

**CHARACTERIZATION OF POLYGALACTURONASE FROM TOMATO  
(*LYCOPERSICON ESCULENTUM* MILL) FRUITS INFECTED BY  
*RHIZOPUS ARRHIZUS* FISHER.**

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**Abstract**

The production of polygalacturonase during the deterioration of tomato fruits by *Rhizopus arrhizus* Fisher was investigated. The enzyme was partially purified by a combination of ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Optimum activity of the polygalacturonase was at 35 °C, pH 4.5 and the substrate concentration at half maximum velocity ( $K_m$  values) for the hydrolysis of pectin by the polygalacturonase fractions (Da, Db and Ea) were 3.8, 2.8 and 2.9 mg/ml. The enzyme was stimulated by Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> but inhibited by EDTA, DNP and HgCl<sub>2</sub>. The enzyme was highly susceptible to heat, losing all its activity within thirty minutes of heating at 70 °C.

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

## 1. Introduction

Pectinases are enzymes responsible for the enzymatic degradation of pectin-containing substrates (Adeyeye and Lashebikan, 2003). They degrade the pectic substances of the middle lamella of plants which is made up of galacturonic acid units joined by  $\alpha$ -1,4-glycosidic linkages (Talboys and Busch, 1970). They are among the cell-wall degrading enzymes produced by various phytopathogens (Walton and Cervone, 1990). Pectic enzymes have been implicated to be very useful in different spheres of life (Laats *et al.*, 1997; Tano-Debrah and Yashiyuki, 1996). Purified pectic enzymes produced by *Rhizoctonia fragariae* and *Botrytis cinerea* were used as a selective tool for in vitro recovery of strawberry plants with fungal disease resistance (Orlando *et al.*, 1997). Igbasan and Guenter (1997) reported that the addition of a combination of pectinase and  $\alpha$ -galactosidase supplement on the nutritive value of peas for broiler chickens improved the growth rate of broiler chickens. Polygalacturonases are produced during the ripening and softening of fruits (Guerrero-Prieto *et al.*, 1996). They are believed to be the most abundant enzyme in ripe fruits (Krama and Keith, 1994).

This present study was therefore carried out to examine the polygalacturonase, a pectic enzyme produced during the deterioration of tomato (*Lycopersicon esculentum* Mill.) fruits by *Rhizopus arrhizus* Fisher. The different physico-chemical

properties of the enzyme examined would help in no small measure in utilizing the enzyme for commercial purposes, especially in tomato processing companies.

## 2. Materials and Methods

### 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 sub cultured 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 work.

### Inoculation of Tomato Fruits:

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 incubation, the deteriorated tomato fruits were weighed and chilled for 30 min inside a

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freezer and homogenized with an MSE homogenizer at full speed (25 cycles per second) with chilled liquid extractant (1:1 w/v) for 2 minutes at 30 seconds interval. The extractant used was 0.5 M NaCl in 0.01 M citrate phosphate buffer, pH 4.5 containing 5 mM  $\text{NaN}_3$  to prevent microbial contamination. The homogenate was initially allowed to percolate through four layers of sterile muslin and thereafter through one layer of sterile glass-fibre filter (Whatman No. 1). 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 x 70 cm) of Sephadex G-100 (Particle size, 40-120  $\mu$ ) 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 milliliters of the pooled enzyme was applied to a CM Sephadex C-50 column (2.5 x 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 0.5 M 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, 1982). 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 and 0.5 ml of the enzyme preparation denatured by heating at 100 °C for 15 min. The experimental and control tubes were incubated in a water bath at 35 °C for 3 h. The reaction was terminated with 3 ml of dinitrosalicylic acid reagent. The total reducing sugars released in the reaction mixtures were determined by the Dinitrosalicylate (DNSA) method (Miller, 1959; Olutiola, 1983). One unit of polygalacturonase activity was defined as the amount of enzyme which releases reducing sugars equivalent to one micromole galacturonic acid per minute under the specified conditions of the reaction.

#### *Effects of Heat, Temperature, pH and Substrate Concentration on Enzyme Activity:*

The influence of temperature on the enzyme was examined at different temperatures of 20 °C, 25 °C, 30 °C, 40 °C and 45 °C. Samples of the partially purified enzyme were heated at 70 °C for different periods of time (0, 2, 5, 10, 15, 20, 25 and 30 minutes), respectively. The effect of pH on the activities of polygalacturonase was also examined for pH ranging from pH 3.0 to pH 7.0. In each case the substrate used 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. Pectin at concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/ml were prepared in 0.01 M citrate phosphate buffer (pH 4.5) and used for polygalacturonase assay.

### 3. RESULTS

The fractions which possessed polygalacturonase activity were used to determine the influence of some factors on the activity of the partially purified enzyme. Optimum pH of 4.5 was obtained for the polygalacturonase fractions (Fig. 1a & 1b). The optimum temperature was at 35 °C (Fig. 2a & 2b). On heating at 70 °C for 5 minutes, the polygalacturonase lost activities of approximately 48%, 30% and 33% for fractions Da, Db and Ea, respectively, while complete inactivation of the enzyme was observed on subjecting the polygalacturonase fractions to heat for 30 minutes (Fig. 3a & 3b). The cations employed in this investigation stimulated the activity of polygalacturonase produced by *R. arrhizus* (Fig. 4a, 4b and 4c). Mercuric chloride ( $\text{HgCl}_2$ ), ethylenediamine tetra acetic acid (EDTA) and 2, 4 dinitrophenol (DNP) inhibited the activity of the enzyme (Fig. 5a, 5b and 5c). The apparent  $K_m$  values for the hydrolysis of pectin obtained from the Lineweaver Burk plot (Fig. 6a, 6b and 6c) were approximately 3.8, 2.8 and 2.9 mg/ml for fractions Da, Db and Ea, respectively.

### 4. DISCUSSION

During the deterioration of tomato fruits by *R. 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 *R. arrhizus* strongly suggests that the enzyme is of fungal origin. The optimum temperature and pH values obtained corroborate the work of other researchers, thus adding to the already available information on polygalacturonase (Blanco *et al.*, 1997; Huang and Mahoney, 1999; Agarwal *et al.*, 1997). The inhibitory actions of EDTA,  $\text{HgCl}_2$ , and DNP coupled with the stimulatory effects of the cations

Fig. 1a: Effect of pH on the activity of partially purified polygalacturonase fractions (Da and Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

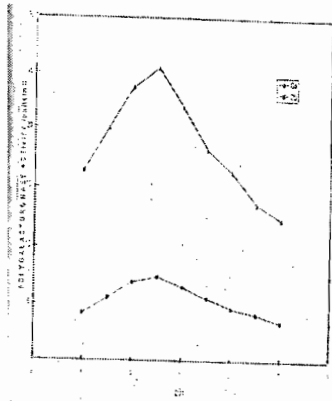


Fig. 1b: Effect of pH on the activity of partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

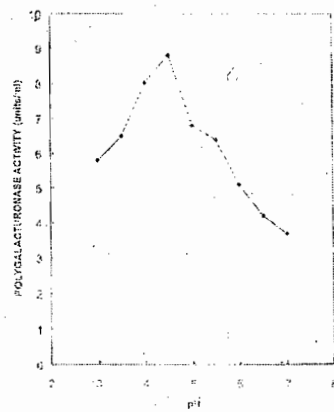


Fig. 2a: Effect of temperature on the activity of partially purified polygalacturonase (fractions Da and Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

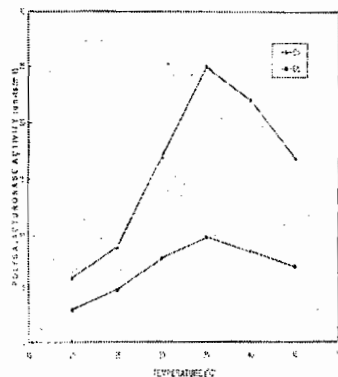


Fig. 2b: Effect of temperature on the activity of partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

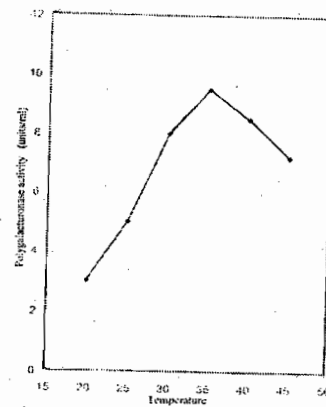


Fig. 3a: Effect of duration of heating (70 °C) on the activity of partially purified polygalacturonase (fractions Da and Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

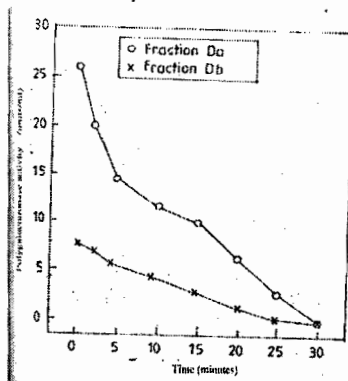


Fig. 3b: Effect of duration of heating (70 °C) on the activity of partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

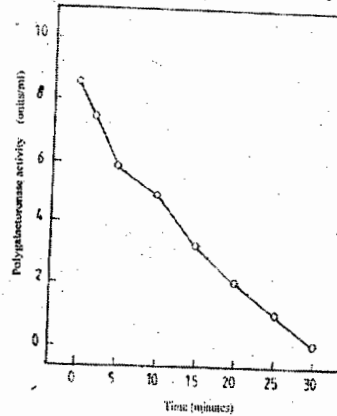


Fig. 4a: Effect of cations on the activity of partially purified polygalacturonase (fraction Da) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

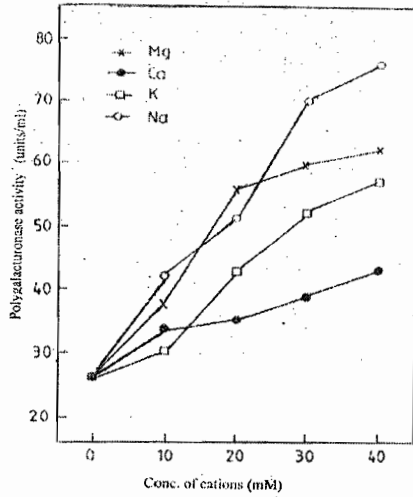


Fig. 4b: Effect of cations on the activity of partially purified polygalacturonase (fraction Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

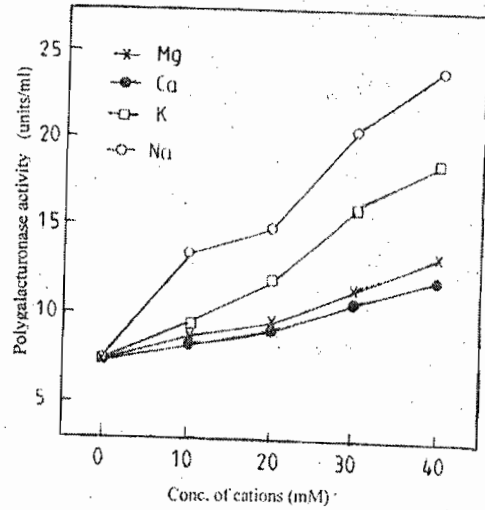


Fig. 4c: Effect of cations on the activity of partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

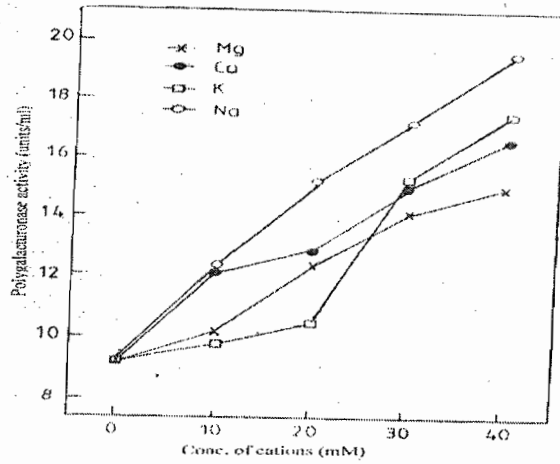


Fig. 5a: Effect of chemicals on the activity of partially purified polygalacturonase (fraction Da) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

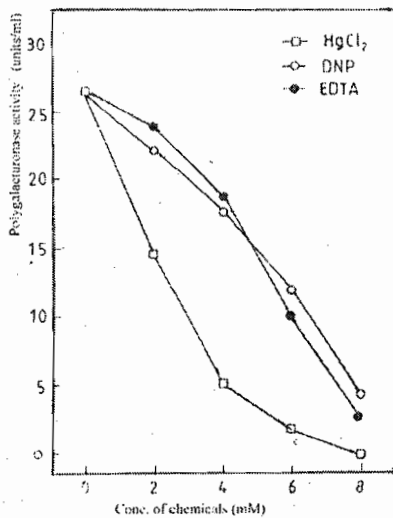


Fig. 5b: Effect of chemicals on the activity of partially purified polygalacturonase (fraction Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

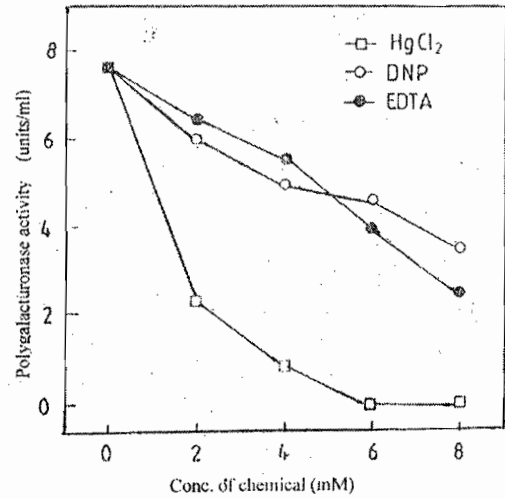


Fig. 5c: Effect of chemicals on the activity of partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

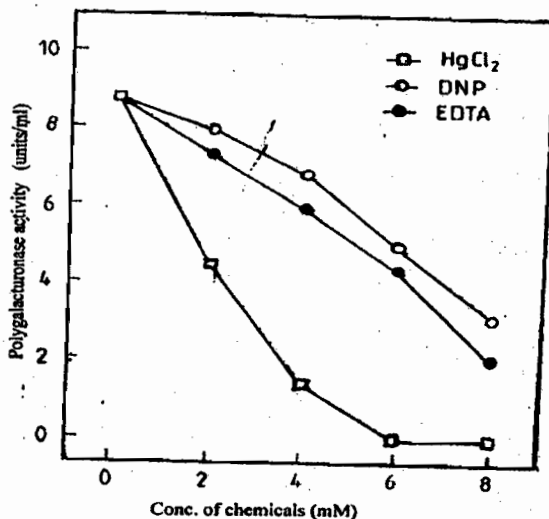


Fig. 6a: Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase (fraction Da) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

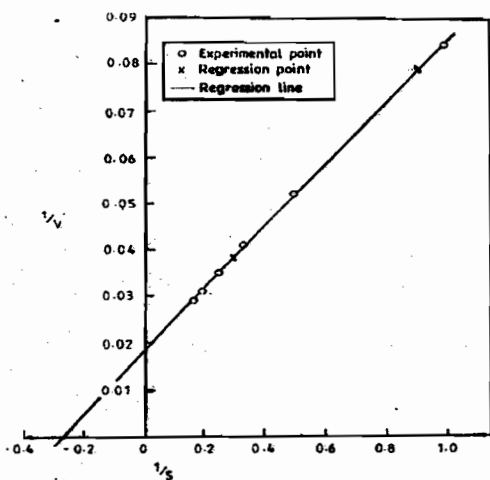


Fig. 6b: Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase (fraction Db) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.

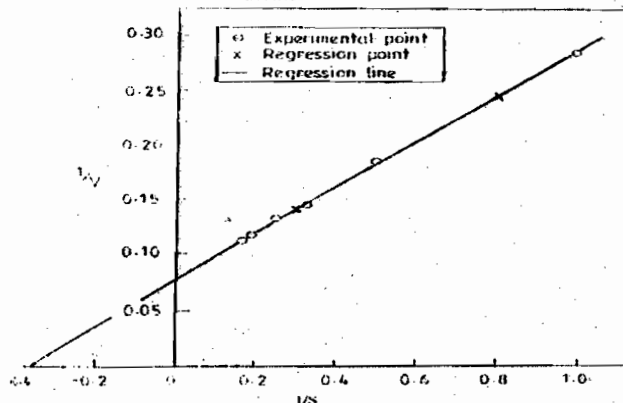
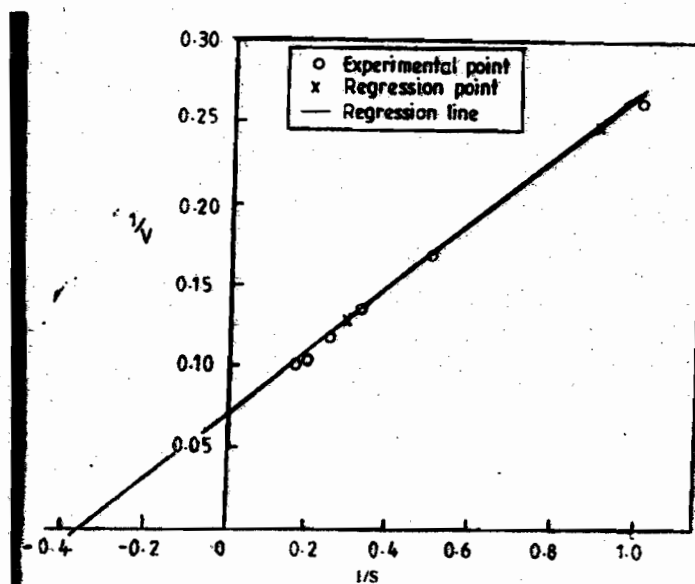


Fig. 6c: Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase (fraction Ea) obtained from tomato fruits deteriorated by *Rhizopus arrhizus*.



(Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, and K<sup>+</sup>) used are comparable with results from other researchers (Famurewa *et al.*, 1993; Oikawa *et al.*, 1994; Sakamoto *et al.*, 1994; Akiba *et al.*, 1995).

In general, the polygalacturonase from tomato fruits deteriorated by *R. arrhizus* is similar in physico-chemical properties to those obtained from other researchers but their results are from other sources, in terms of different organisms from which they are obtained or from different hosts.

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