Pesticide Biochemistry and Physiology 131 (2016) 46–52

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Insecticidal potential of an endophytic *Cladosporium velox* against *Spodoptera litura* mediated through inhibition of alpha glycosidases

Bahaderjeet Singh [a], Tamanreet Kaur [b], Sanehdeep Kaur [b], Rajesh K. Manhas [a], Amarjeet Kaur [a,*]

[a] Department of Microbiology, Guru Nanak Dev University, Amritsar 143005, India
[b] Department of Zoology, Guru Nanak Dev University, Amritsar 143005, India

ARTICLE INFO

Article history:
Received 30 July 2015
Received in revised form 18 January 2016
Accepted 18 January 2016
Available online 19 January 2016

Keywords:
Endophytic fungi
Cladosporium velox
Alpha glycosidase inhibition
Biocontrol
Chlorogenic acid
Spodoptera litura

ABSTRACT

Alpha glucosidase inhibitory activity was exhibited by partially purified fractions obtained from an endophytic *Cladosporium velox*, isolated from *Tinospora cordifolia*. Taking into account the increasing importance of digestive enzyme inhibitors as insecticidal agents, the entomopathogenic potential of the fractions obtained was evaluated against *Spodoptera litura* (Fab.), a polyphagous pest. Considerable mortality was obtained when the larvae were fed on diet supplemented with the partially purified extract. All the concentrations of the extract significantly prolonged the overall developmental period of *S. litura*. At higher concentrations, the extract influenced the longevity of females as well as their reproductive potential. Phytochemical analysis revealed the presence of phenolic compounds in the active fraction. The phenolic compound responsible for the bioactivities was purified and identified to be chlorogenic acid using HPLC and MS analysis. The content of chlorogenic acid in the extract was quantified to be 250 μg/ml. The purified compound also demonstrated inhibition of alpha glycosidases *in vivo*. The present study indicates that the endophyte imparted resistance to the insects in the plants could be mediated through chlorogenic acid targeting the alpha glycosidases present in the gut of the insect. The isolate obtained can be exploited for the production of chlorogenic acid, which has the potential to be exploited as a biocontrol agent against *S. litura*.

1. Introduction

One of the major threats encountered by agriculturalists is insect pests. Fifteen percent of the crops worldwide are currently lost to insects on an average every year. The threat of insects to agriculture is set to increase with global warming [1]. Usage of chemical pesticides has led to contamination of water and food sources, poisoning of non-target beneficial insects and development of insect populations resistant to the chemical insecticides [2,3]. The undesirable effect of chemical insecticides on the environment has increased the global public concern to look for alternative methods of insect control. Entomopathogenic microorganisms are an option that may contribute to reduce the use of chemical products in agriculture. An alternative strategy could be to take advantage of plants' own defense mechanisms. Endophytes are microorganisms that reside asymptomatically in the tissues of higher plants and are a promising source of novel organic natural metabolites exhibiting a variety of biological activities [4,5]. One of the major benefits which endophytes confer on the plants is providing protection against insect pests. Toxins produced by them have been shown to adversely affect the insect pests, inducing weight loss, retarding growth and development and leading to increase in pest death rate [6,7,8]. These adverse effects could be mediated through inhibition of the enzymes of the pests. Alpha-glucosidase is the enzyme responsible for the hydrolysis of dietary saccharides into absorbable monosaccharide in the digestive system. The inhibitors of this enzyme compete with the binding of oligosaccharides and prevent their cleavage to monosaccharides, thereby slowing the digestion process. In insects, alpha glucosidases are found in the alimentary canal, salivary secretions and hemolymph. Alpha glucosidase inhibitors (AGIs) produced by plants are a part of their natural defense mechanism [9,10]. It is possible that the biomolecules produced by endophytes could be acting as inhibitors of digestive enzymes of insects, thus providing protection to the plant. Although AGIs from plants have been reported for their insecticidal potential, there are no detailed studies on the production and effect of endophytic fungal AGIs on insects. A microbial source of a bioactive compound is easier and economically viable to produce. The biomolecules from these endophytes having enzyme inhibitory activity may prove to be an ideal component for the development of bioinsecticides which would help to reduce the load of insecticides on the environment.

An endophytic *Cladosporium velox* isolated from *Tinospora cordifolia* in our laboratory was found to exhibit AGI activity. The aim of this study

* Corresponding author.
E-mail addresses: sidhubahaderjit@yahoo.in (B. Singh), tamanreetkaur@gmail.com (T. Kaur), sanehsaini@gmail.com (S. Kaur), rkmanhas@rediffmail.com (R.K. Manhas), amarjeet_b@rediffmail.com (A. Kaur).

http://dx.doi.org/10.1016/j.pestbp.2016.01.004
0048-3575/

was to evaluate the effect of this extract on various growth and development parameters *viz.* larval mortality, percent pupation, adult emergence, fecundity *etc.* against the insect *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). *S. litura*, commonly known as tobacco caterpillar, is one of the most destructive polyphagous pest causing economic losses to many crops like cauliflower, cabbage, groundnut, maize, sunflower, and pulses and is shifting to new target crops. Moreover it has also developed resistance to most of the commercially available insecticides [11], necessitating the need for novel ecofriendly approach for its control.

2. Materials and methods

2.1. Production and partial purification of AGI

An endophytic *C. velox* (MTCC 12032) exhibiting alpha glucosidase inhibitory activity isolated from *T. cordifolia* in our laboratory was used in the present study [12]. Production of the inhibitor was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of liquid production medium (malt extract 20 g/l, dextrose 20 g/l, peptone 1 g/l) inoculated with one plug (8 mm diameter) taken from periphery of the actively growing culture. The flasks were incubated at 250 rpm on a rotary shaker at 30 °C for 10 days. Thereafter, the culture broth along with the fungal biomass was extracted with 50 ml of ethyl acetate under shaking conditions at 120 rpm and 40 °C for 2 h. The upper organic phase thus obtained was separated and concentrated on rotary evaporator (BUCHI). The concentrated samples were then re-suspended in 1 ml of HPLC grade water for further studies. The concentrated ethyl acetate extract was loaded onto a silica gel (60–120 mesh size) column (2 × 25.0 cm). The column was eluted with 270 ml of chloroform: methanol: formaldehyde (8.5:1:0.). Activity was observed in fraction no. 16 [12]. Subsequently, the same column was subjected to elution with ethyl acetate: methanol (60:40) and fraction nos. 22–25 (15 ml each) exhibiting alpha glucosidase inhibitory activity and showing similar TLC profile were pooled and selected for further studies.

2.2. Inhibition assay for alpha glucosidase

Alpha glucosidase inhibitory activity was determined by modified method as given by Bachhawat et al. [13]. In a 96-well plate reader, a reaction mixture containing 50 μl of phosphate buffer (50 mM; pH 6.8), 10 μl of alpha-glucosidase from *Saccharomyces* sp. (1 U/ml) and 20 μl of partially purified fungal extract was pre-incubated for 5 min at 37 °C, and then 20 μl of 2 mM para-nitrophenyl-alpha-D-glucopyranoside (pNPG) (prepared in 50 mM phosphate buffer pH 6.8) was added to the mixture as a substrate. After further incubation at 37 °C for 30 min, the reaction was stopped by addition of 50 μl of sodium carbonate (0.1 M). The yellow color produced (due to *p*-nitrophenol formation) was quantitated by colorimetric analysis and reading the absorbance at 405 nm. Controls were conducted in an identical manner replacing the fungal extracts with water. Acarbose (a commercially used AGI) was used as a positive control. The % inhibition was obtained using the formula:

$$\%\text{inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.3. Evaluation of insecticidal activity

2.3.1. Insect rearing

Different larval stages of *S. litura* were collected from the cauliflower fields around Amritsar (Punjab), India, and their subsequent generations were reared in the laboratory under controlled temperature of 25 ± 2 °C and 65 ± 5% relative humidity (RH). The larvae were reared on castor leaves which were changed daily. Glass cylindrical containers (15 × 10 cm) were used for insect rearing. The pupae were transferred to pupation jars containing a 2–3 cm layer of moist sterilized sand covered with filter paper. Just after emergence, the adults were shifted to oviposition jars similar to pupation jars. The adults were provided with water and honey solution (4:1 v/v) as food, soaked on cotton swab hanging from the muslin cloth covering the jar. To facilitate egg laying the oviposition jars were lined with filter paper. For experimental studies the newly hatched larvae were transferred to artificial diet which was prepared as recommended by Koul et al. [14] with slight modifications.

2.3.2. Bioassay studies

The artificial diet was supplemented with five concentrations (0, 100, 200, 300, 400, 500 μg/ml) of partially purified extract in order to evaluate the effect on survival, growth and development of *S. litura*. Second instar larvae were reared on amended and unamended diets at 25 ± 2 °C and 65 ± 5% R.H., respectively. Each experiment was replicated five times with six larvae per replication (n = 30). To avoid cannibalism the larvae were kept individually in plastic containers (4 × 6 cm) and diet was refreshed regularly after two days. The observations on larval and pupal mortality, larval and pupal development period, adult emergence, adult longevity, fecundity and egg viability were recorded daily. Additionally, sublethal effects like larval, pupal and adult deformities were also recorded.

2.4. Characterization of the active compound

2.4.1. Phytochemical tests for functional group identification

The partially purified extract was subjected to TLC using chloroform: methanol: formic acid (8.5:1:0.5) as the solvent system. Phytochemical analysis was performed using identifying visualization reagents *i.e.* Dragendroff's reagent for alkaloids, $FeCl_3$ and fast blue B for phenols, ninhydrin for amine group, p-anisaldehyde for the detection of steroids and terpenoids to determine the nature of the inhibitor [15].

2.4.2. Determination of total phenolic content (TPC)

TPC of the extract was determined using Folin–Ciocalteu assay as described by Mayur et al. [16] with minor modifications. 10 μl of extract and 100 μl Folin–Ciocalteu reagent were incubated for 5 min followed by addition of 80 μl of 7.5% sodium carbonate solution and mixed well. This was kept in dark at room temperature for 30 min. The absorbance was measured at 750 nm on ELISA plate reader. TPC was expressed as gallic acid equivalent (GAE).

2.4.3. Purification and identification of active component

HPLC based purification of active compound was carried out on semi preparative HPLC (Shimadzu). The partially purified extract was chromatographed on Microsorb MV C18 (100 × 10 mm ID, 10 μm) column (enable, USA), with a mobile phase of 5 mM potassium dihydrogenphosphate solution (pH 2.5)–acetonitrile (41:9) at a flow-rate of 3.0 ml/min with detection wavelength at 319 nm [17]. Three peaks were obtained and collected manually in separate test tubes. AGI activity was observed in fraction no. 2 which was identified to be chlorogenic acid on comparison with standard compounds.

Mass spectrum of compound obtained was recorded in ACN–H_2O solution on Bruker microTOF QII mass spectrometer in +ve ESI mode at source temperature, 180 °C; dry gas N_2 was used with flow of 4 l/min; pressure of the nebulizer was 0.4 Bar, and the species formed were identified from their respective m/z. The mass spectrum was acquired with scan range of m/z from 100 to 1000.

Further confirmation for chlorogenic acid was made by using starch–iodine inhibitory activity assay of the purified inhibitor according to Xiao et al. [18] with minor modifications. The total assay mixture comprising of 40 μl of 0.02 M sodium phosphate buffer (pH 6.9) solution and purified inhibitor (20 μl) was incubated at 37 °C for 10 min. Soluble starch (1%, w/v) was added to each reaction well