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Partial Purification of Polygalacturonase from Tomato Fruits Infected by *Rhizopus arrhizus* Fisher

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Abstract: The production of polygalacturonase during the deterioration of tomato (*Lycopersicon esculentum* Mill.) by *Rhizopus arrhizus* Fisher was investigated. The enzyme was partially purified by a combination of ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Two peaks of absorption, with molecular weight estimates of approximately 166 000 Daltons and 60 260 Daltons were obtained.

Key words: Polygalacturonase, tomato fruits, *Rhizopus arrhizus*, gel filtration, ion-exchange chromatography

INTRODUCTION

One of the essential substances primarily found in tomato fruits is lycopene. This substance, apart from the fact that it gives tomatoes their vibrant color, it is of health value as it helps to protect against diseases such as cancer and heart diseases (Rao *et al*., 1999). Audisio *et al*. (1993) reported that tomato is very rich in vitamin C and this serves as a good index in estimating the freshness of the product. However, a large percentage of the tomato fruits produced in Nigeria is lost to post harvest infections caused by microorganisms. Fungi are most of these microorganisms (Ajayi *et al*., 2003).

Rhizopus arrhizus is a known pathogenic fungus (Stevens, 1974; Orabi *et al*., 1999). It establishes itself in its host by secreting extracellular enzymes which break down host tissues (Wheeler, 1975; Virto *et al*., 1999; Elibol and Dursun, 2002). Martak *et al*. (2003) reported the semi continuous fungal fermentation of lactic acid by *Rhizopus arrhizus*. The ability of pathogenic fungi to produce enzymes responsible for the degradation of host tissues is one of the major factors influencing their virulence (Agrios, 1978; Lehtinen, 1993). Pectinases are a group of cell wall degrading enzymes produced by many phytopathogens (Hagar and Mclntyre, 1972; Bruno *et al*., 2001). Pectinases are enzymes useful in the degradation of such host tissues (Alabi and Naqvi, 1977; Walton and Cervone, 1990).

Very little has been reported about the involvement of *Rhizopus arrhizus* in the deterioration of tomato fruits in Nigeria. This study therefore describes the production of polygalacturonase, a pectic enzyme, during the infection of tomato fruits by *Rhizopus arrhizus*. Attempts were made to determine the molecular weight of the enzyme during purification. It is believed that this study would be very useful in providing solutions to the problems of tomato wastage in our markets.

MATERIALS AND METHODS

The experiment described in this paper was carried out between December, 1999 and January, 2005 in the Department of Microbiology, Obafemi Awolowo University, Ile Ife, Osun state, Nigeria.

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Organism

The isolate of *Rhizopus arrhizus* Fisher employed for this research work was isolated from tomato fruits in the Department of Microbiology, Obafemi Awolowo University, Ile Ife, Osun state, Nigeria. The organism was routinely grown and maintained on 1% (w/v) Sabouraud dextrose agar slants. The organism was subcultured from the old culture onto fresh Sabouraud dextrose agar plates whenever it was to be used. Seventy-two-hour-old culture was used as inoculum for this research.

Inoculation and Cultivation

The inoculation techniques employed were as earlier described (Ajayi *et al*., 2003). The medium used in this case was Sabouraud dextrose agar. The experimental and control tomato fruits were placed individually in sterile Petri dishes under surface sterilized bell jars. Incubation was at room temperature. The fruits were examined daily for deterioration.

Extraction of Enzyme from Tomato Fruits

Ten days after inoculation, the deteriorated tomato fruits were weighed and chilled for 30 min inside a freezer and homogenized with an MSE homogenizer at full speed (25 cycles per second) with chilled liquid extractant (1:1w/v) for 2 min at 30 sec. interval. The extractant was 0.01M citrate phosphate buffer, pH 4.5 containing 5 mM NaN_3 to prevent microbial contamination. The homogenate was initially allowed to percolate through four layers of sterile glass fibre. This was used as the crude enzyme.

Preparation of Enzyme for Column Chromatography

The crude enzyme preparation was dialyzed using acetylated cellophane tubing (Whitaker *et al*., 1963). Analysis was performed using a Multiple dialyser (Pope Scientific Inc., Model 220, U.S.A) at 4°C. Proteins in the crude enzyme preparation were precipitated by adding solid ammonium sulphate (Sigma) to 90% saturation.

Fractionation of Enzyme on Sephadex G-100

The vertical glass tube chromatography column (2.5×70 cm) of Sephadex G-100 (Particle size, 40-120 μ) was prepared and calibrated as previously described (Ajayi *et al*., 2003). Ten millimeters of the enzyme concentrate was applied to the column and eluted with 0.05 M citrate phosphate buffer (pH 4.5). Each of the fractions was analyzed for polygalacturonase activity.

Fractionation by Ion-Exchange Chromatography

Fractions from the Sephadex G-100 column which showed appreciable polygalacturonase activity were pooled. Ten milliliter of the pooled enzyme was applied to a CM Sephadex C-50 column (2.5×40 cm) which was prepared as described above for Sephadex G-100 column. Fractions were eluted with 0.05 M citrate phosphate buffer (pH 4.5) containing 0.1, 0.2, 0.4 and 0.5M gradients of NaCl. Fractions (5 mL per tube) were collected and assayed for polygalacturonase activity.

Enzyme Assay

Polygalacturonase activity was assayed according to the method described (Olutiola, 1982a). The reaction mixture was 1 mL of 0.1% (w/v) pectin (Sigma) in 0.01 M citrate phosphate buffer (pH 4.5) and 0.5 mL of the enzyme. Each control tube contained 1 mL of the substrate. The experimental and control tube were incubated in a water bath at 37°C for 3 h. The total reducing sugar was determined by the Dinitrosalicylic acid (DNSA) method (Miller, 1959; Olutiola, 1983).One unit of polygalacturonase activity was defined as the amount of enzyme which released 1 μ mole galacturonic acid per minute.

RESULTS

Gel-filtration of the dialyzed enzyme on Sephadex G-100 column gave four peaks of absorption designated D, E, F and G (Fig. 1). Components of peaks D and E exhibited polygalacturonase activity whereas peaks F and G lacked polygalacturonase activity. The molecular weights of components of peaks D and E were approximately 166,000 and 60,260 Daltons respectively. Elution of components of peak D on CM Sephadex C-50 column produced four peaks of absorption designated Da, Db, Dc and Dd (Fig. 2). Components of peaks Da and Db possessed polygalacturonase activity while the components of peaks Dc and Dd lacked polygalacturonase activity. Purification folds of approximately 30 and 14 were obtained for the components of peaks Da and Db respectively (Table 1). Components of peak E produced three peaks of absorption when separated on CM Sephadex C-50 column. These peaks were designated Ea, Eb and Ec (Fig. 3). Components of peak Ea possessed polygalacturonase activity with a purification fold of approximately 12 (Table 1) while the components of peaks Eb and Ec possessed no detectable polygalacturonase activity.

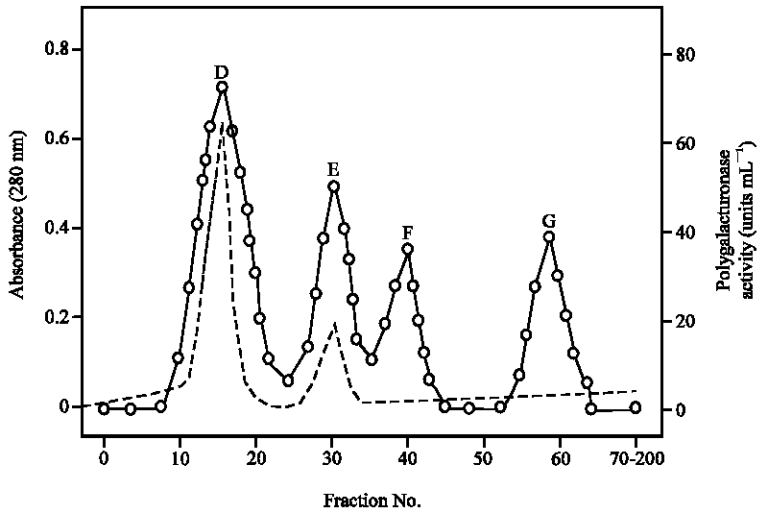

Fig. 1: Separation by gel filtration of proteins obtained from tomato fruits deteriorated by *Rhizopus arrhizus* and the enzymic activity of the fractions towards pectin. (O,) protein (E_{280}); ---, polygalacturonase

Table 1: Partial purification steps of polygalacturonase from tomato fruits infected by *Rhizopus arrhizus*

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1} protein)	Yield (%)	Purification fold
Crude extract	1840	390.0	4.7	100.0	1.00
$(NH_4)_2SO_4$	1600	155.0	10.3	87.0	2.19
Sephadex G-100					
Peak D	960	18.5	51.9	52.2	11.04
E	290	11.8	24.5	15.8	5.21
CM Sephadex C-50					
Peak Da	380	2.7	140.7	20.7	29.90
Db	110	1.7	64.7	6.0	13.80
CM Sephadex C-50					
Peak Ea	132	2.3	57.4	7.2	12.20

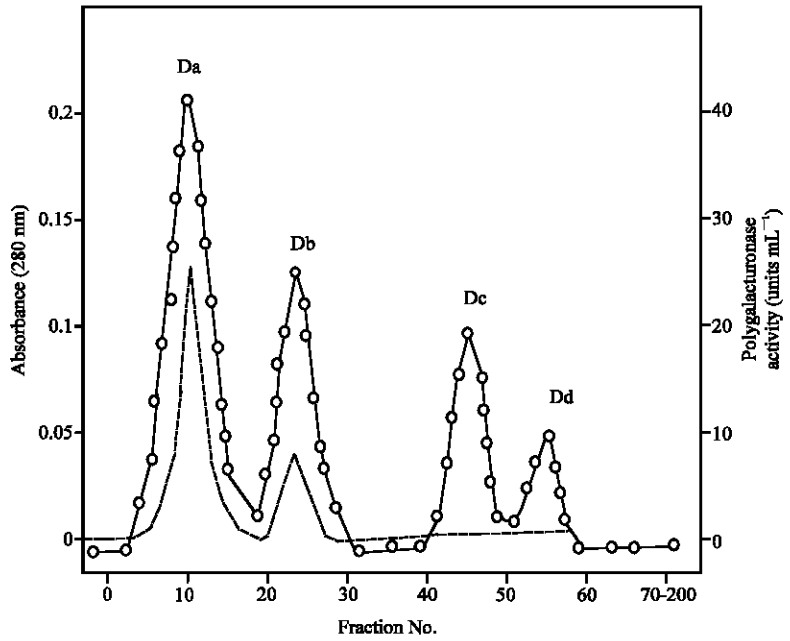

Fig. 2: Separation by ion-exchange chromatography (CM Sephadex C-50) of proteins (fraction D) separated by gel filtration, Fig. 1) and enzymic activity of the fraction towards pectin. (O), protein (E_{280}); ---, polygalacturonase

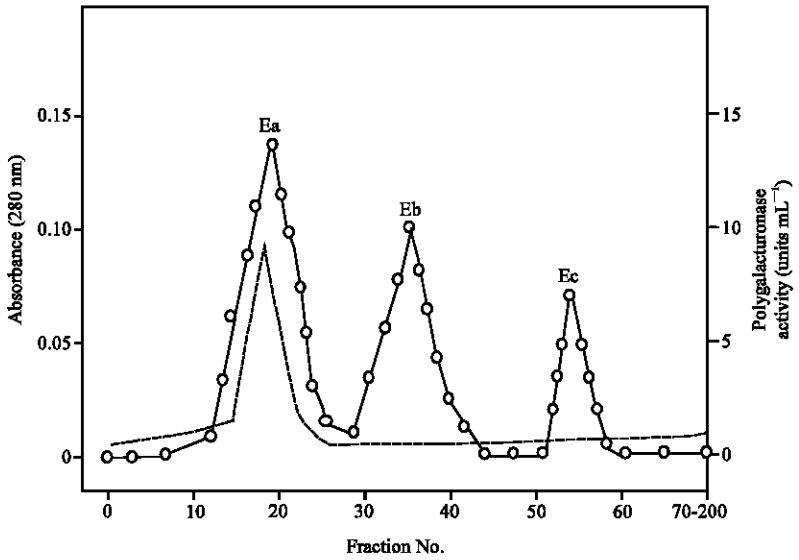

Fig. 3: Separation by ion-exchange chromatography (CM Sephadex C-50) of proteins (fraction E) separated by gel filtration, Fig. 1) and enzymic activity of the fractions towards pectin. (O), protein (E_{280}); ---, polygalacturonase

Table 2: Molecular weights of polygalacturonase from other microorganisms

Species investigated	Molecular weight	References
Botryodiplodia thebromæ	12,000 Daltons (peak Aa) 56,200 Daltons (peak Ab)	Ajayi *et al.* (2003)
Aspergillus niger	82 kDa (exo-PG 1)	Sakamoto *et al.* (2002)
A polygalacturonase inhibitor protein (PGIP) characterized from tomato fruit.	34 kDa	Stotz *et al.* (1994)
Ripe tomatoes	42 kDa PG 1 (Heat stable form). 42 kDa PG 2 (Heat labile form).	Fachin *et al.* (2004)
Aspergillus niger	Multiple form of polygalacturonase with molecular mass ranging from 30-60 kDa	Jyothi *et al.* (2005)

DISCUSSION

During the deterioration of tomato fruits by *Rhizopus arrhizus*, proteins which exhibited polygalacturonase activity were produced. However, similar extracts from uninfected tomato fruits possessed traces of polygalacturonase activity. The occurrence of appreciable quantities of the enzyme in tomato tissues infected by *Rhizopus arrhizus* strongly suggest that the enzyme is of fungal origin. It has been reported that plant cell wall polysaccharide degrading enzymes play fundamental role in host-pathogen interaction (Alana *et al*., 1990). Previous researchers have implicated the involvement of fungi in the deterioration of tomato fruits and fruits from other plants (Adejuwon and Olutiola, 2005; Olutiola, 1982b) but there is paucity of information on the deterioration of tomato fruits by *Rhizopus arrhizus* in Nigeria.

The protein and total reducing sugars of the tomato fruits decreased with infection, corroborating research findings from previous researchers, but the molecular weights of the enzyme fractions differ from the work of previous researchers (Table 2). It has been shown that the differences in molecular weights of enzymes have been associated with a number of factors (Sevillano and Zarra, 1997).

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