Peptides 34 (2012) 251–257

Contents lists available at ScienceDirect

Peptides

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

Growth-inhibition effects of pacifastin-like peptides on a pest insect: The desert locust, *Schistocerca gregaria*

Jornt Spit[1], Bert Breugelmans[*,1], Vincent van Hoef, Gert Simonet, Sven Zels, Jozef Vanden Broeck

Department of Animal Physiology and Neurobiology, Zoological Institute K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 28 January 2011
Received in revised form 21 June 2011
Accepted 21 June 2011
Available online 28 June 2011

Keywords:
Adaptation
Digestion
Enzyme
Feeding
Peptidase
Protease
Inhibitor

ABSTRACT

The main reason for the varying degrees of success of peptidase inhibitors (PI) as biological insecticides is the existence of a poorly understood mechanism, which allows pest insects to compensate for PI present in their diet. To challenge this highly flexible physiological mechanism and to prolong the inhibitory effect of PI on insect growth, a number of measures were taken into account before and during experiments with a notorious pest insect, the desert locust, *Schistocerca gregaria*: (i) **non-plant PI** (pacifastin-related inhibitors) were used to reduce the risk of a specific co-evolutionary adaptation of the pest insect, (ii) based on the main types of digestive enzymes present in the midgut, mixtures of **multiple PI** with different enzyme specificity were selected, allowing for a **maximal inhibition** of the proteolytic activity and (iii) digestive peptidase samples were taken during oral administration experiments to study compensatory mechanisms. Contrary to larvae fed on a diet containing plant-derived PI, a significant growth impediment was observed in larvae that were fed a mixture of different pacifastin-like PI. Nevertheless, the growth inhibition effect of this PI mixture attenuated after a few days, Moreover, a comprehensive study of the observed responses after oral administration of PI revealed that *S. gregaria* larvae can adjust their secreted digestive enzyme activities in two distinct ways depending on the composition/concentration of the PI-mixture.

1. Introduction

The significant increase in agricultural productivity during the recent decades has been realized to a great extent by the use of chemical insecticides. The negative side effects of these chemicals for mankind and environment on the one hand and the increase in resistant insect populations on the other hand, call for the development of alternative insect pest management strategies that are less harmful to the environment but can maintain this high productivity. The advances in the area of plant biotechnology and genetic engineering have led to the development and application of transgenic plants [21]. By incorporating one (or multiple) gene(s) into the plant genome, transgenic plants gain additional traits, such as tolerance against pest insects.

Although the introduction of delta endotoxin genes of the bacterium, *Bacillus thuringiensis* (Bt), in several economically important crops was very successful [1,24,31], studies under laboratory conditions have already shown the occurrence of resistance in certain insect populations [11,14,31]. Therefore, the study of potential alternative 'resistance' genes, such as peptidase inhibitors (PI), is of vital importance. Many plants express PI as part of their natural defense mechanism against phytophagous insects [13,19]. These inhibitors are present in most seeds and are induced in certain plant tissues when damage is inflicted by feeding [26,27]. Upon ingestion, PI are capable of inhibiting the proteolytic activity of gut peptidases in the digestive tract of insects. This may lead to an amino acid deficiency that, in turn, can result in a stunted growth or even in the death of the insect [13]. Polyphagous insects however, often have a higher tolerance to a wide range of plant PI [3,9,17], since a variety of adaptation mechanisms to evade these plant defense peptides have evolved: (i) mutations that make the gut peptidases less sensitive for plant PI without the loss of digestive proteolytic activity, (ii) peptidases that degrade the plant PI, and (iii) the ability to physiologically adjust the 'activity spectrum' of the gut peptidases by an elevated expression of PI sensitive or insensitive peptidases [8,13,18]. The transcriptional regulation of the expression of digestive peptidases in function of the diet

* Corresponding author. Tel.: +32 16 32 42 60; fax: +32 16 32 39 02.
E-mail addresses: Jornt.Spit@bio.kuleuven.be (J. Spit), Bert.Breugelmans@bio.kuleuven.be (B. Breugelmans), Vincent.vanHoef@bio.kuleuven.be (V. van Hoef), Gert.Simonet@bio.kuleuven.be (G. Simonet), Sven.Zels@bio.kuleuven.be (S. Zels), Jozef.VandenBroeck@bio.kuleuven.be (J.V. Broeck).
[1] Equally contributing authors.

0196-9781/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.06.019

allows the insect to compensate in a fast and flexible way for the PI-dependent loss of peptidase activity [9,18,28]. Many economically important crops have been genetically modified to express (additional) plant PI, resulting in a higher tolerance against different pest insects [15,16]. Moreover, the combination of Bt toxins with PI resulted in synergistic effects including a lower lethal Bt dose and delayed insect resistance [12]. However, in most cases, the inhibitory effects of PI on insect digestion were found to attenuate, or even disappear completely, after long-term exposure, because the digestive physiology of many insects is highly flexible [3,4,13,20]. These studies pointed out that the successful introduction of transgenic PI plants to the market depends on a well thought-out approach that focuses on the prevention of these physiological compensation mechanisms for PI in the diet. In practice, this means that, in contrast to most previous studies, different PI will have to be combined in such a way that the complete and possibly changing gut peptidase spectrum of insects remains inhibited. Some studies have already shown that combining different plant PI, or using PI with multiple inhibitory domains, can induce a higher growth inhibition/mortality as compared to individual PI [28].

Inspired by these observations and based on the strong inhibitory activity and specificity of pacifastin-like PI toward locust digestive serine peptidases [6], the insecticidal potency of members of a family of serine peptidase inhibitors, the pacifastin PI-family [5,29], was studied in detail and is reported in the presented manuscript. Contrary to plant PI, which are part of the host plant defense mechanism, pacifastin-like PI are not expressed by plants. In addition, pacifastin-like precursor genes appear to be expressed in many insect tissues, but there is no evidence for their expression in midgut [29]. Therefore, it is not very likely that specific co-evolutionary adaptations have already led to the selection of intestinal digestive peptidases which are insensitive to pacifastin-like PI. The effects of oral administration of pacifastin-like peptides were studied in the desert locust, *Schistocerca gregaria*, a notorious swarming insect that presents a threat to agriculture. These locusts are found from north-west Africa over the Mediterranean region and the Middle East to India and have become resistant to many chemical insecticides, thus demonstrating the need for alternative control strategies (*cf.* FAO website: http://www.fao.org/ag/locusts/en/info/info/index.html).

To facilitate the selection of potential PI that can inhibit the total gut peptidase activity, the overall peptidase 'profile' of *S. gregaria* midguts was studied. High amounts of pacifastin-like inhibitors were produced by means of a recombinant production system. Next, the individual and combined in vitro inhibitory potential of the produced PI on digestive enzymes was determined. Based on these studies, the most promising PI-combinations were selected for in vivo experiments. The growth inhibition effect generated by oral administration of pacifastin-like PI mixtures was compared to that of plant-derived PI. In addition, during these bio-assays, midgut samples were collected to study the PI-induced compensatory mechanism(s) in the polyphagous desert locust.

2. Materials and methods

2.1. Rearing of animals and enzyme sampling

Gregarious desert locusts, *S. gregaria* (Forskål), were reared under crowded conditions with controlled temperature (32 ± 1 °C), light (14 h photoperiod) and relative humidity (40–60%). Depending on the experimental conditions, locusts were developmentally synchronized at the time of ecdysis.

In order to obtain biologically active digestive peptidases, midguts of 5th instar larvae were dissected, cleaned and incubated separately during 1 h in Ringer's solution. Subsequently, the tissues were removed and the solution containing secreted enzymes was used for in vitro assays.

2.2. Recombinant production of locust derived pacifastin-like inhibitors

Pacifastin-related peptides from the desert locust (SGPI-1, SGPI-2, SGPI-4A and SGPI-4A-L30K) were successfully produced and purified via an expression system (pMAL Protein Fusion and Purification System, New England Biolabs) as described previously by Breugelmans et al. [6].

2.3. Peptidase activity and inhibitor assays

In vitro assays were performed to measure peptidase activity and/or inhibitory potency of PI. Azocasein, a general peptidase substrate was used, providing a measure to assess all peptidase activity regardless of the mechanistic class and or specificity ('total' peptidase activity). The optimal pH was determined by measuring the midgut enzyme activity at a range of different (5–10) pH values. Additionally, different PI concentrations (1–50 μM) were tested. For each azocasein assay, a solution, containing secreted midgut enzymes was pre-incubated with buffer (control) or PI (total volume 100 μl) for 10 min (32 °C). Then, 100 μl azocasein (1%, Sigma) was added and incubated at 32 °C (45 min). The reaction was terminated by addition of 75 μl of 10% trichloroacetic acid and cooled on ice (10 min). After centrifugation (16,000 × *g*, 10 min, 4 °C), 14 μl of 5 M NaOH was added to 90 μl of the supernatant and absorbance was measured at 405 nm (3 technical replicates). Enzyme activity is expressed as A_{405}/min/mg protein, or is calculated to percentage when comparing different inhibitory potencies. Inhibitory activity (in%) was calculated as defined below:

$$\text{Inhibitory activity}\,(\%) = 100 - \frac{\Delta \text{Abs}_{\text{PI}}/\,\text{min}}{\Delta \text{Abs}_{\text{Control}}/\,\text{min}} \times 100$$

For these experiments, peptidase inhibitors of enzymes of different mechanistic classes were used. In addition to the locust PI, the plant derived serine peptidase inhibitors, Soy Bean Trypsin-like Inhibitor (SBTI, Sigma) and Soy Bean Bowman–Burk inhibitor (SBBI, Sigma), a potato derived carboxypeptidase inhibitor (PCI, Sigma), a bovine inhibitor of cysteine peptidases (cystatin, Sigma), a chemical inhibitor of metallopeptidases PTMH (1,10-Phenanthrolin Monohydrate) and a chemical inhibitor of serine peptidases AEBSF [4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride, Sigma] were tested routinely. The final concentration of PI of biological origin was 25 μM. The concentrations of the chemical PI were 10 mM for AEBSF and 1 mM for PTMH.

2.4. Oral administration experiments

The most potent PI mixtures that were selected based on the in vitro assays and the individual midgut peptidase profiles, were tested in vivo on last instar larvae of *S. gregaria*. The effect of these PI on larval growth (bodyweight) was monitored over a period of 5–7 days.

Desert locust larvae were synchronized at the day of the fifth larval molt (Day 0). Then, each animal was numbered and individually weighted. Subsequently, three experimental conditions were formed by random selection ($n = 25$/group). Starting from day 2, insects were fed over a period of six days on a diet containing (i) plant derived PI (SBTI, SBBI), (ii) a combination of pacifastin-like