ISSN Online: 2349-7785

Evaluation of Cuticle Degrading Collagenase of *Pseudomonas* sp. as Biocontrol Agent Against Nematodes

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Received 10 September 2014; accepted in revised form 14 October 2014

Abstract: The ability to digest native collagen makes bacterial collagenases perfect for the degradation of the extracellular matrices of animal cells. These enzymes act as important virulence factors in a variety of pathological processes. Collagens being the most abundant proteins in all higher organisms, a diverse spectrum of therapeutic and biotechnological applications exists for bacterial collagenases. An extracellular collagenolytic enzyme isolated from *Pseudomonas* sp. has found to be effective in degrading cuticular proteins of nematodes. The present study demonstrates the digestion of intact cuticles of fish nematode and plant rootknot nematode *Meloidogyne javanica,* by *Pseudomonas* sp. collagenase. The degradation of cuticular proteins could prove to be an attractive way in controlling pre- and post- parasitic forms of nematodes in the near future.

Key words: Collagen, Collagenase, Pathological processes, *Pseudomonas* sp., Rootknot nematode, Cuticular protein, *Meloidogyne javanica.*

Introduction

Collagens are the main structural proteins responsible for the structural integrity of vertebrates and many other multicellular organisms [23,25]. The fibrillar collagens degraded *in vivo* by enzymes known as collagenases, which belongs to the larger matrix metalloprotenases family [2,18]. Collagenase present in the intercellular spaces, acts upon the collagen and contributes to degradation and liquefaction of the host's tissues in particular conditions [8]. Due to the ability to digest native collagen, bacterial collagenases are involved in the degradation of the extracellular matrices of animal cells and serve as important virulence factors in a variety of pathogenic bacteria [6,21,27].

Collagenases have been isolated and characterized from microbial cells and animal tissues and Clostridial collagenases were the first to be identified and characterized and are the reference enzymes for comparison of newly discovered collagenolytic enzymes [6].

Collagenase from predatory bug *Podisus nigrispinus* (Hemiptera: Pentatomidae) leads to the disintegration of the prey's internal organs by acting on peptide bonds of collagen and basement membrane proteins [11]. In addition, the cuticles degradation of nematodes *Caenorhabditis elegans* [5] and *Panagrellus silusiae* [16] and animal parasitic Ascaris species [28], by bacteria has been attempted. A multilayered cuticle covers the entire surface of these parasitic nematodes and major structural components of this cuticle layer are collagens [3]. Hence, cuticle degradation could be an effective way of controlling pre-parasitic and post-parasitic forms of nematodes [12].

Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests [15,26]. The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil

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and usually attack the underground parts of the plants [24]. The chemical use of nematicides leads to the release of toxic substances, the build-up of soil antagonists to parasitic nematodes and the lethal effect of nitrogen build up in soil like as amomonia [1,7,22]. The search for novel, environmentally friendly alternatives to manage plant-parasitic nematode populations has therefore become increasingly important. This report describes the nematocidal effect of collagenase isolated from *Pseudomonas* sp.; which found to be one of the most dominant microorganisms in the soil enriched with protenaceous waste.

Materials and methods

Animal nematode

Fish nematode *Procamallanus*, (extracted from Eel *Mastacembelus armatus*) used to see the effect of collagenase on degradation of cuticle proteins of animal nematodes.

Plant parasitic nematodes

Root-knot nematode (*M. javonica*) is a major plant-parasitic nematode species affecting the tomato crop production, used to evaluate the nematocidal effect of collagenase.

Microorganism

The *Pseudomonas* sp. used in this study was isolated from sewage samples of meat market of Bilaspur, Himachal Pradesh, India. The serially diluted samples were spreaded on selective medium (pH 7.5) and selection of hyperproducer was done on the basis of gelatine hydrolysing activity of isolates [29]. The culture was maintained on the medium containing (%, w/v) NaCl 0.01; Peptone 0.05; KH_2PO_4 0.05; $MgSO_4.7H_20$ 0.02; Gelatin 2.0 and Agar 1.5 at pH 7.5. One colony was transferred to 50ml flask containing (%, w/v) sucrose 1.0; tryptone 1.0; yeast extract 0.25; meat extract 0.2; and gelatin 0.3, medium (pH 7.0) and grown aerobically on a rotating shaker (150 rpm), overnight at 37°C. The crude enzyme was obtained after centrifugation of 14h old fermentation broth at 15,000 g for 15 min at 4ºC and the supernatant (2.52 U/mL) was purified by ammonium sulphate precipitation and Octyl Sepharose column chromatography.

Isolation of collagens from nematode cuticle

Collagenic proteins were isolated from cuticles of fish nematode (*Procamallanus*, extracted from Eel *Mastacembelus armatus*) as described by Reddigari *et. al*., [20] with some modifications. Nematode suspension of 1mL was sonicated (59 sec pulses for 30 min) to obtain intact cuticle fragments. These fragments were pelleted and resuspended in two volumes of SDS Tris-PMSF buffer, pH 6.8, consisting of 17 mM SDS, 50 mM Tris-HCl, pH 7.4 consisting of 5 mM $CaCl_2.2H_2O$ and 1 mM PMSF (a protease inhibitor). The Tris buffer contained 10% (w/v) β-mercaptoethanol. The fragments boiled in a boiling water bath at 100ºC for 15 min and held overnight at room temperature. The β-mercaptoethanol soluble proteins in the supernatant precipitated with nine volumes of cold acetone, stored at 4ºC overnight for precipitation. The pellet, made up of β-mercaptoethanol free collagens, resuspended in Tris-HCl buffer and stored at 4ºC.

Digestion of nematode cuticle by collagenase

β-mercaptoethanol soluble proteins (1 mg/mL) incubated with 0.165 U/mg of commercial collagenase from *C. histolyticum* as well as with purified enzyme obtained from *Pseudomonas* sp. The reaction mixture incubated for 1-5 h at 37ºC. As a control, the collagenases were incubated under identical conditions and 1.5 mg/mL commercial collagen (positive control) from bovine achilles tendon (Sigma) was used. To end the reaction, the mixture added to a sample buffer, heated for 5 min at 100ºC, separated by 10 % SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

Effect of *Pseudomonas* sp. collagenase on female of *Meloidogyne javanica*

Root-knot nematode, *M. javonica* is a major plant-parasitic nematode species affecting the quantity and quality of the tomato crop production. Infected plants show typical symptoms including root galling, stunting and nutrient deficiency, particularly nitrogen deficiency. The tested nematode species, *M. javonica* harvested according to the method as described by Barker, [2] with some modifications. The roots of about 3 months

old tomato plants, infected with the nematode washed in distilled water. The roots cut into 1-2 cm pieces and soaked in the water overnight and nematode were isolated from the moistened roots by vigoursly shaking. These, isolated nematodes were treated with purified collagenase of *Pseudomonas* sp. (0.165 U/mg) for 48 h at 37°C and the effect of enzyme on cuticle disintegration was observed microscopically.

Results

Digestion of nematode cuticle by collagenase

β-mercaptoethanol soluble proteins obtained from nematode cuticles (1mg/mL) incubated with commercial collagenase from *C. histolyticum* (0.165 mg/mL) and with purified enzyme of *Pseudomonas* sp. (0.165 mg/mL). The collagenases were incubated under identical conditions as for control and 2 mg/mL commercial collagen form (Sigma) was used as positive control. The *Pseudomonas* sp. collagenase exhibited some degree of specificity. Lane 2 of Figure 1a, showed the pattern of hydrolysed commercial collagen form (Sigma) and β-mercaptoethanol soluble proteins obtained from nematode cuticles run in lane 4 of Figure 1c. The purified enzyme of *Pseudomonas* sp. run in lane 2, 3 in Figure 1b respectively, and lane 1 of Figure 1c showed the pattern of commercial collagenase from *C. histolyticum* on SDS-PAGE. The middle range molecular weight SDS-protein marker was used for molecular mass analysis of collagenase of *Pseudomonas* sp. The molecular weight of the protein marker ranged from 11 kDa to 245 kDa (Figure 1b, lane 5). Although the collagenase production by *Pseudomonas* sp. was carried out on gelatin containing medium, it could digest hydrolysed collagens from Bovine Achilles tendon (Figure 1a; Lane 1, and Figure 1b; Lane 1) after 1h and 5h, respectively as well as the extracted from fish nematodes. However, *Pseudomonas* sp. collagenase was not able to digest native form of collagen as it require relatively more time and higher temperature for solublization. The cuticular proteins isolated from fish nematode (*Procamallanus*) extracted from Eel, were digested by purified collagenase from *Pseudomonas* sp. and *C. histolyticum* collagenase (Figure 1c, lane 3, 4,

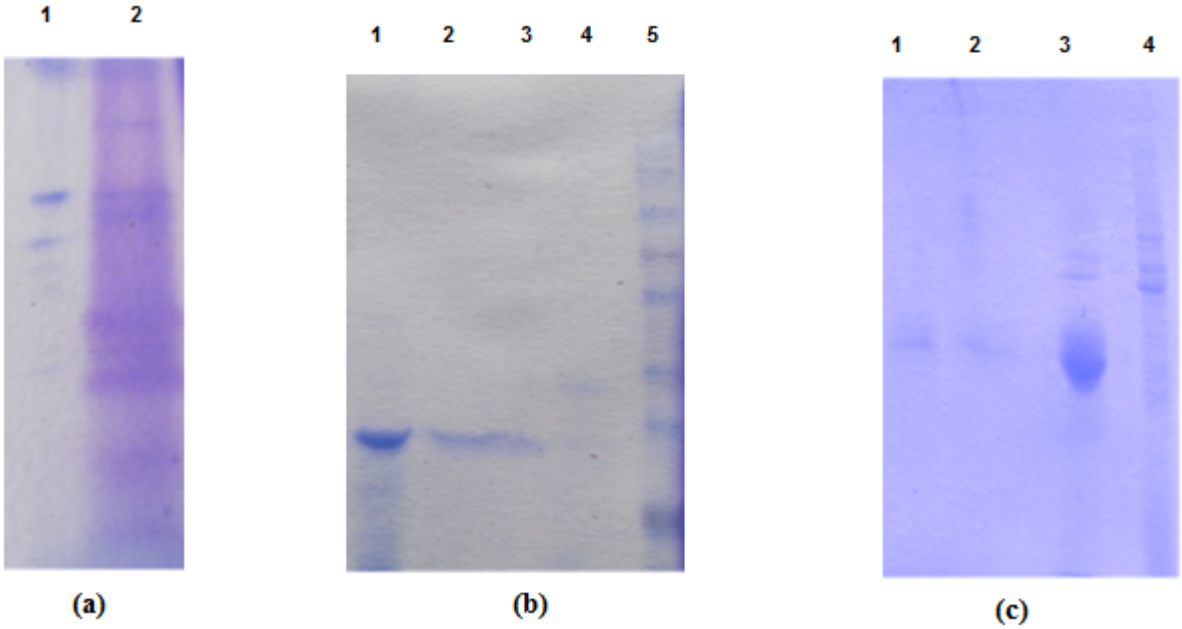

Figure 1. SDS-PAGE profile for the digestion of hydrolysed collagen and β-mercaptoethanol soluble collagenic proteins extraxted from fish nematode (*Procamallanus*, extracted from Eel), (a) Digestion of hydrolysed collagen after 1 h of treatment with enzyme (b) Digestion hydrolysed collagen of after 5 h of treatment with enzyme (c) Digestion of soluble β-mercaptoethanol extracted proteins from the cuticle of fish nematode

respectively), suggesting that these proteins are collagenic. The digestion of hydrolysed collagen and collagenic proteins extracted from fish nematodes by *Pseudomonas* sp. collagenase within 5h at 37°C, revealed its strong proteolytic nature. This explains why the collagens of nematode origin, only partially digested by *C. histolyticum* after 5 h (Figure 1c, lane 3), whereas the collagenase from *Pseudomonas* sp. completed their digestion within 5 h (Figure 1c, lane 2).

Effect of *Pseudomonas* sp. collagenase on the female of *Meloidogyne javanica*

The nematode isolated from the infected tomato plant, treated with purified collagenase as well as heat inactivated *Pseudomonas* sp. collagenase and observed under compound microscope for 24-48 h respectively (Figure 2). It was observed from the microscopic study that the cuticle layer of nematode partially digested by *Pseudomonas* sp. collagenase after 24th hour of treatment (Figure 2e) and there was no effect seen on the nema-todes treated with heat inactivated *Pseudomonas* sp. collagenase even after the same incubation time given for the treatment (Figure 2f). The complete digestion of nematode body was seen after 48th hour of treatment with *Pseudomonas* sp. collagenase (Figure 2g) and heat inactivated enzyme did not show any effect on the nematode even after 48 h of treatment period (Figure 2h).

Discussion

Collagenases are the enzymes which bring about the degradation of collagen into small peptides, which is one of the extremely hard structural matrix proteins to degrade [14]. The major structural components of nematode's cuticles reported to be collagens [3]. Moreover, the information on rootknot nematode cuticular collagens is scarce but it reported that the rootknot nematode cuticle is composed of several collagenic/proteinaceous layers [13,19]. Polyclonal antiserum rose against the major 76-kDa collagen protein extracted from cuticles of *M. javonica* adult females and used in immunogold electron microscopy to localize collagen in the cuticles of different life stages [19]. As the collagens are the major component of rootknot nematode body, its disruption may affect the nematode cuticle, resulting in disruption of the nematode's life cycle. Thus, the *Pseudomonas* sp. collagenolytic enzyme may prove to be an efficient agent for attacking and disrupting this nematode's cuticular collagens in both pre and postparasitic forms [10].

The present research describes the effectiveness of the collagenase enzyme isolated from *Pseudomonas* sp. in degradation of cuticle proteins extracted from the fish nematode and digestion of the collagenic membranes of the female of plant rootknot nematode *M. javanica.* The role of collagenolytic activity on animal (Fig. 1) and plant parasitic nematodes (Fig. 2) evaluated by applying collagenase of *Pseudomonas* sp., respectively. The treatment of soil by collagenolytic microorganisms drastically reduced the number of galls caused by *M. javanica* on tomato roots [9]. Collagenases and proteases reduced the motility of a *Tylenchorhynchus dubius* population up to 75 %, when added directly to the soil. Moreover, they were also effective against *Pratylenchus penetrans*. The root galling caused by *M. javanica* further decreased when the collagen-amended soil supplemented with a new isolate of the collagenolytic and elastolytic fungus *Cunninghamella elegans*. Culture filtrate of this fungus immobilized *M. javanica* second stage juveniles, inhibited egg hatch and substantially reduced the motility of *Rotylenchulus reniformis* and *Xiphinema index* [10].

Conclusion

The present research describes the effectiveness of the collagenase enzyme isolated from *Pseudomonas* sp. in degradation of cuticle proteins extracted from the fish nematode and digestion of the collagenic membranes of the plant rootknot nematode *M. javanica*.

Conflict of interest

The authors have declared no conflict of interest.

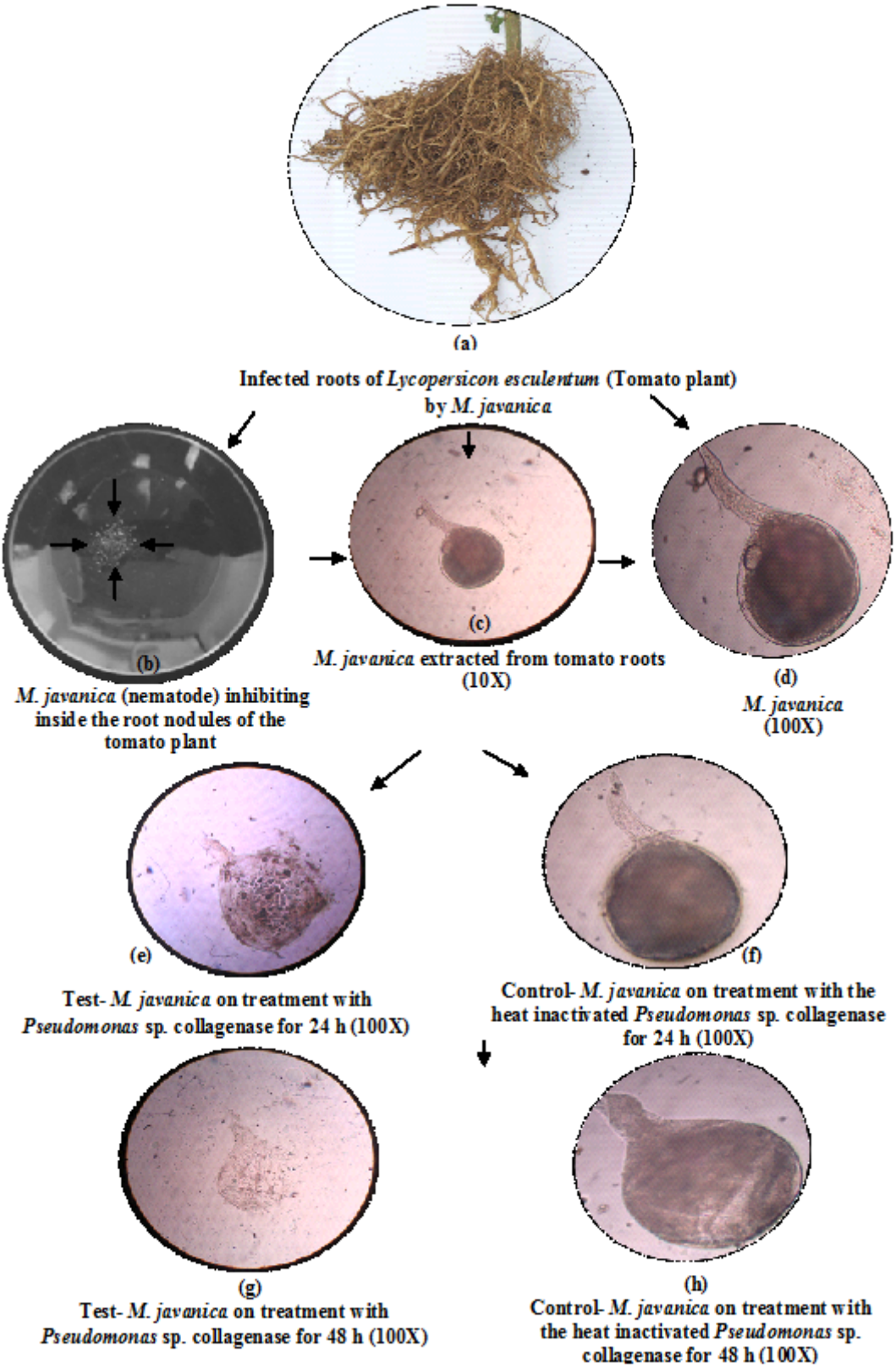

Figure 2. Effect of *Pseudomonas* sp. collagenase on the female of *M. javanica* extracted from the roots of *Lycopersicon esculentum* (Tomato plant)

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