Process Biochemistry 46 (2011) 498–504

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Evaluation of seed coagulant *Moringa oleifera* lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects

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ARTICLE INFO

Article history:
Received 13 July 2010
Received in revised form
26 September 2010
Accepted 28 September 2010

Keywords:
Lectin
Moringa oleifera
Anagasta kuehniella
Insecticidal activity
Bioinsecticidal
Nutritional parameters

ABSTRACT

Lectins have demonstrated significant levels of protection against different pests when expressed in transgenic plants. The effects of the coagulant *Moringa oleifera* lectin (cMoL) on moth flour (*Anagasta kuehniella*) were tested by incorporating the protein in an artificial diet at levels of 0.5%, 1% and 2% (w/w), respectively. cMoL showed a dose-dependent effect on average larval weight and a series of nutritional disturbances. A significant increase in total development time of 15 days was observed in the group fed with cMoL at 1%, increasing the rate of pupal mortality by 27.6%. The *A. kuehniella* midgut proteases were unable to digest cMoL for up to 12 h of incubation. The lectin presented a tight binding to a chitin column, suggesting that the insecticidal activity of cMoL involves carbohydrate–lectin interactions on the surface of the digestive tract, with glycoproteins and others glycolsylated structures in the midgut and resistance to enzymatic digestion.

1. Introduction

The steady increase in worldwide agricultural production and the creation of large crop areas has facilitated the spreading of insect pests that increase production costs, in addition to reducing farming productivity and causing losses in stored products. Problems associated with widespread insecticide usage, together with the development of insect resistance to *Bacillus thuringiensis* (Bt) toxins in genetically engineered crops, have resulted in a greater interest of scientists in exploiting the potential of using plant defensive proteins, such as lectins, to help in combating crop damage [1].

Plant seeds contain several biologically active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes, inhibitors, lectins and the ribosome inactivating proteins [2,3]. Plant lectins are defined as proteins possessing at least one non-catalytic domain, which binds reversibly to specific mono or oligosaccharides [4]. Indeed, many highly-abundant plant lectins have been found to combine a role in storage with a role in plant defense whenever the plant is under attack by predators [5]. Several lectins have shown effects during different life stages of many insect orders such as Coleoptera [6–8], Diptera [9], Hemiptera [10], Homoptera [11], Hymenoptera [12], Isoptera [13] and Lepidoptera [14–16]. This feature demonstrates the potential of using plant lectins as naturally occurring insecticide agents against the pests that diminish crop production [16]. Generally, *in vitro* bioassays are undertaken to judge this biological characteristic, which consists of inclusion of the studied lectin into artificial diets offered to the target insect during a given period of time [8].

Lectins are naturally occurring proteins/glycoproteins with substantial structural diversity [17]. Transgenic crops expressing genes that confer resistance against insect herbivory have been produced for most of the world's economically important crops [18] and several studies have shown the efficiency of lectin expression in transformed crops, such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), which is one of the most studied lectins. Genes that encode GNA have been incorporated into a range of crops, including potato [19,20], rice [21–23], maize [24], tobacco [25], wheat [26], tomato [27] and sugarcane [28–30]. In addition to GNA (mannose specifies), ConA (mannose/glucose specifies), PNA (galactose specifies), morniga-G (galactose/N-acetylgalactosamine specifies) and WGA (N-acetylgalactosamine specifies) are examples of other lectins that have also proven insecticidal activity and are widely studied. These observations support the search for new

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doi:10.1016/j.procbio.2010.09.025

lectins with insecticidal activity of different carbohydrate specificities, to act against the most distinct pest species.

cMoL (coagulant lectin from *Moringa oleifera*), belongs to a group of lectins isolated from *Moringa oleifera* seeds (Moringaceae family) [31,32]. This lectin is constituted of a monomeric protein with an approximate weight of 26.5 kDa, as revealed by SDS-PAGE and showed coagulant activity, similar to aluminium sulphate, the coagulant most widely used in water treatment [31]. Another lectin from *M. oleifera*, WSMoL (water-soluble *M. oleifera* lectin), presented larvicidal activity to *Aedes aegypti*, the vector of dengue [33]. The aim of this study was to evaluate the insecticidal activity of cMoL, as judged by its effect on the survival and growth of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae). This is a polyphagous pest that feeds on a wide variety of stored products, particularly in stored grains, such as dried cocoa beans, dried grains, nuts, tobacco, coconut and dried fruits [34]. *A. kuehniella* is of major economic importance as a flour and grain feeder; it is often a severe pest in flour mills [16,35].

2. Materials and methods

2.1. cMoL purification

Seeds of *M. oleifera* were collected in Recife city, northeastern Brazil and purified according to Santos et al. [31]. Seed flours of *M. oleifera* were extracted with 0.15 M NaCl for 6 h at room temperature. The proteins in this extract were precipitated using 0–60% ammonium sulphate fractionation for 4 h at room temperature. The 0-60F was dialyzed with distilled water (two changes) and 0.15 M NaCl, overnight. The 0-60F was chromatographed (10 mg of protein) on a guar gel column (10.0 cm × 1.0 cm), previously equilibrated with 0.15 M NaCl (20 ml/h flow rate). cMoL was eluted with 1.0 M NaCl.

2.2. Insects and effects of cMoL feeding on insect growth, survival and development

The eggs of flour moths (*A. kuehniella* (Zeller); Phycitinae, Pyralidae, Lepidoptera) were supplied by the Laboratório de Purificação de Proteínas e suas Funções Biológicas (LPPFB), Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil. The colony was housed in standard conditions of 28 ± 1 °C, 65–75% relative humidity and a 16:8 (light:dark) photoperiod and routinely maintained on an artificial diet prepared by mixing whole wheat flour, whole wheat husks, whole wheat, and yeast (8:2:1.9:0.1, w/w), according to Macedo et al. [16].

To examine the effects of cMoL on *A. kuehniella* development, neonate first instar larvae were selected and fed an artificial diet containing 0.5%, 1% and 2% of cMoL (w/w). Controls were fed with untreated diet. Each treatment was set up in 250-mg clear plastic, airtight containers and five larvae were transferred to each plastic container ($n = 60$). After the larvae reached the 4th instar at standard conditions, the relationship between protein content and the weight and number of larvae were determined. Larval consumption and faecal production were analyzed on a dry mass basis. The protein content and tryptic activity of the faecal and midgut samples were also determined.

Linear regression analysis was used to evaluate the response of *A. kuehniella* to the concentrations of cMoL. The treatment that provided the effective dose for a 50% response (ED_{50}), defined as the concentration of cMoL that reduced the larval mass by 50% compared to the control larvae, was utilized for further assays. The fresh weight of the resulting pupae and adults was determined on the first day following pupation and upon adult eclosion, respectively. The number of adults that emerged was counted in order to determine the percentage survival to adult emergence (S). The time at which the adults emerged was also recorded to allow estimation of the mean time of development (T). Howe's index [36] was calculated by dividing the log10 of the percentage survival to adult emergence ($\%S$) by the mean time of development (T).

2.3. Measurement of nutritional parameters

Several nutritional parameters were used to compare fourth instars fed on the control diet with those fed on a diet containing 1% cMoL. The larvae, faeces and remaining uneaten food were separated, dried and weighed. The indices of consumption, digestion and food utilization were calculated as described by Scriber and Slansky [37]:

The efficiency of the conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass, and was calculated as: [biomass gained (mg fresh mass)/food ingested (mg dry mass)] × 100.

The efficiency of the conversion of digested food (ECD) estimates the efficiency with which digested food is converted to biomass, and was calculated as: biomass gained (mg fresh mass)/[food ingested (mg dry mass) – feces (mg dry mass)] × 100. Approximate digestibility (AD) estimates the amount of ingested food that is digested, and was calculated as: [food ingested (mg dry mass) – feces (mg dry mass)]/food ingested (mg dry mass) × 100.

Metabolic cost (MC) was calculated as: 100 – ECD.

2.4. Midgut preparation

Homogenates of the larval guts were prepared according to Macedo et al. [38]. Fourth instar larvae were cold-immobilized and dissected in cold 150 mM NaCl. The midguts were surgically removed from the larvae using tweezers. The gut portion taken was posterior to the proventriculus and anterior to the malpighian tubules. After removing all extraneous tissue and freeing the lumen of its contents by rinsing in 150 mM NaCl, the midgut tissues were homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer immersed in ice. Midgut homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used immediately as enzymes sources for enzymatic assays or stored at −20 °C.

2.5. Faecal pellet preparations

Faeces were prepared according to Ramos et al. [34]. Faeces were collected and homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer. The homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used as a source of enzymes for enzymatic assays or stored at −20 °C.

2.6. Protein quantification

Protein concentrations were determined by the dye-binding method of Bradford [39], with bovine serum albumin (1 mg/mL) as the standard.

2.7. Enzymatic assays

Trypsin-like enzymes from gut extracts and faecal samples from *A. kuehniella* larvae were determined using the chromogenic substrate, N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), prepared in 1% (v/v) DMSO (dimethyl sulfoxide) and 100 mM Tris–HCl, pH 8.0 buffer. Samples of 10 μL of trypsin-like enzymes were added to 60 μL of assay buffer, and then 200 μL of BAPNA was added a final concentration of 1 mM. Six replicates were made for each assay. The reaction rate was determined by monitoring the absorbance change at 410 nm for 30 min in a VersaMax Microplate Reader (Molecular Devices, US).

2.8. Digestion of cMoL

The digestion of lectin was carried out according to Macedo et al. [16]. cMoL (2 mg/mL) was incubated with midgut homogenate in Tris–HCl 100 mM pH 8.0 buffer. The cMoL/midgut protein ratio was 1:10 (w/w). Digestion was performed for 1, 3, 6, 12, 24 and 48 h at 37 °C and was stopped by immersing the tubes in boiling water for 2 min. The degradation of bovine serum albumin was used as a positive control for protease activity. The proteins were subsequently separated by SDS-PAGE on 12.5% as described by Laemmli [40]. The proteins were detected by silver staining.

2.9. Protease activity of midgut and faecal extracts in polyacrylamide gels containing 0.1% gelatin

Proteins extracted from the midguts and faecal extracts of *A. kuehniella* larvae fed on control diet and diet containing 1% cMoL were run on 12.5% SDS-PAGE containing 0.1% gelatin [41]. The samples were incubated without prior boiling for 30 min at 37 °C. The samples had also been incubated with TLCK, a synthetic trypsin inhibitor (N-p-tosyl-lysine chloroketone, 1 mM) for 30 min at 37 °C to inhibit proteolytic activity prior to SDS-PAGE. Following electrophoresis at 4 °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 100 mM Tris–HCl buffer, pH 8.0, for 2 h. The gels were subsequently stained with Coomassie brilliant blue R-250. Bands of proteolytic activity appeared as clear (white) zones against a blue background.

2.10. Chitin-column chromatography

To examine the possible interaction of cMoL with chitin, cMoL was chromatographed on a chitin column (2 ml) and equilibrated with 150 mM NaCl. After adsorption of the protein (2 mg), the column was washed with 150 mM and 1 M NaCl, respectively. The bound cMoL was then eluted with 1 M acetic acid. The bound cMoL was then eluted with 0.1 M acetic acid. Fractions (2 ml) were collected, dialyzed against water and used in assays of hemagglutinating activity and protein quantification.