African Journal of Plant Science Vol. 3 (5), pp. 113-116, May 2009
Available online at http://www.academicjournals.org/AJPS
ISSN 1996-0824 © 2009 Academic Journals

Full Length Research Paper

Cellulase activity in tomato fruits infected with *Penicillium funiculosum* Thom.

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Accepted 14 May, 2009

Within eight days of incubation at room temperature (27°C), tomato (*Lycopersicon esculentum* Mill.) fruits infected with *Penicillium funiculosum* Thom. had deteriorated. Extracts from the infected fruits exhibited cellulase activity. Uninfected fruits lacked cellulase activity. The enzyme was partially purified by a combination of gel filtration and ion-exchange chromatography. On separation by molecular exclusion chromatography, two peaks of absorption with molecular weight estimates of 223,800 Daltons and 89,100 Daltons were obtained. Only the components of the peak with the lighter weight exhibited cellulase activity. The enzyme showed optimum activity at pH 4.5 and 40°C. Na^+ and Ca^{++} ions stimulated enzyme activity while EDTA and Hg^{++} were inhibitory. The apparent k_m for the hydrolysis of carboxymethylcellulose was approximately $0.53 mgml^{-1}$. The occurrence of cellulase in tomato fruits infected with *P. funiculosum* Thom. and its absence in uninfected fruits suggests a role of this enzyme in pathogenicity of the fungus. Cellulolytic components of the fruits are degraded, the fruits are deteriorated and lost to this post harvest pathogen. Knowledge of the conditions of growth of this fungus and properties of this enzyme will assist the farmer in optimizing production of these fruits and engaging the best conditions for preservation.

Key word: Gel filtration, ion-exchange chromatography, enzyme activity, molecular weight, carboxymethyl-cellulose, deteriorated, infected.

INTRODUCTION

Tomato fruits in Europe and Asia originated from seeds brought from Latin America by Spanish and Portuguese merchants during the sixteenth century. African tomato fruits were introduced by European merchants or colonizers (Villareal, 1973; 1980). Tomato, one of the most important vegetable in many countries has a world wide economic and nutritive importance. It contains an energy value of 20 kcal per 100 grams of edible product (Esquinas-Alkazar, 1981). Quality of tomato fruits could be reduced by various diseases in the field (Walker, 1957). Fungi are responsible for spoilage of the fruits during storage (McColloch et al., 1968). *Penicillium funiculosum* has been found to cause the crown rot of tomato (Marois et al., 1981).

A wide range of cell wall degrading enzymes are produced by most phytopathogens (Cooper, 1981). An alteration in plant cell wall can be brought about by these enzymes (Nicole et al., 1990). Successful plant infection largely depends on the ability of the phytopathogen to gain access into the internal tissue components. A central role in this regard is played by the chain split-ting cellulase enzymes (Ronalt and Donald, 1986). The breakdown of cellulose to soluble sugars by phytopathogens involves the action of a multi-enzyme system (Berghein and Pettesson, 1973). A proposal made by Resse et al. (1950), suggests that a cellulase C_1 initiates the degradation of native cellulose into soluble form of cellulose by breaking down the poly-β-1, 4 glucan chains, while another form of cellulase referred to as C_x catalyses the hydrolysis of these soluble celluloses into soluble sugars.

.This paper describes how *P. funiculosum* Thom. Produces a cellulase in infected tomato (*Lycopersicon esculentum* Mill.) fruits. Attempts to purify and charac-

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terize the enzyme were made.

MATERIALS AND METHODS

Organism and culture conditions

The isolate, *P. funiculosum Thom*. (PEN 02) was part of a culture collection of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria. It was sub cultured from a stock on to 1% malt yeast extract-glucose agar. Five day old culture of the organism served as inoculum.

Inoculation of tomato fruits

Apparently healthy tomato (*L. esculentum* Mill.) fruits were obtained from the Ile-Ife main market. They were surface sterilized using 3% sodium hypochlorite solution for 30 min. The fruits were later rinsed with several changes of distilled water to remove the residual sodium hypochlorite solution. Tissue discs were removed from the tomato fruits using a cork borer (3 mm). The fruits were inoculated with discs (3 mm) (removed from the edge) of five-day old plate culture of the organism. The point of inoculation was sealed with paraffin wax. Controls were steriled tomato fruits inoculated in the same manner with sterile malt yeast agar discs. Both the experimental and control sets of fruits were placed in sterile Petri dishes with bell jars inverted over them. The rims of the bell jars were sealed with vaseline for air tight contact. Incubation was at room temperature (27°C). Observation for deterioration was made on a daily basis.

Extraction of enzyme

Within eight days of incubation, the inoculated tomato fruits had collapsed. The fruits were weighed, chilled for 30 min inside a refrigerator and then homogenized with cold (4°C) liquid extractant (1:1 w/v). The extractant was 0.5 M NaCl in 0.01 M citrate phosphate buffer pH 5.0 with 5 mM Sodium azide (NaN_3) to prevent microbial contamination. The homogenate was filtered through four layers of muslin cloth and further clarified by filtering through filter paper (Whatman No.1). The protein content of this crude filtrate was determined using the method of Lowry et al. (1951). Cellulase activity was analysed by a modified dinitrosalicylic acid reagent method of Miller (1959).

Preparation of extract for fractionation

The crude enzyme was concentrated to about one seventh of its originnal volume in a rotary evaporator (Quickfit, Rotavapor-R, Buchi, Switzerland) at 30°C under low vacuum and slow evaporation, to avoid frothing.

Fractionation on Sephadex G-25 column

The column (1.5 x 30 cm) of Sephadex G-25 was prepared as previously described by Olutiola and Cole (1980). It was equi-librated with 0.01 M citrate phosphate buffer pH 5.0 and surrounded by a constant temperature water jacket (4°C). Five millilitre of the concentrated crude enzyme was applied to the column. The eluting buffer was 0.01 M citrate phosphate buffer pH 5.0 containing 5 mM NaN_3. Column fractions were collected (5 ml/tube). Optical density of the fractions was measured spectrophotometrically at 280 nm. Each fraction was analysed for cellulase activity.

Fractionation on Sephadex C-50 column

Fractions (11-15) with appreciable cellulase activity, from Sephadex G-25 column were pooled together. Six millilitre of the mixture was applied to a column (2.5 x 40 cm) of Sephadex C-50 surrounded by a constant temperature water jacket (4°C), supplied by Pharmacia fine Chemicals, Uppsala, Sweden. Fractions (5 ml /tube) were eluted with 0.01 M citrate phosphate buffer pH 5.0 containing a gradient of 0.1-0.5 M NaCl to weigh down the protein. Optical densities of fractions were measured at 280 nm. Fractions were analysed for cellulase activity.

Fractionation on Sephadex G-100 column

Fractions (9 – 12) from the first peak of Sephadex C-50 column with appreciable cellulase activity were pooled together. Four milliliter of the mixture was applied to a column (2.5 x 70 cm) of Sephadex G-100 surrounded by a constant temperature water jacket (4°C), supplied by Pharmacia fine Chemicals, Uppsala Sweden. It had been previously calibrated with proteins of known molecular weights (Andrews, 1964). Fractions (5 ml/tube) were eluted with 0.01 M citrate phosphate buffer pH 5.0. Optical densities of fractions were measured at 280 nm. Fractions were analysed for cellulase activity.

Enzyme assay

Cellulase activity was analysed using a modified dinitrosalicylic acid reagent method of Miller (1959). The substrate, 0.6% (w/v) high viscosity carboxymethylcellulose (Sigma) was dissolved in 0.01 M citrate phosphate buffer pH 5.0.

The reaction mixture was 0.5 ml of the enzyme added to 1 ml of the substrate. Incubation was at 35°C for 1 h. in a water bath. Enzyme activity was terminated by adding 3 ml of dinitrosalicylic acid (DNSA) reagent. Controls which initially contained only 1 ml of the substrate were incubated with the experimental tubes at 35°C for 1 h. 3 ml of the dinitrosalicylic acid (DNSA) reagent was added to the control tubes after which 0.5 ml of the enzyme was added. The reducing sugars released were then measured. One unit of cellulase activity was arbitrarily defined as the amount of enzyme in 1 ml of reaction mixture that liberated reducing sugars equivalent to 10 µg glucose per minute under assay conditions.

RESULTS

Apparently healthy tomato fruits inoculated with *P. funiculosum* Thom. had collapsed within eight days of incubation at room temperature (27°C). The fruits were overgrown with mycelia and greenish spores of the organism. Extracts of the infected tomato fruits exhibited cellulase activity. Uninfected fruits did not show cellulase activity. Fractionation of the concentrated crude extract on Sephadex G-25 column gave two peaks of absorption. Only the components of the first peak showed cellulase activity. Fractionation of the components of this first peak on Sephadex C-50 column gave two new peaks of absorption. Components of the first peak showed cellulase activity. When the components of this peak were further fractionated on Sephadex G-100 column, two peaks of absorption were obtained. The molecular weight estimates of these peaks were approximately 223,800 Daltons and 89,100 Daltons. Only the components of the second peak with lighter weight showed cellulase activity. The steps of purification are represented in Table 1. Investigations on the

Table 1. Partial purification of cellulase from tomato fruits infected with Penicillium funiculosum Thom.

Fraction	Total activity (u)	Total Protein (mg)	Specific activity (u/mg protein)	Yield (%)	Purification (fold)
Crude enzyme	9450	169.5	55.8	100	1
Sephadex G-25	5693	12.8	444.8	60.2	8.0
Sephadex C-50	4217	3.8	1109.7	44.6	19.9
Sephadex G-100	3374	1.6	2108.8	35.7	37.8

properties of the partially purified cellulase were carried out. Within a temperature range of 20- 50°C, optimum cellulase activity was observed at 40°C. Using 0.01 M citrate phosphate buffer with pH ranges of 3.0–7.0, optimum cellulase activity was observed at pH 4.5. The cellulase was stimulated by both Na^+ and Ca^{++}. Optimum activity was recorded at 20 mM concentrations for both ions. With different concentrations of high viscosity carboxymethylcellulose as substrate (ranges of 2-10 mg ml^{-1}), optimum activity was observed at the range of 8 and 9 mg ml^{-1}.

The rate of enzyme reaction seemed to follow the Michalis-Menten Kinetics. From the Lineweaver Burk plot, the apparent K_m for the hydrolysis of carboxymethylcellulose was approximately 0.53 mg ml^{-1}.

The cellulase was inhibited by EDTA and Hg^{++}. Total inhibition was at 6 and 2 mM concentrations respectively.

DISCUSSION

Apparently, healthy tomato (*L. esculentum* Mill.) fruits inoculated with *P. funiculosum* Thom., incubated at room temperature (27°C), became deteriorated within eight days. Cellulase activity was detected in the extracts of the fruits. Osagie (1980) made a similar observation during infection of tomato fruits by *Aspergillus niger*.

Temperature changes had an effect on the activity of the enzyme. Optimum activity was observed at 40°C. Oyede (1989) reported an optimum activity at a temperature of 40°C for cellulase produced by *Aspergillus niger* during deterioration of maize grains. The enzyme showed optimum activity of pH 4.5. This shows that acidic conditions favoured activity of the cellulase.

A similar observation was reported by Olutiola (1983) for cellulase produced by *Penicillium steckii* associated with the deterioration of cocoa beans. The cellulase was able to degrade carboxymethylcellulose with optimum activity at the range 8 and 9 mg ml^{-1} concentrations. An almost similar report has been made by Bagga and Sandhu (1982). The cellulase was stimulated by the monovalent Na^+ ions and divalent ca^{++} ions. Popoola (1987) made a similar report. Wang and Pinchard (1971) made a report on the ability of Ca^{++} and Na^+ ions to stimulate enzyme activity. The cellulase was inhibited by EDTA and Hg^{++} ions. Similar inhibitory effects have been reported (Osagie, 1980; Oyede, 1989 and Olutiola, 1983).

The occurrence of cellulase in tomato fruits infected with *P. funiculosum* Thom. suggests a role of this enzyme in deterioration of the fruits and pathogenicity of the fungus. The results suggest that the enzyme is produced by the organism in order to hydrolyse the complex cellulolytic portions of the fruit cell wall. Simpler forms of compounds such as glucose are the result of such hydrolysis. Though these simple sugars are absorbed and metabolised by the organism for its growth, the hosts which are the tomato fruits however become deteriorated. These processes result in structural modifications in the components of the fruits.

Tomato fruits in the tropics are frequently lost to post harvest pathogens. *P. funiculosum* is one of such pathogens. Knowledge of the conditions of growth of this fungus and properties of this enzyme will assist the farmer in optimizing production of these fruits and engaging the best conditions for preservation.

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