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ScienceDirect
Pesticide Biochemistry and Physiology 86 (2006) 23–29
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

Protein digestibility, protease activity, and post-embryonic development of the velvetbean caterpillar (*Anticarsia gemmatalis*) exposed to the trypsin-inhibitor benzamidine

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Received 19 August 2005; accepted 2 November 2005
Available online 28 February 2006

Abstract

Protein digestibility, proteolytic activity, and post-embryonic development of *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae) were assessed in larvae reared on artificial diet containing 0.00, 0.25, 0.50, and 0.75% (w/w) of the synthetic trypsin inhibitor benzamidine. Diet consumption was affected by the inhibitor when the insects were exposed to 0.50% benzamidine showing a 4-day delay and a 70%-higher peak of consumption. Larva weight gain was also affected by benzamidine and again the results of 0.50% benzamidine were unexpected due to the worst performance of the insects at this inhibitor concentration and not at 0.75% benzamidine. These patterns of consumption and weight gain were however consistent with the results of protein digestibility, which affects larvae mortality and adult emergence. The insect proteolytic activity was also affected by benzamidine, particularly at 0.50%. These results indicate that the insects are able to circumvent the potentially harmful effects of the inhibitor since at the highest concentration the negative impact is mitigated.

Keywords: Insect digestion; Serine proteases; Protease inhibition; Adaptation to protease inhibitors; Noctuidae

1. Introduction

Plants are generally well suited to defend themselves against insects [1]. Legumes for instance are particularly rich in nitrogen leading to a greater reliance on the production of a variety of nitrogen-rich defensive compounds [1–3]. The proteins arcelin and lectin, besides α-amylase and protease inhibitors are some of these compounds commonly found in legumes [3–5]. Earlier reports on plant proteins active against insect proteases sparked investigations of interactions between insect digestive proteases and plant protease inhibitors, with consequences for biotechnology and plant breeding [4,6–8].

Protease inhibitors can inhibit insect midgut proteases leading to death and such alternative control method may be exploited in two different ways [9,10]. First, by plant genome transformation for increased expression of potent protease inhibitors [7,8,11,12]. Second, by applying peptides or synthetic compounds, which are potent inhibitors of insect midgut proteases, like insecticides [4,10]. Benzamidine is an example of a synthetic protease inhibitor [13] that may have potential for use as insecticide and may be useful for deciphering the insect response to dietary protease inhibitors.

Serine proteases are the best-studied proteases and are present in viruses, prokaryotes and eukaryotes, suggesting their vital role for the survival of organisms. They are also the prevailing proteases reported in the majority of insect species, especially within Lepidoptera [14–16]. Among serine proteases, trypsins, and chymotrypsins are the most

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doi:10.1016/j.pestbp.2005.11.005

commonly reported enzymes acting in a wide range of physiological processes including digestion, protein activation in the melanization cascade, antibacterial activity, and insect immune response [16–18].

Trypsins preferentially cleave internal peptide bonds on the carboxyl side of the basic L-amino acids, like arginine and lysine. The digestive activity of trypsin-like enzymes was widely reported among the insect species studied and their specificity resembles vertebrate trypsins [15,19]. However, trypsins from both these groups may differ in optimum pH, molecular weight, ion sensibility, isoelectric point, and sensibility to plant protease inhibitors [19,20].

Inhibition of mammalian and insect digestive trypsins may differ indicating that the disruption of insect protein digestion when fed on diet containing protease inhibitors requires the prior selection of the appropriate inhibitors for the digestive proteases of the insect pest to be targeted [9,16]. In addition, insect adaptation to plant protease inhibitors has been reported in some insect species [21–25] what may compromise their potential as an alternative control method of insect pest species. The mechanisms by which such adaptation takes place are therefore of importance and began receiving recent attention [26–29].

Soybean (*Glicine max* (L.) Merrill) is a crop of worldwide importance, which has inducible serine protease inhibitors as defense mechanism against insect pests [30]. Despite this, the velvetbean caterpillar (*Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae)) remains as a key pest of soybean in warm climates [31]. Recent characterizations of digestive proteases of the velvetbean caterpillar indicated the prevalence of digestive trypsin-like enzymes in this species [9,10]. This fact makes this species a suitable model for studies assessing the potential of insect adaptation to protease inhibitors not only of natural occurrence, such as the soybean trypsin inhibitor (SBTI) and soybean Bowman–Birk inhibitor (SBBI), but also to synthetic trypsin inhibitors such as benzamidine.

Here, we report the response of the velvetbean caterpillar to increasing diet concentrations of benzamidine in terms of protein digestibility, protease activity, and post-embryonic development of this pest species. Benzamidine is expected to impair protein digestibility of caterpillars with negative consequences to the insect post-embryonic development. However, a soybean pest such as the velvetbean caterpillar should be able to somehow circumvent this problem what may compromise the potential use of trypsin inhibitors in its control.

2. Materials and methods

2.1. Insects and chemicals

The laboratory colony of *A. gemmatalis* was started from eggs obtained from the National Research Center of Soybean (CNP–Soja, EMBRAPA, Londrina, PR, Brazil). The insects were reared on the artificial diet [32] and maintained under controlled conditions of 25 ± 5 °C, 70 ± 10% r.h. and 14:10 (L:D) photoperiod. All reagents were purchased from Sigma–Aldrich Química Brasil (São Paulo, SP, Brazil), except agar, which was obtained from Isofar Ind. Com. (Jacaré, RJ, Brazil), and ascorbic acid and nipagin, which were obtained from Synth-LabSynth (São Paulo, SP, Brazil). Beans, yeast, wheat germ, soybean protein, and vitamins were obtained from the local market.

2.2. Exposure to benzamidine

The experiment was established in a randomized blocks design using four concentrations of the trypsin inhibitor benzamidine (0.00, 0.25, 0.50, and 0.75% w/w) mixed to the artificial diet during its preparation. Each replicate was constituted of 10 newly emerged caterpillars individualized in plastic containers at the same rearing conditions reported above. Five replicates were used in the study. Diet, containing benzamidine or not, was provided ad libitum to the insects according to each established treatment. The post-embryonic development of each insect was daily followed until the pupa stage. Diet consumption and feces production were also recorded in each developmental stage using an analytical balance.

2.3. Protein concentration and digestibility

Weight of diet consumed and feces produced per insect were used for determining protein concentration aiming to calculate the protein digestibility. Protein determination in the non-consumed diet and in the feces produced per insect was determined using the micro-Kjeldahl method to quantify total nitrogen [33]. Nitrogen from benzamidine does not interfere with the determination not only because of its low concentration in the diet, but also because this element constitutes a highly stable functional group in the synthetic inhibitor that is stabilized by resonance between its amino groups linked to the non-aromatic carbon [34], preventing its release during the Kjeldahl [34]. The material was initially dried (at 105 °C for 24 h), cooled, weighted, and grained before running the method. After the sample digestion, hydrogen peroxide (30%) was added and the mixture was heated for 30 min. After distillation, the ammonia released was recovered in a 4% boric acid solution and subsequently titrated with 0.01 N HCl. Nitrogen concentration was converted to protein concentration using the factor 6.25. The digestibility was calculated by the formulae: $\%\ \text{digestibility} = [(\text{ingested protein} - \text{excreted protein}) \times 100] / \text{ingested protein}$.

2.4. Preparation of midgut extracts

Larva from each developmental phase (i.e., from 1st, 2nd, 3rd, 4th, 5th, and 6th instars), maintained at each benzamidine concentration, were water rinsed and ice-chilled for dissection in 10^{-3} M HCl at 4 °C. The midguts extracted were used as enzyme source for determination of proteolytic and amidolytic activity after their removal from the