Inhibition of insect pest α-amylases by little and finger millet inhibitors

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Received 22 July 2005; accepted 14 November 2005
Available online 6 March 2006

Abstract

The inhibitory effect of three proteinaceous inhibitors isolated from little and finger millet was examined on gut α-amylases for four stored grain and four phytophagous insect-pests. Additionally, using native PAGE, several α-amylases isozymes were observed in all insect-pests studied. Furthermore, thermostabilities and the pH optimum for insect-pests α-amylases, which varied from acidic to alkaline, were also determined. On the other hand, proteinaceous inhibitors from little millet seeds Panicum sumatrense (LMCO3) and from finger millet (FMCO11 and FMCO13) inhibited insect-pests α-amylases with different proportions. The highest inhibition percent was recorded for LMCO3 and FMCO13 against Callosobruchus chinensis α-amylase, where the inhibition percent was approximately 70 and 50%, respectively. Furthermore, millet α-amylase inhibitors also reduced significantly digestive α-amylolytic activities of Acaea janata, C. cephalonica, Sitophilus oryzae, and Tribolium castaneum, indicating that these α-amylase inhibitors could be used toward crop insect-pests.

Keywords: α-Amylase inhibitor; Eleusine coracana; Panicum sumatrense; Phytophagous and storage pests

1. Introduction

Several starchy dependent insect-pests utilize α-amylases for carbohydrate metabolism, releasing mixture of oligosaccharides for energy production. Due to their importance, different forms of α-amylases can be found in a unique insect species, to guarantee the digestive process efficiency [1]. Otherwise, as these insects are totally dependent on α-amylases for their survival, these enzymes are good target candidates for bio-insecticides by using α-amylase inhibitors [2,3].

α-Amylase inhibitors are extensively found in many plant seeds and tubers, being particularly abundant in cereals and legumes [2–5]. These molecules play a key role in plant defense toward pests and pathogens [6], which cause severe damages to field crops and stored grains [2,7–10]. As these inhibitors could show different specificities against α-amylases from different sources [6], inhibitors with a wide specificity spectrum are strongly favored for insect control [2].

In recent years, α-amylase and proteinase inhibitors were available as genetic sources for production of insect-resistant transgenic crops [11]. Since large scale pesticide utilization caused deleterious effects to human health and environment [12], enzyme inhibitors could be an alternative strategy on control of phytophagous and storage seed insect-pests [5,11]. Several studies demonstrated the efficiency of proteinaceous inhibitors against digestive enzymes of important economic Lepidopteran [13] and Coleopteran pests [14], especially when utilized in plants genetic engineered [15–18]. Once seeds of little (Panicum sumatrense) and finger millet (Eleusine coracana) are known to be practically free from insect attack after long time storage, probably due the presence of some toxic and anti-metabolite chemical compounds [19], this report aims to identify, in both seeds, α-amylase inhibitors with insecticidal potentiality. On the other hand, α-amylases from various economically important insect species were also
characterized and their interaction with $\alpha$-amylase inhibitors was studied.

2. Material and methods

2.1. Extraction of millets $\alpha$-amylase inhibitors

$\alpha$-Amylase inhibitors from seeds of $P.~spp.$ were extracted according to Feng et al. [21]. The ground seeds (100 g each) were homogenized using pestle and mortar in 20 mM sodium phosphate buffer pH 7.5, followed by centrifugation at 10,000 g for 10 min at room temperature and supernatant was discarded. Pellet was resuspended in 20 mM sodium phosphate buffer pH 6.9, containing 300 mM NaCl, homogenized for 30 min, and centrifuged at 10,000 g for 20 min. Supernatant was incubated at 70 °C for 20 min to inactivate major endogenous enzymes and centrifuged at 10,000 g for 15 min. Pellet, containing the rich fraction of $\alpha$-amylase inhibitors, was dissolved in ice-cold 20 mM sodium phosphate buffer (pH 6.9) containing 300 mM NaCl and dialyzed overnight against same buffer.

2.2. Isolation of larval soluble fraction midgut homogenates

To analyze inhibitory activity of millet seeds against insect $\alpha$-amylases, pest midguts were surgically removed under cold in an iso-osmotic solution of 0.9% NaCl according to Franco et al. [6]. Midguts were homogenized using ice-cold pestle and mortar with 20 mM sodium phosphate buffer pH 7.2 containing 20 mM NaCl and 0.1 mM CaCl$_2$. The homogenate was centrifuged at 10,000 g for 20 min at 4 °C. The ammonium sulphate concentration of the supernatant was increased to 55%, kept for 12 h at 4 °C, and followed by centrifugation at 10,000 g for 10 min. The precipitate was dissolved with the homogenate buffer and dialyzed overnight against same buffer. This extract was lyophilized and stored at ~20 °C. Crude extract was used as enzyme source for further experiments.

2.3. Determination of optimum conditions for $\alpha$-amylase activity

Optimum conditions (pH and temperature) for different insect-pests $\alpha$-amylases were determined. For determination of pH activity curve, 50 mM sodium acetate buffers ($I = 0.05$; pH 3.5–3.5); 50 mM sodium phosphate buffers ($I = 0.05$; pH 6.5 and 7.5); 50 mM Tris–HCl buffer ($I = 0.05$; pH 8.5); 50 mM glycine–NaOH buffers ($I = 0.05$; pH 9.5 and 10.5), and 50 mM sodium carbonate buffer ($I = 0.05$; pH 11.5) [1] were used. Enzymes dissolved in each buffer were pre-incubated for 15 min at 30 °C, in the presence of 1% starch as substrate according to Bernfeld [20] for 20 min at 37 °C. Enzymatic reaction was stopped by adding 1.0 ml of 3.5 DNS$^1$ (1% dinitrosalicilic acid dissolved in 0.2 M NaOH and 30% sodium potassium tartarate) and evaluated by optical density at 530 nm. Subsequently, optimum temperature was also determined, pre-incubating $\alpha$-amylases in a wide range of 10–80 °C for 15 min, using optimum pH observed for each pest $\alpha$-amylase (50 mM sodium acetate buffer pH 4.5 for $C.~chinensis$, pH 5.5 for $S.~oryzae$ and $T.~castaneum$ and Tris–HCl buffer pH 9.0 for all lepidopteran insect $\alpha$-amylases). One unit of $\alpha$-amylase activity was defined as one μg of maltose liberated under the assay conditions. Each assay was carried out in triplicate.

2.4. Assays of $\alpha$-amylase inhibitors

The effect of $\alpha$-amylase inhibitors on insect-pest $\alpha$-amylases was examined according to Feng et al. [21] with minor modifications as described before. Aliquots of inhibitor sample containing 50 μg of protein were pre-incubated with 10 μg of insect-pest total gut protein (approximately 10 enzymatic units) for 60 min at 30 °C. Protein concentration of enzymes and enzyme inhibitors was determined according to Bradford [22], using bovine serum albumin as protein standard. After pre-incubation in 50 mM sodium phosphate buffer, pH 6.5, containing 10 mM CaCl$_2$, 5 mg soluble starch was added and incubated for 60 min at 30 °C. Enzymatic reaction was stopped by adding 1.0 ml of 3.5 DNS (1% dinitrosalicilic acid dissolved in 0.2 M NaOH and 30% sodium potassium tartarate) and, after boiling for 10 min, the absorbance was evaluated at 530 nm. Enzyme inhibitory activities were determined in triplicate by their reduction on enzyme activity units.

2.5. Zymograms

The inhibitory effect of prepared $\alpha$-amylase inhibitors on insect-pest $\alpha$-amylases was detected on native gel electrophoresis (PAGE) at 12% under non-denaturing conditions. Pre-incubated sample (10 μg of midgut crude extract + 50 μg $\alpha$-amylase inhibitors, incubated for 60 min at 30 °C) and the native gels were run under non-denaturing conditions in the presence of sodium dodecyl sulphate (SDS) and mercaptoethanol (ME) and stained with Coomassie blue.

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$^1$ Abbreviations used: 3,5 DNS, 3,5 dinitrosalicilic acid; FMCO11 finger millet CO11 crude extract; FMCO13, finger millet CO13 crude extract LMCO3, little millet CO3 crude extract; PAGE, polyacrylamide gel electrophoresis; RH, relative humidity.
trol, which consist in sample broth of LMCO3, FMCO11 and FMCO13, respectively.

Insect species

| Insect species | α-Amylasea | Specific activity (U/µg/min) | Optimum pH | α-Amylase + LMCO3 | Inhibition (%) | α-Amylase + FMCO10 | Inhibition (%) | α-Amylase + FMCO13 | Inhibition (%) |
|----------------|------------|----------------------------|------------|-----------------|---------------|-----------------|---------------|-----------------|---------------|
| S. litura      | 38.61      | 8.5                        | 8.03       | 22.58           | 23.05         | 34.85           | 19.61         | 33.38           | 33.38         |
| A. janata      | 14.43      | 8.5                        | 25.09      | 34.85           | 19.61         | 34.85           | 19.61         | 33.38           | 33.38         |
| P. xylostella  | 43.1       | 8.5                        | 30.81      | 39.35           | 11.74         | 39.35           | 11.74         | 33.38           | 33.38         |
| C. cephalonica | 39.87      | 5.5                        | 35.99      | 39.35           | 11.74         | 39.35           | 11.74         | 33.38           | 33.38         |
| S. oryzae      | 29.0       | 5.5                        | 21.41      | 31.69           | 39.07         | 31.69           | 39.07         | 33.38           | 33.38         |
| T. castaneum   | 71.2       | 5.5                        | 15.29      | 36.13           | 30.03         | 36.13           | 30.03         | 33.38           | 33.38         |
| H. armiger      | 41.03      | 8.5                        | 9.73       | 24.40           | NAb           | 24.40           | NAb           | 30.03           | NAb           |
| C. chinensis   | 59.85      | 4.5                        | 69.92      | 37.51           | 50.04         | 37.51           | 50.04         | 33.38           | 33.38         |

LMCO3 corresponds to little millet var. CO3 rich fraction, FMCO10 corresponds to finger millet var. CO10 and FMCO103 to finger millet var. CO13.

Each assay was carried in triplicate, do not differing more than 10%.

a One α-amylase unit (1 UI) was defined as the amount of this enzyme that increased the absorbance at 530 nm by 0.1 OD during 60 min of the assay.

b Not assayed.
insects were biochemically characterized, showing higher activity at pH 8.5. Alkaline pH optima for α-amylases have also been reported in other insect-pests such as Plodia interpunctella and Ephesia kuehniella [27]. In C. cephalonica larvae, the α-amylases showed a pH optimum of 10.5. Otherwise, Coleopteran insect α-amylases are well adapted to acidic physiological environment of larval midgut, with optimum pHs between 4.5 and 5.5. Acidic nature of digestive α-amylases from C. chinensis (pH 5.2–5.4) and T. castaneum (pH 4.6 to 5.2) was previously reported [28,29]. Furthermore, thermostabilities of α-amylases from representative Coleopteran and Lepidopteran insects were analyzed, showing a pronounced increased between 30 and 40 °C (data not shown). Similar results were also observed for α-amylases from Zabrotes subfasciatus, T. castaneum, and T. molitor, which showed higher activities at 37°C [30,31]. It is important to remember that physico-chemical midgut conditions might be crucial to modulate α-amylase/inhibitor specificity. Therefore, the inhibitory effect of α-amylase inhibitors from millets on insect α-amylases rich fractions was carried out. The results indicated that millet inhibitors could probably contribute especially to C. chinensis, S. oryzae and C. cephlonica control. In the present study, α-amylase inhibitors prepared from finger and little millet showed a potential as pest resistance factor against a variety of insect pests. Similarly, α-amylase inhibitors have been identified in cereal kernels [4,32,33] as well wheat [6,34], rye [35,36], and maize [37].

To determine the isoenzymes of α-amylase for each insect-pest as target/s for inhibiting by inhibitors, the zymograms of α-amylases in absence and presence of α-amylase inhibitors were carried out using starch as substrate in PAGE under non-denaturing conditions. Several insect-pests synthesize at least two α-amylases isozymes at digestive tract [33,37]. PAGE under non-denaturing conditions revealed that more than one isoform of α-amylase were detected in midgut crude extracts of the eight insect-pests examined (Fig. 1). Nevertheless, one single major form of α-amylase as observed for T. molitor [31] and Bombyx mori [38], while several isozymes were detected for Drosophila melanogaster [39], Sitophilus zeamais [1], Callosobruchus maculatus, Z. subfasciatus and Acanthoscelides obtectus [33,37]. Silva et al. [37] reported the presence of a large number of α-amylases isozymes is an efficient insect strategy to escape from inhibitor toxicity. α-Amalase inhibitors were not capable of inhibiting efficiently α-amylase isozymes completely as shown in Fig. 1. Furthermore, genomic sequence differences in α-amylase isozymes may lead to the production of a different isoform, which could originate insect resistance. As observed before, proteinaceous inhibitors that inhibit digestive α-amylases could induce the expression of insensitive α-amylases as observed for H. armigera guts treated with pigeon pea inhibitors [40].

Due to existence of more than one α-amylase isoform in a given insect species, the inhibitory specificity is an important initial step in discovery of molecules that could be useful for generating insect resistant transgenic plants [15]. The α-amylase diversity found in insect studied here indicates that unless an α-amylase inhibitor with reasonably broad specificity, capable of inhibiting all the α-amylase isozymes produced by target insect, their expression in transgenic plants would probably have no impact on starch digestion.
by insect-pests. So, the use of α-amylase inhibitors with different insect specificities could be combined to improve pest control [6]. The complexity of α-amylases expression in each insect species and their varying inhibition degree by millet compounds observed in this report might provide novel information that could contribute to pest management programs.

Acknowledgments

The authors are thankful to Dr. A.K. Fazullah Khan, Professor and Head, Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India, for providing seeds of finger and little millets. Dr. M. Mohan gratefully acknowledges the award of postdoctoral fellowship awarded to him by the Department of Biotechnology, Government of India. The authors are also thankful to Alinne Pereira de Castro for critical reading and for his help in the preparation of the manuscript.

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