequence encoding a peptide of 198 amino acids. The deduced peptide presented high similarity to a typical reserve protein, the 22-kDa protein in lychee

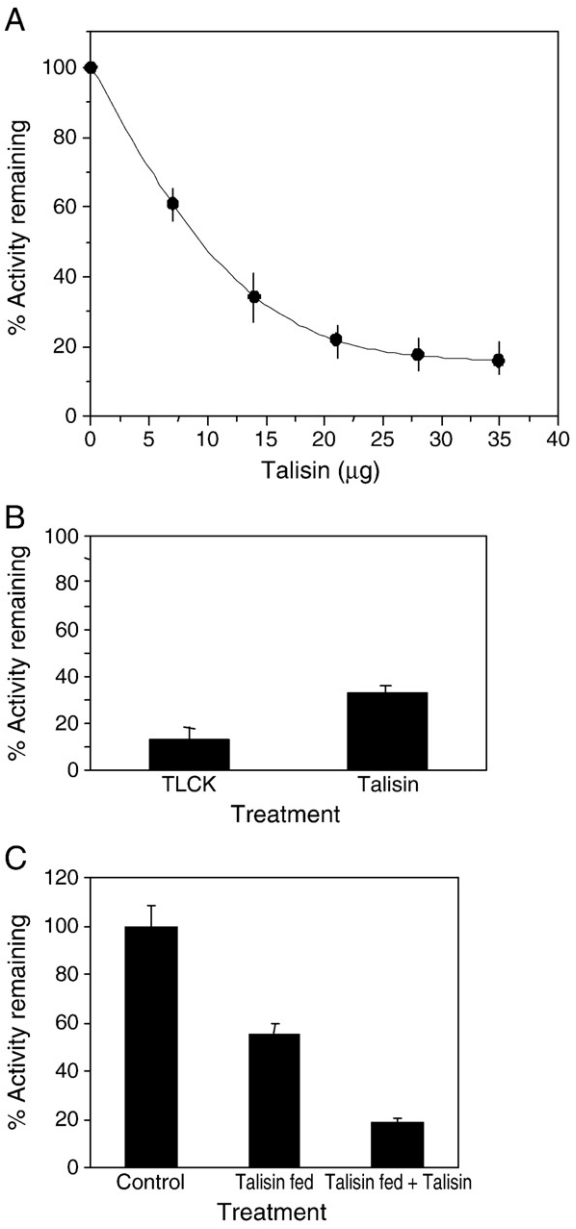

Fig. 5. (A) Inhibition of *A. gemmatalis* proteinase by increasing amounts of Talisin. (B) Inhibition of *A. gemmatalis* proteinase by TLCK (1 mM) and Talisin (10 μg). (C) Inhibition of *A. gemmatalis* proteinase to Talisin ingestion: (Control) — larvae fed a control diet; (Talisin-fed) — larvae fed 1.5% Talisin in the diet. The assays were done using 10 μg of midgut protein from *A. gemmatalis* larvae.

(73%) and 50.0% identity to *T. bicolor* reserve protein. There were identities of 52.0% and 44.0% to trypsin inhibitors from *T. mammosum* and *P. tremula* respectively (Freire et al., 2009).

Comparatively few storage proteins have been tested by bioassay using artificial diets (Macedo et al., 2003; Souza et al., 2010), and there are no reports about the effects of storage protein on *A. gemmatalis*. Talisin is a novel storage protein fom *T. esculenta* seeds, the first to be characterized from the family Sapindaceae (Freire et al., 2009).

Feeding talisin (1.5% and 2%, w/w, of dietary proteins) in an artificial diet for 16 days reduced the weight of *A. gemmatalis* larvae by 50% and 76%, respectively (Fig. 1A), but had no effect on survival (Fig. 1B). These findings were similar to those of vicilins, 7 S storage globulins (*V. unguiculata*) on *C. maculatus* (Macedo et al., 1993). *Arabidopsis thaliana* VSP when incorporated into the diets of three coleopteran and dipteran insects delayed the development of the insects and increased their mortality (Liu et al., 2005). Sporamin, a

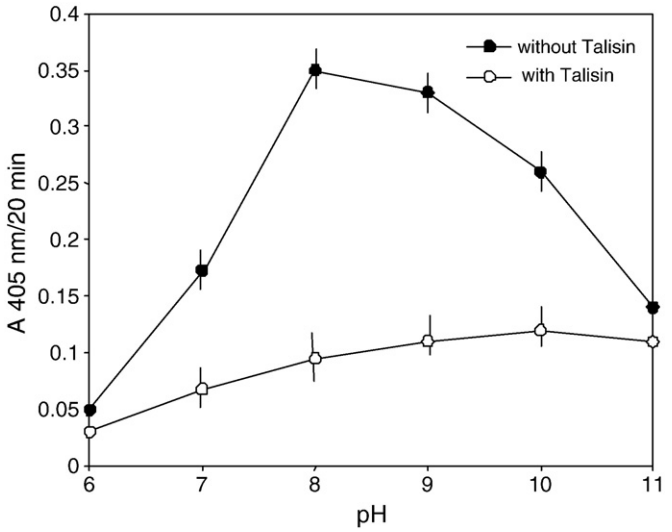

Fig. 6. Effect of pH on the hydrolysis of BApNA by *A. gemmatalis* proteinases and on the inhibition of *A. gemmatalis* proteinases by Talisin. Residual *A. gemmatalis* proteinase activity was measured after preincubation at the indicated pH.

tuberous storage protein (Maeshima et al., 1985), was introduced into tobacco plants (*Nicotiana tabaccum* cv. W38) by *Agrobacterium tumefaciens* — mediated transformation. Two transformed tobacco plants (Tb-1 and Tb-2) showed a high degree of resistance to *Spodoptera litura* (Yeh et al., 1997).

Talisin markedly affected pupal weight, mortality, and malformation. The viability of adult insects was also severely affected by the lectin. Talisin reduced the survival and longevity of the moths, produced malformations in all individuals examined and affected the fertility of *A. gemmatalis* (Table 1), decreasing the amount of eggs produced, an important parameter with respect to insect control. Talisin is a bioactive protein important in the defense processes of many species of plants (Macedo et al., 2004). This protein is characterized by strong affinity to chitin (Freire et al., 2002). These proteins have activities involving biopesticides on a variety of pests to structures of the epithelium and peritrophic membrane in the gut of various insects, principally Lepidoptera. We believe that the morphological abnormalities of the insects fed talisin be due in part to binding of the peritrophic membrane and protein structures or chitinous insect.

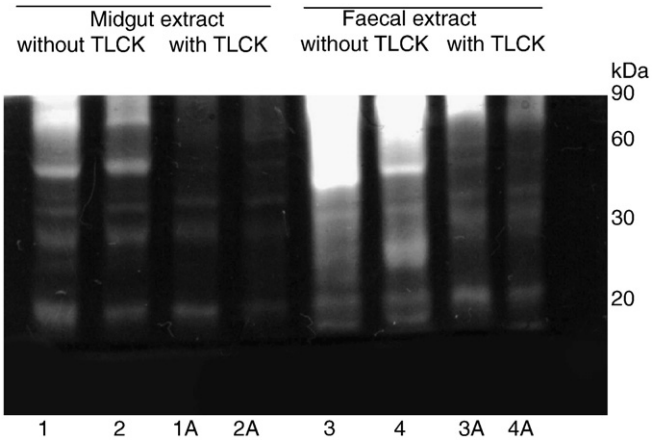

Fig. 7. Proteinase assays after PAGE with SDS. (Lanes 1 and 2) Midgut extract of control diet and 1.5% talisin-fed larvae, respectively, without TLCK; (lanes 3 and 4) Faecal extract of control diet and 1.5% talisin-fed larvae, respectively, without TLCK; (lanes 1A and 2A) Midgut extract of control diet and 1.5% talisin-fed larvae, respectively, with TLCK; (lanes 3A and 4A) Faecal extract of control diet and 1.5% talisin-fed larvae, respectively, with TLCK. Values on the right of the gel are for molecular mass markers.

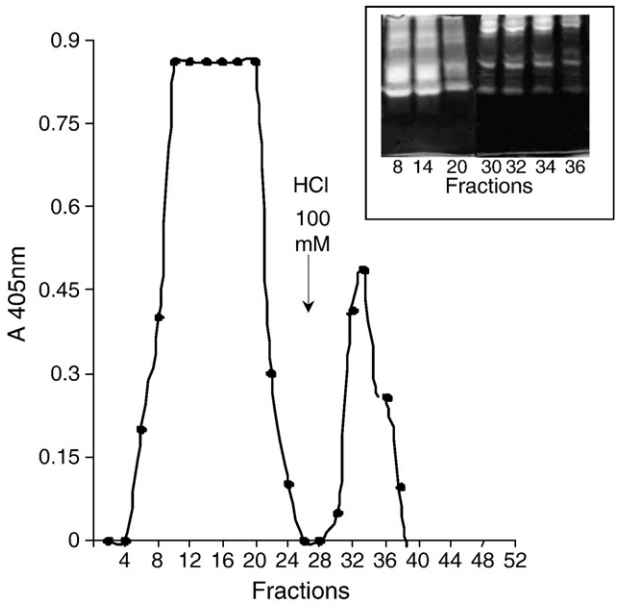

Fig. 8. Chromatography of *A. gemmatalis* midgut extract on a Talisin-Sepharose column. The column (5 mL) was equilibrated with 100 mM potassium phosphate pH 7.6, containing 100 mM NaCl, and was eluted with 100 mM HCl. (●) trypsin-like activity. Insert: 0.1% gelatin SDS-PAGE of the chromatography fractions.

Carbohydrate-binding activity for some proteins is a determining factor for its insecticidal activity (Shahidi-Noghabi et al., 2008). Ohizumi et al. (2009) showed that mannose-binding lectin, named DB1, from the yam (*Dioscorea batatas*) tubers DB1 presented insecticidal activity against *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae at different stages of development. Ye et al. (2009) showed transgenic tobacco expressing *Zephyranthes grandiflora* agglutinin, mannose-binding, confers enhanced resistance to tobacco aphids (*Myzus nicotianae*) presented no effect on reducing the survivability and fecundity of tobacco aphids.

The feeding experiments with an artificial diet provide the strongest evidence for deleterious effects caused by talisin. Larvae reared on a talisin-containing diet showed a 40% reduction in feeding and a 30% reduction in frass (Fig. 3A). The reduced feeding was a consequence of the smaller larval size since when consumption was expressed as a ratio of body weight, the talisin-fed larvae consumed 25% more than the controls (Fig. 3B). Thus, talisin apparently did not affect the feeding pattern, as also observed for the rice brown planthopper (Powell et al., 1995).

The reduction in larval weight (Fig. 1A), despite the 25% increase in consumption noted above for talisin-fed larvae, suggests that talisin

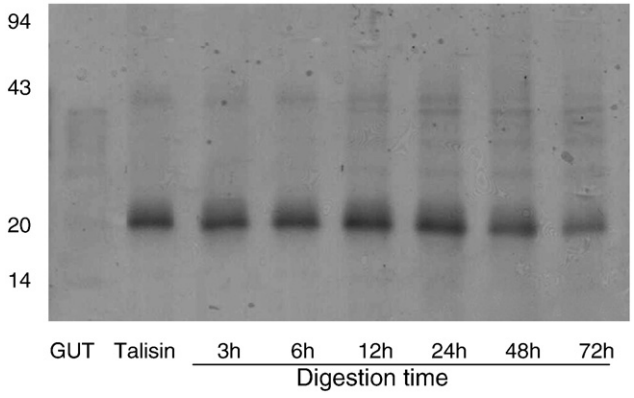

Fig. 9. SDS-PAGE patterns of Talisin digested *A. gemmatalis* midgut proteinase. (E) Trypsin. (GUT) *A. gemmatalis* midgut extracts.

inhibits nutrient uptake in *A. gemmatalis*. The mechanism for this remains to be established, but by analogy to mammals, the insecticidal effects of talisin may be determined primarily by binding to suitably glycosylated targets in the insect gut, thereby inhibiting nutrient absorption and/or midgut cell disruption (Eisemann et al., 1994).

Our results for the effect of talisin on the trypsin and chymotrypsin activities of A. *gemmatalis* larvae fed a talisin-containing diet for 16 days showed that talisin did not alter the chymotrypsin activity of midgut and faecal extracts when compared with control larvae (Fig. 3C,D). However, larvae exposed to talisin had lower trypsin activity in midgut (ca. 55%) and faecal (ca. 30%) extracts (Figs. 4A, B and 7A, B). The lack of an increase in the chymotrypsin and trypsin activities of faeces from talisin-fed larvae suggests that talisin did not cause rupture of the peritrophic membrane of *A. gemmatalis*.

The inhibition of enzyme activity by reserve proteins has previously been reported. Sporamins are tuberous storage proteins and account to 80% of soluble protein in sweet potato tubers (Maeshima et al., 1985). Based on nucleotide homology this protein can be grouped into two gene subfamilies, sporamin A and B (Hattori et al., 1989). *SpTI-1* belongs to the sporamin A subfamily, the *SpTI-1* product functions as a serine-protease-inhibitor (Kunitz-type) with trypsin-inhibitory activity (Yao et al., 2001). Its defensive role in protecting plants from herbivorous damage was confirmed in transgenic against tobacco cutworm (*Spodoptera litura*; Yeh et al., 1997). In another study, the *SpTI-1* gene was expressed in Taiwan cauliflower (*Brassica oleracea* var. *botrytis* L.) cultivars, which resulted in transgenic plants showing resistance to *Spodoptera spp.* (Ding et al., 1998).

Because of the unusual action of talisin on the trypsin activity of *A. gemmatalis* larvae, we investigated the effect of talisin on endogenous proteinases of *A. gemmatalis* and on bovine trypsin. Fig. 5 shows clearly that talisin was capable of inhibiting the trypsin acitivity of *A. gemmatalis* in a manner proportional to the amount of talisin used (Fig. 5A). TLCK, a specific inhibitor of trypsin, confirmed the presence of this enzyme in midgut extract since this inhibitor reduced the trypsin activity by about 87% (Fig. 5B). The presence of trypsin and chymotrypsin (Fig. 4A and C) in *A. gemmatalis* agrees with literature reports showing that lepidopterans contain serine-type endopeptidases, particularly trypsin and chymotrypsin (Lam et al., 1999; Terra et al., 1996). The pH optimum of 8–9 for the trypsin activity of *A. gemmatalis* (Fig. 6) agreed with that for other noctuids (Marchetti et al., 1998).

The mechanism by which talisin inhibited trypsin activity was not examined here, but may have involved binding through hydrophobic and/or electrostatic interactions at sites distinct from the sugar binding site of talisin.

Since talisin was able to inhibit the trypsin activity of *A. gemmatalis* larvae, we examined the action of talisin as a proteinase inhibitor (PI). The ability of PIs to interfere with the growth and development of insects has been attributed to their capacity to bind to and inhibit the action of insect digestive proteinases (Carlini and Grossi-de-Sá, 2002). However, biochemical and molecular evidence indicates that some insects, such as *Spodoptera littoralis*, adapt to the presence of protease-inhibitors by overproducing existing digestive proteases (De Leo et al., 2001), while some, such as *Spodoptera frugiperda*, adapt to the presence of soybean proteinase inhibitor by altering the expression of trypsin and chymotrypsin activities (Paulillo et al., 2000; Macedo et al., 2010). Still others selectively induce inhibitor-insensitive proteases, as in the case of Colorado potato beetles, *Spodoptera exigua*, and other insect species (Jongsman et al., 1995). The trypsin activity in talisin-fed larvae was sensitive to talisin, as confirmed using BApNA as substrate (Fig. 5C) and by the detection of activity in gels (Fig. 7A and B). The finding that all of the trypsin activity in talisin-fed larvae was sensitive to talisin indicated that no novel proteolytic forms resistant to talisin were induced in larvae reared on a diet containing this lectin (Fig. 7).

In addition to binding to glyconjugates exposed on the surface of epithelial cells in the digestive tract, insectide proteins may also bind to digestive enzymes and to chitin in the peritrophic membranes

(Macedo et al., 2004, 2007). Affinity chromatography was used to examine the ability of talisin to bind to *A. gemmatalis* proteinases. As shown here, proteinases present in midgut homogenates of *A. gemmatalis* larvae bound partially to a Talisin-Sepharose column (Fig. 8). The trypsin-like activity was confirmed towards BApNa and zymogram.

A prerequisite for toxicity is that the lectin should be able to survive the hostile proteolytic environment of the insect midgut (Murdock and Shade, 2002). Macedo et al. (2004) showed that Talisin is resistant to hydrolysis by the digestive enzymes present in the midgut of *C. maculatus* larvae. Numerous insecticidal lectins, such as those of *Bauhinia monandra*, *Annona coriaceae*, *Datura stramonium*, *Solanum tuberosum*, *Griffonia simplicifolia*, are resistant to insect digestive enzymes (Zhu-Salzman and Salzman, 2001; Coelho et al., 2007; Macedo et al., 2002, 2007). The production of protease-resistant defensive proteins represents an effective strategy developed by some plants. The incubation of talisin with proteases of *A. gemmatalis* midgut homogenates resulted in only slight degradation of the lectin (Fig. 9), indicating that talisin is resistant to hydrolysis by *A. gemmatalis* enzymes.

The insect midgut often contains a noncellular semipermeable membrane, the peritrofic membrane (PM). Several functions have been attributed to the PM, including the prevention of non-specific binding to cell surfaces, the prevention of excretion by allowing enzyme recycling and the removal of oligomeric molecules from the PM (Terra, 2001). Freire et al. (2002) reported that talisin bound to a chitin column, suggesting that talisin can associate with the PM of insects.

In conclusion, the results reported here show that the insecticidal activity of talisin on *A. gemmatalis* may involve (1) the inhibition of trypsin-like proteases, (2) binding to digestive enzymes present in the midgut, (3) interaction with glycoconjugates on the surface of epithelial cells along the digestive tract, (4) binding to chitin components in the insect gut, and (5) resistance to enzymatic digestion by midgut larval proteinases.

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