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Full Length Research Paper

The role of pectinase enzyme in the development of soft rot caused by *Pseudomonas fluorescens* in the purple variety of onions (*Allium cepa*)

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Pectic enzyme production was induced *in vitro* by culturing the pathogen (*P flourescens*) in minimal salt medium containing citrus pectin as the only carbon source and *in vivo* by inoculating the pathogen into healthy onion bulbs. The spent broth and onion rot extract were obtained and enzyme detection was done using viscometric method and cup plate assay. The crude enzyme was partially purified by precipitating with ammonium sulphate and dialyzed against water at 4^{o}C for 24 h before use. The activity of the purified enzyme was determined based on the macerating ability on Onion and Potato discs and later by estimation of reducing sugars (galacturonic acid) level. The type of cleavage reaction of the enzyme was determined using the thiobarbituric acid reaction and the presence of antimicrobial substance in the rot tissue was also investigated. The pathogen produced a hydrolytic polygalacturonase *in vitro* and *in vivo*. The purified enzyme showed appreciable activity with all the assay methods used. Activity was highest at pH 5 in the culture filtrate sample and at pH 4 in the rot extract. Maceration was observed in potato and onion discs. The detection of increasing concentration of polyphenols in the rot tissue during rot development demonstrated the plant's response to the pathogen.

Key words: Onion, maceration, pectinase, polyphenol and rot.

INTRODUCTION

A characteristic feature of many phytopathogenic organisms is their ability to produce an array of enzymes capable of degrading the complex polysaccharides of the plant cell wall and membrane constituents. These enzymes are usually produced inductively and are extracellular, highly stable and present in infected host tissue (Arinze, 1978). The most widely studied of these enzymes are those that can degrade pectic substances and cellulose (Arinze, 1978). The cementing substances in vegetables induce the formation of pectinase which hydrolyze pectin to produce a mushy consistency (Jay, 2005).

The involvement of pectic enzymes in the degradation of pectic constituents of cell walls and of the middle lamella of plant tissues has been reported for diverse types of diseases such as soft rot, dry rot, wilts, blights and leaf spots which are caused by pathogenic agents such as fungi, bacteria and nematodes (Bateman and Millar, 1966). Pectic enzyme activities of bacteria have been reported during rot development in onions (Obi and Umezurike, 1981). In most cases, pectic substances are the main inducers in culture media and they can be differentiated based on the mechanism by which the 0 - 1.4 glycosidic bond is split, i.e. hydrolytic or trans-eliminative cleavage (Bateman, 1966). The activity of the type of induced enzyme may be influenced by environmental factors.

It has been found that *Botrytis cinerea* was capable of producing different 0 – 1.4 pectic-degrading enzymes *in vitro* depending on the pH of the culture medium (Damle, 1952).

The production of pectic enzyme *in vivo* is usually proven by the removal of infected tissue from the sick plant, purification of the crude enzyme and determination of its ability to reproduce disease symptoms in a healthy plant.

Evidence of pectinase activity can sometimes be obtained microscopically as the invaded cells will be seen to separate along the line of the middle lamella and subsequent loss of their staining ability (Woods, 1972). Physically, this manifests as tissue maceration as injury and death of unplasmolysed cells would have occurred.

Bashan (1974) found that polygalacturonase lyase from Erwinia chyrsanthemi, purified to homogeneity, caused cell death and maceration in disks of potato tissue.

Plants however, resist invasion of pathogenic agents

through different mechanisms. One of these is the production of antimicrobial substances, usually phenolic compounds. There is evidence that phytoalexins are produced during soft rot development of sweet potato caused by *Botryodiplodu theobromae* and other fungi (Arinze, 1978). In this study, the ability of *Pseudomonas fluorescens*, a plant pathogen implicated in the soft rot of onion bulbs to produce pectic enzymes *in vitro* and *in vivo* was investigated.

MATERIALS AND METHODS

Preparation of crude enzyme sample *in vitro*

The method of Arinze (1978) was used. Briefly, *P. flourescens* (10^9 cfu/ml) was inoculated into conical flasks that contained minimal salt medium of the following composition: $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.2 g; NaCl, 5.0 g; $Ca(NO_3)_2.2H_2O$, 0.2 g; $FeSO_4$, 0.01 g; K_2PO_4, 5.5 g; KH_2PO_4, 0.5 g; 2% citrus pectin and 1000 ml distilled water. The flasks were incubated in a rotary shaker (60 rpm) at 25°C for 7 days. The cultures were then filtered through a millipore filter (pore size of 45 µm) and the filtrate was dialysed against distilled water at 4°C for 24 h using a visking cellulose tube, before being stored at -20°C.

Preparation of crude enzyme sample *in vivo*

Healthy onion bulbs were surface sterilized according to the method of Oguntuyo (1981). Discs (0.5 cm in diameter) were cut on the first leave base of the bulb using a sterile cork borer. *P. fluorescens* (10^9 cfu/ml) was introduced into the cavities using a sterile disposable syringe. The bulbs were placed in sterile plastic jars and incubated at room temperature for 7 days. The rot extract was obtained by removing the rot tissue and mixing it with 0.1 M phosphate buffer (pH 7), containing 0.2 M NaCl and 0.001 M dithiothreitol in a ratio of 1:10 (tissue: buffer). NaCl was used to extract the protein in the tissue and dithiothreitol to prevent oxidation by oxidative enzymes present in the tissue (Arinze, 1978). The tissue was homogenized and filtered under vacuum. The sample was then dialyzed and stored as described above.

Enzyme purification

To purify the enzyme, ammonium sulphate was added to the previously prepared *in vitro* and *in vivo* enzyme samples to obtain saturation levels of 40, 60, 80, 90 and 100% (Green and Hughes, 1955). Addition of salt was carried out at 25°C in a water bath. The precipitate obtained at each saturation level was removed after allowing it to stand for 30 min followed by centrifugation at 10 000 g for 30 min at 4°C. The supernatant was used for the next higher saturation level. The precipitate at each level was dissolved in a small volume of water and dialysed against water at 4°C for 24 h before being stored at -20°C.

Enzyme activity assays

A viscometric method was used as described by Oguntuyo (1981). Viscometers were calibrated against water, the flow of which represented 100% viscosity reduction. The autoclaved enzyme served as the control. Enzyme activity was expressed in viscometric units defined as 100/t where t is time in minutes taken for 50% loss in viscosity of the reaction mixture. In addition, cup-plate assays were also performed, as described by Oguntuyo (1981). Agar medium containing 2% pectin in 0.1 M citrate buffer was prepared. Wells were made in the agar medium into which the enzyme sample was pipetted. The development and the diameter of a white precipitate surrounding the well within 5 min was used as a measure of enzyme activity. Autoclaved enzyme samples were used as control.

Effect of pH on enzyme activity

The effect of pH on enzyme activity was determined by performing the enzyme assay with reaction mixtures of which the pH ranged from 4 - 9. The enzyme activity was expressed as viscometric units.

Thiobabituric acid reaction

The reaction mixture was prepared as described above and incubated at 25°C for 1 h. Following incubation, 2.5 ml of a mixture comprising 2.5 ml of 1 NHCl and 5 ml of 0.04 M thiobarbituric acid was added and the reaction mixture was mixed thoroughly. The capped test tube was then placed into a water bath for 30 min, cooled and the absorbance was read at 515 νm for pectin hydrolase activity and at 550 νm for transeliminase activity. An autoclaved enzyme sample was used for blank reading.

Macerating enzyme activity

The ability of the enzyme samples to separate cells in the tissue was determined in a histological study using potato and onion tissues, as described by Oguntuyo (1981).

Release of reducing groups

The method of Nelson and Somogyi (1944) was used to determine the split of the 0 - 1.4 glycosidic linkages in a pectic substance by a hydrolytic reaction and the release of reducing groups were determined both quantitatively and qualitatively. The results were obtained from a standard curve of galacturonic acid (5 – 50 µg/ml). The affinity of the substrate for the enzyme both *in vitro* and *in vivo*, and the ability to release free galacturonic acids were determined.

Determination of total polyphenol

Total polyphenol was obtained from healthy and diseased onion tissues by homogenizing the tissues with methanol. A filtrate was obtained under vacuum to which the same volume of petroleum ether was added. This process was repeated four times and the ether was evaporated to 10 ml in a rotary evaporator (Model Bibby, RE 200). The extract was finally shaken with the same volume of petroleum ether and stored at 4°C prior to use. For polyphenol estimation, the method of Oguntuyo (1982) was used. The methanol extract (1 ml) was diluted in 7.5 ml of distilled water in a test tube and 1 ml of saturated Na_2CO_3 and 0.5 ml of Folin-Denis reagent was added. The mixture was left to stand for 1 h after which it was centrifuged at 10 000 × g for 5 min and the precipitate was removed. The extinction value was read at 700 νm (Zeiss PM6 digital spectrophotometer) against a reagent blank prepared with methanol. The result was expressed as µg tannic acid equivalent/g fresh weight. The effect of incubation period (in days) and *P. fluorescens* inoculum concentration on the polyphenol content were likewise assessed.

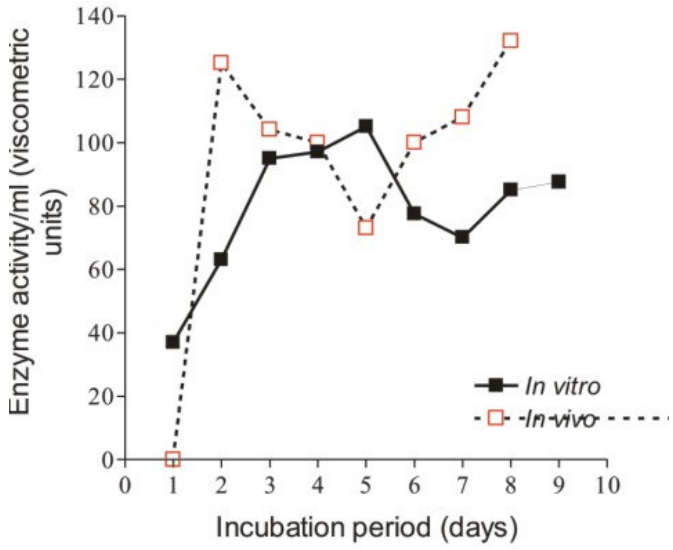

Figure 1. Pectic enzyme activity over a 10 day incubation period.

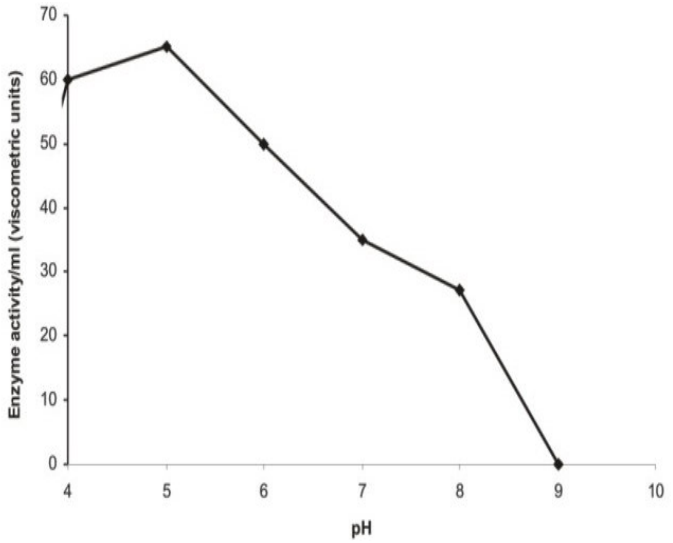

Figure 2. Effect of pH on enzyme activity.

RESULTS

Detection of pectic enzyme activity

In vitro studies showed that a pectic enzyme was inductively produced by *P. fluorescens* in culture medium containing citrus pectin as shown by viscometry. Optimal activity was achieved with pectin as the sole carbon source. The enzyme was also detected in the rot tissue extract as it could reduce viscosity of pectin after 30 min. The activity of the enzyme produced *in vitro* and *in vivo* every 24 h during a 10 day incubation period showed that there was an initial increase, followed by a slight decrease and then an increase towards the end of the incubation period (Figure 1). The development of a white precipitate in the cup-plate assay also confirmed the presence of the enzyme produced by *P. fluorescens* (result not shown).

Classification of the enzyme

The thiobarbituric acid reaction showed that the enzyme is a hydrolytic polygalacturonase, as there was maximum absorbance at 515νm. The cup-plate assay and the increasing concentration of reducing sugars (galacturonic acid) in the culture filtrate confirmed this. The hydrolase was active at pH 5 for the culture filtrate and at pH 4 for rot extract, as determined by viscometry (Figure 2).

Macerating activity

The maceration of onion and potato discs within 1 h showed the activity of the enzyme (Plate 1).

Release of reducing groups

Enzyme activity of the *in vitro* enzyme sample exhibited maximum activity at a substrate concentration of 0.2% (w/v) pectin (14.1 μm km value) and 0.5% (w/v) pectin concentration (22.5 μm km value) for the *in vivo* enzyme sample (Figure 3)

Polyphenol detection

Polyphenols were detected in healthy and infected onion tissues. The rot tissue from artificially inoculated bulbs showed an appreciable increase from 59.28 μg tannic acid equiv/g at time of inoculation to 112.20 μg tannic acid equiv/g tissue after 7 days of incubation (Figure 4). It was also observed that the polyphenol content increased as the *P. fluorescens* inoculum size increased (43 μg tannic acid equiv/g with 10^2 cfu/ml to 110 μg tannic acid equiv/g with 10^{10} cfu/ml) (results not shown).

DISCUSSION

The ability of *P. fluorescens*, implicated in the soft rot of onion bulbs to produce pectic enzymes *in vitro* and *in vivo* was investigated. The bacterium produced a proteineous substance in the culture medium containing pectin as the only carbon source. Reducing sugars were also detected in the medium after incubation. This gave an indication of the presence of a pectic enzyme that showed optimal activity at 2% (w/v) pectin concentration and in rot tissue extract during *in vivo* studies. The viscometric assay confirmed this and suggests a role for the pectic enzyme in soft rot development in plants.

Pectic substances are the primary constituents of the middle lamella and are structural elements in the primary cell wall and microorganisms therefore require specific enzymes for the degradation of cell wall to penetrate it.

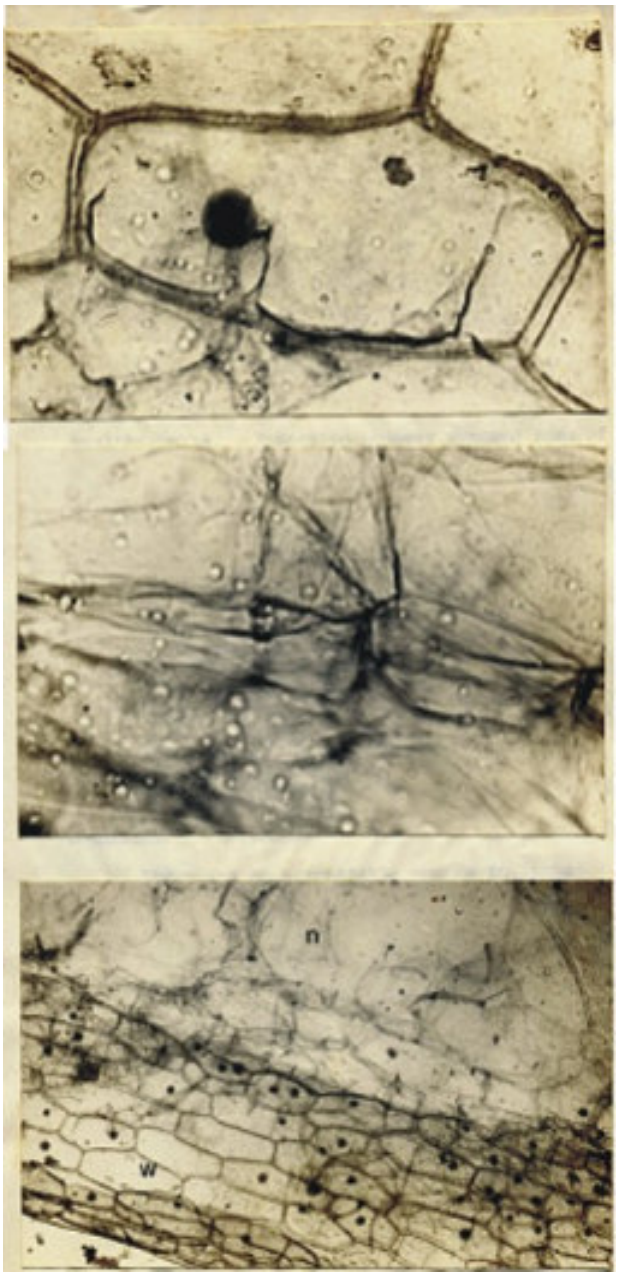

Plate 1. Photomicrograph showing intact cells (top slide), collapsed cells (middle slide) and area of advancing lesion (infected tissue and intact cells) (bottom slide).

Plant pathogens have evolved a means to recognize the chemical structure in the plant cell wall and elaborate enzymes on the various cell wall components (Bateman and Basham, 1976). Many pectolytic bacteria have been implicated in soft rot diseases of plants. *P. fluorescens* was found to produce a pectic enzyme causing storage soft rot of potato tubes. Obi and Umezurike (1981) also reported the elaboration of a pectic enzyme by *Burkholderia* (*Pseudomonas*) *cepacia* in diseased onions. In addition, such enzymes are produced by a variety of phytopathogens (Doyle et al., 2001).

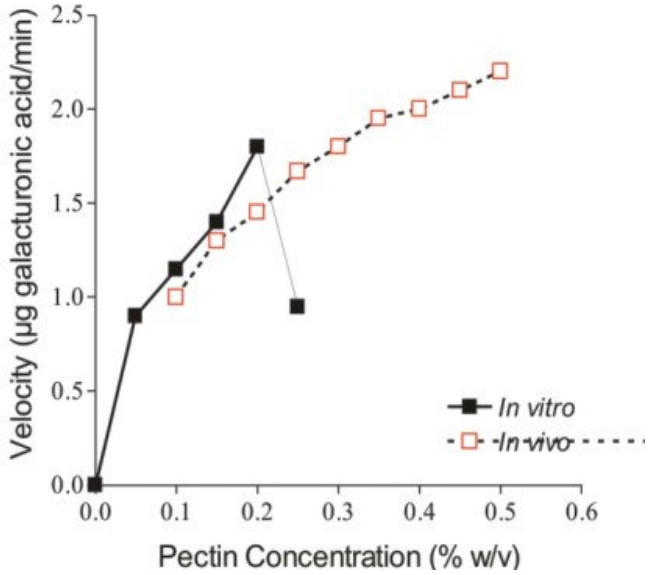

Figure 3. Rate of release of free galacturonic acid at different substrate concentrations.

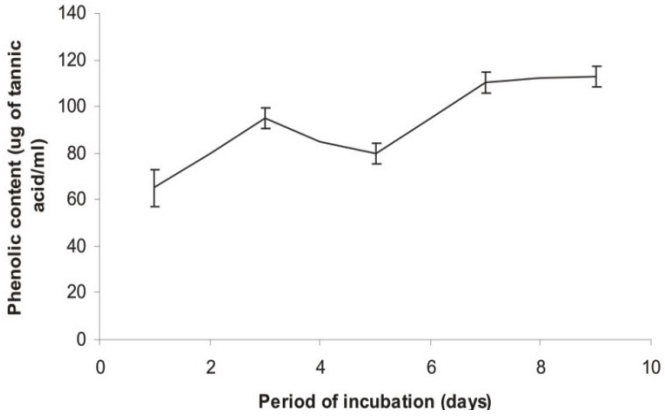

Figure 4. Polyphenol content in infected tissue.

In this study, the purified *P. fluorescens* enzyme was found to be a hydrolytic polygalacturonase as there was clearing around wells in the cup-plate assay and it showed macerating activity on potato and onion discs.

This hydrolase was active at pH 5 in the culture filtrate and at pH 4 in the onion rot extract. Ulrich (1975) found an endo-polygalacturonase produced by *B. cepacia* to have an optimum pH range of 4 – 4.6. The increasing amount of reducing sugars in the medium during growth confirmed the presence and activity of the enzyme, a manifestation of its involvement in soft rot development. This was not surprising as enzyme activity was found to increase during growth in the shake cultures and in the onion bulbs as the storage period increased. Inoculation size was also found to be proportional to extent of rot development. The histological study of rot tissue at areas of advancing lesions showed complete disintegration of

the tissue (Plate 1). Pectic enzymes that have the ability to split the 1, 4 bonds between the galacturonic acid moieties in the pectic fraction of the cell wall remain the only enzyme confirmed to cause plant tissue maceration. The polygalacturonate lyase from *Erwinia chrysanthemi* caused maceration and cell death in discs of potato tissue (Basham, 1974).

The role of pectic enzymes in many plant diseases cannot be over-emphasized (Oguntuyo, 1981; Obi and Umezurike, 1981). The control of such conditions therefore can be solved by appropriate enzyme inhibition strategies. It is known that plants produce antagonistic substances as defense mechanisms against plant pathogens. The ability to overpower the pathogen depends on many factors. Extracts of plant tissues have been found to contain substances known to inhibit pectic enzymes *in vitro* (Bell et al., 1962). These substances have been found to be mostly phenols and their oxidation products. Their free radicals react readily with biochemical constituents such as $–NH_2$ and OH groups, thereby preventing or limiting some infections (Mahadevan et al., 1965). Extracts of plant tissues have been found to contain substances known to inhibit pectic enzymes *in vitro*. Omidiji (1985) reported that phenolic extract from the inner scales of purple onions can inhibit growth of pathogenic *P. fluorescens* in broth and on agar plates.

In this study, polyphenols were detected in healthy and diseased tissues, but were at a higher concentration in infected tissue. Huang and Agrios (1979) had found higher amounts of phenols in infected than healthy tissue in skin disease of apples. It is also believed that the change in colour that accompanies injury or infection may be due to the presence of polyphenols. Onion juice and vapors are one of the best characterized antimicrobial systems in plants. They contain the phenolic compounds protocatechnic acid and catechol, which are believed to contribute to their antimicrobial activity (Doyle et al., 2001).

In conclusion, it is clear from this study that although *P. fluorescens* had the ability to produce pectic enzyme used for invasion of healthy tissue, it could not be controlled by the antimicrobial polyphenols of the plant tissue.

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