Purification of Cellulase obtained from Tomato fruits (*Lycopersicon lycopersicum* (L.) Karst) deteriorated by Aspergillus Flavus Linn

[1]Ajayi A. A, [2]Adebiyi M. O, [1]Olasehinde G. I and [3]Olutiola P.O
[1]Department of Biological Sciences, College of Science and Technology, Covenant university, ota,Ogun State, Nigeria
[2]Department of Computer and Information Sciences, College of Science and Technology, Covenant university, ota, Ogun State, Nigeria
[3]Department of Microbiology, Faculty of Science, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria
[1]quietasever@yahoo.com,[2]onlimarion@yahoo.com,[1]golasehinde@yahoo.com
Corresponding Author: onlimarion@yahoo.com

***Abstract*- Tomato fruits infected by *Aspergillus flavus* Linn produced proteins with cellulolytic activity. The enzyme was partially purified by Ammonium Sulphate Precipitation, Gel filtration and ion-exchange chromatography. Three peaks of absorption A, B and C were obtained. Peak B had Cellulase activity with molecular weight of approximately 30,200 Daltons while Peaks A and C lacked Cellulase activity. Elution of components of Peak B on CM Sephadex C-25 produced four peaks of absorption designated Ba, Bb, Bc and Bd. Only components of Peaks Bb and Bc possessed Cellulase activity. Purification folds of approximately 80 and 81 were obtained for components of Peaks Bb and Bc respectively for Cellulase of *A. flavus.* The apparent Km values for the hydrolysis of carboxymethylcellulose by *A.flavus* Cellulase fractions, Bb and Bc were approximately 16.7 and 15.4mg/ml respectively. The partially purified enzyme preparations obtained from A.flavus during the deterioration of tomato fruits caused tissue maceration and cellular death. This result can be very useful in splitting and solubilization of pectic substances and pathogenicity.**

***Index Term*-- Purification, Cellulase, Tomato fruits, *Aspergillus flavus* linn .**

I. INTRODUCTION

Cellulases are enzymes that are very important in Phytopathogenesis and they have been associated with different plants and fruits (Adejuwon *et al. 2009;* Danesh *et al., 1999;* Ronalt and Donald, 1986). It has been reported severally that a major pre-requisite for phytopathogenicity is the ability of the causal agents to secrete enzymes necessary to degrade the cellulolytic and also the pectinolytic components of the cell wall of plants (Famurewa and Olutiola, 1991; Ajayi *et al., 2007).* Different microorganisms produce Cellulase (Yadav and Prasad, 1998). Most of these organisms are fungi (Olutiola, 1983; Bagga and Sandhu, 1982). *Aspergillus flavus* has been reported as one of these fungi (Ojumu *et al*., 2003). *Aspergillus flavus* Linn is a prevalent air-borne fungus that has been identified to be the causative agent of human allergic disorders (Chou *et al.*, 1999). The fungus gives off carcinogenic by-products called aflatoxins that have been implicated in liver cancer when crops are contaminated (Brown, 1999). *A. flavus* is a filamentous fungus that can be isolated from the soil (de Souza *et al.*, 1999). *Streptomyces transformant* T3-1 produces thermostable Cellulase (Jan and Chen, 2003). Abu-shady *et al*. (2002) reported the production of Cellulase by a local strain of *Trichoderma longibrachiatum* under solid substrate cultivation on corn stalks fortified with czapek's solution. Van Walsum and Lynd (1998) reported the production of Cellulase by the aerobic fungus *Trichoderma reesei*. Two strains of ectomycorrhizal edible mushroom *Cantherallus cibrius*, one from a coniferous forest and the other a deciduous forest produced Cellulase in culture (Dahm *et al*., 1999).

The experiment described in this paper was therefore carried out to report the Cellulase produced during the deterioration of tomato (*Lycopersicon lycopersicum* (L.) Karst) fruits by *Aspergillus flavus* Linn.

II. MATERIALS AND METHODS

The experiment in this investigation was carried out in the Microbiology Laboratory of the Department of Microbiology, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria between January 2000 and January 2005.

A. Organism and Culture Conditions

The isolate (NSPRI.101) of *Aspergillus flavus* Linn used for this work were as previously described (Ajayi *et al*., 2007). Seventy two-hour-old culture of the organism was employed in inoculating fresh tomatoes.

B. Inoculation and Cultivation

The inoculation techniques were as earlier described (Ajayi *et al*., 2003). The experimental and control tomato fruits were placed individually and control tomato fruits were placed individually in sterile petridishes in tied polythene bags under surface sterilized bell jars. The fruits were incubated at room temperature (27^0C) and they were examined daily for rot.

C. Extraction of Enzyme

After incubation for ten days the fruits was weighed and chilled in a refrigerator. They were then homogenized with cooled liquid extractant for ten minutes. The liquid extractant consisted of 0.5m Nacl in 0.1M-citrate phosphate buffer (pH 5.0) with 5mM sodium azide (NaN_3). The homogenate was filtered using the glass fiber filter paper (Whatman GF/A) and it was thereafter clarified by centrifugation at 15,000g for 15min at 4ºC. This was employed as the crude enzyme solution for the enzyme assay. The protein content of this crude enzyme was determined by the method of Lowry *et al.*(1951). Cellulase activity was analyzed by the Dinitrosacylic acid reagent method of Miller (1959).

D. Preparation of Enzyme for column chromatography.

The crude enzyme preparation was dialyzed using the acetylated cellophane tubing (Whitaker *et al*., 1963). This analysis was carried out using the multiple Dialyzer (Pope scientific Inc. model 220, U.S.A) at 4ºC. The proteins contained in the preparation were precipitated by adding solid Ammonium Sulphate (Sigma) to 90% saturation.

E. Fractionation on Sephadex G-100

The column (2.5x 70cm) of Sephadex G- 100 (particle size, 40- 120 µ) was prepared and calibrated as previously described (Ajayi *et al. 2007).* Ten millimeters of the enzyme concentrate was applied to the column and eluted with 0.05M Citrate phosphate buffer (pH 4.5). Measurement of the protein content of the eluted fraction and calibration of the column with proteins of known molecular weight were as previously described (Olutiola and Cole, 1977). Each eluted fraction was analyzed for Cellulase activity.

F. Fractionation on CM Sephadex C-50 column

The CM Sephadex C-50 column used for this fractionation was prepared as previously described (Ajayi *et al. 2003).* A shorter column (2.5x40cm) by Pharmacia (Sweden) was used. Fraction from the gel filtration, which exhibited appreciable enzyme activities, were pooled and concentrated to a small volume using a rotary- evaporator (Buchi Rotavapor- R) at 30ºC. The enzyme concentrate was eluted in a gradient elution fashion with 0.01m citrate phosphate buffer (pH 4.5) containing 0, 0.1, 0.2, 0.4 and 0.5M Nacl (Andrews, 1964). Fraction (5ml per tube) was collected and the protein content in each tube determined at 280nm. Using a UV spectrophotometer (Cecil ultraviolet spectrophotometer, 272 Linear Readout Spectrophotometer). Each fraction was analyzed for Cellulase activity.

G. Enzyme Assay

Cellulase activity was assayed according to the method described by (Miller 1959) and Olutiola 1983). The reaction mixture consisted of 1ml of 0.6% (w/v) Carboxymethyl Cellulose (Sigma) in 0.01M citrate phosphate buffer, pH 4.5 and 0.5ml of the enzyme solution. One unit of activity (U) is defined as the amount of enzyme in 1ml of reaction mixture that liberates 1µg glucose in one minute under the specified conditions of the reaction. The total reducing sugar was determined by the Dinitrosalicylic acid (DNSA) method (Miller, 1959).

III. RESULTS

Gel filtration of the dialyzed enzyme on Sephadex G-100 column gave three peaks of absorption designated, A, B and C for *A.flavus* (Fig.1). Components of peaks B exhibited Cellulase activity whereas peaks A and C lacked Cellulase activity. Elution of components of peak B on CM Sephadex C-25 produced four peaks of absorption designated (Ba, Bb, Bc and Bd) (fig. 2) Component of peak Bb and Bc possessed Cellulase activity while components of peak Ba and Bd lacked Cellulase activity. Purification fold of approximately 80 and 81 were obtained for component of peaks Bb and Bc respectively (Table I)

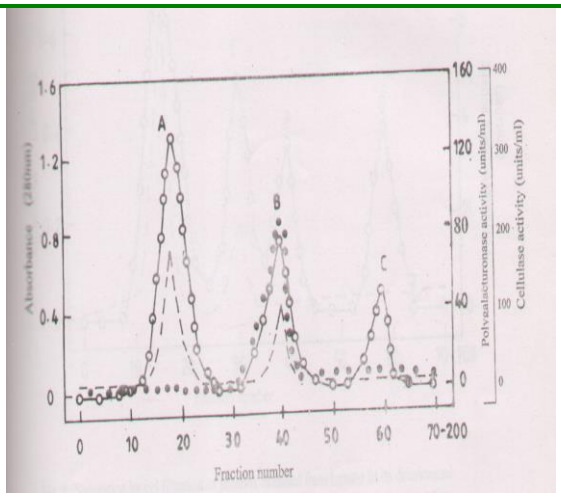

Fig. 1. Separation by gel filtration (Sephadex G-100) of protein obtained from tomato fruits deteriorated by *Aspergillus flavus* and the enzymic activity of fractions towards Carboxymethyl cellulose. (O,) protein (E. 280). Cellulase.

Table I
Partial purification steps of Cellulase from tomatoes fruits deteriorated by *Aspergillus flavus.*

Fraction	Total activity (u)	Total protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	3900	520	7.5	100	1
$(NH_4)SO_4$	3640	210	17.3	93.33	2.3
G-100 Peak B	640	17.3	37.0	32.3	9.7
CM (C-25) Peak Bb	900	1.5	600.0	23.08	80.0
CM (C-25) Peak Bc	975	1.6	609.4	25.0	81.3

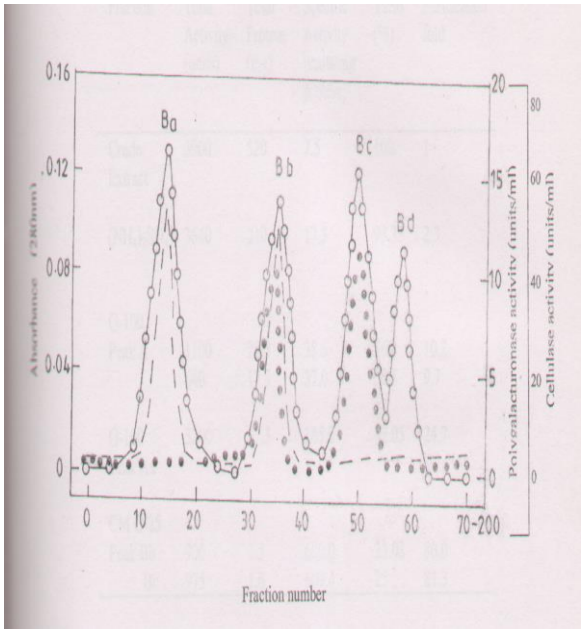

Fig. 2. Separation by ion- exchange chromatography (CM Sephadex C-25) of protein (fraction B separated by gel filtration, Fig. 1) and enzymatic activity of the fraction towards Carboxymethyl Cellulase, (O), protein (E280); Cellulase.

IV. DISCUSSION

Freshly ripe tomato (*lycopersicon lycopersicum* karst*)* fruits inoculated with *A.flavus* Linn incubated at room temperature (27ºC) collapsed extensively within ten days of incubation. Cellulase activity was exhibited in extracts from the deteriorated fruits. Similar results have been reported in infection of tomato fruits by microorganisms.

During the deterioration of the proteins contained in the tomato fruits by *A.flavus*, proteins that exhibited Cellulase activity was produced. The protein content increased with infection. However, similar extracts from uninfected tomato fruits possessed no detectable Cellulase activity. This suggests a role for the enzyme in the infection process. Several workers have implicated Cellulase in pathogenicity (1997)). The molecular weights obtained for the Cellulase from *A.flavus* is similar for some researchers while it differs for others.

Enzymes causing tissue maceration and cellular death are enzymes that are capable of splitting the 1, 4-α-D glycosidic bonds in pectic substances and they are also able to cause extensive solubilization of pectic substance (Famurewa *et.al.* (1993) ; Chesson and Codner (1978)).

Several workers have implicated Cellulase using different assays in pathogenicity.

REFERENCES

[1] Adegoke GO, Moyosade JO (1987). Spoilage microflora of tomatoes and onions in a tropical marketing system. Nig. Food J. 5: 80-84.

[2] Ajayi AA, Olutiola PO, Fakunle JB (2003). Studies on Polygalacturonase associated with the deterioration of tomato *(Lycopersicon esculentum* Mill) Fruits infected by *Botryodiplodia theobromae* PAT. Science Focus, 5: 68-77

[3] Anyanwu CO, Komolafe BT (2003). Agricultural Science for Schools and Colleges. Longman Publishers, Ibadan, Nigeria.

[4] Bakai-Golan R, Karadavid R (1991). Cellulolytic activity of *Penicillum digitatum* and *Penicillum italicum* related to fungal growth and pathogenesis in citrus fruit. J. Phytopathol. 131: 65-72.

[5] Decuypere JD (2006). Food and industrial value of tomato. United State Department of Agriculture (USDA) food and Nutrition centre. Annual Publication, pp. 100-106.

[6] Famurewa O, Olutiola PO (1991). Comparison of growth and cellulolytic enzyme production in *Aspergillus chevailieri* and *Penicillum steckii* from mouldy cocoa beans. Folia Microbiol. 36: 347-352.

[7] FAO(2002). Production of Nigeria, Principal Crops, pp. 26-28. Fiedurek J, Szczodrak J, Roalski J (1995). Seeds as natural matrices for immobilization of Aspergillus niger mycelium producing pectinase. J.Appl.Bacteriol. 78: 409–412.

[8] Glick BR, Pasternak JJ (1998). Principles and Applications of Recombinant DNA. .ASM, Washington D.C. p. 683.

[9] Jan H-D, Chen K-S (2003). Production and characterization of thermostable cellulases from Streptomyces transformant T3-1. World J. Microbiol. Biotec. 19: 263-268.

[10] Kalogeris E, Christakopeulos P, Katapodis P, Alexiou A, Vlachou S, Kekos D, Macris BJ (2003). Production and characterization of cellulolytic enzymes from the thermophilic fungus Thermoascus auranticus under solid state cultivation of agricultural wastes. Process Biochem. 38: 1099-1104.

[11] Kollar A (1998). Characterization of an endo-polygalacturonase produced by the apple slab fungus, *Venturia inaequalis.*Mycological Res. 102: 313- 319.

[12] Lang C, Cornelia G, Milan P, Ulf S (1997). Optimization of fungal polygalacturonase synthesis by *Saccharomyces cerevisiae* in fed-batch culture. Chem. Engine. J. 65: 219-266.

[13] Lowry OH, Rosebrough NJ, Parr AL, Randal RJ (1951). Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 219: 13-25.

[14] McGlasson B (1993). Tomatoes in Encyclopedia of Food Science Technology and Nutrition. Academic Press. 7: 4579-4589.

[15] Olutiola PO (1982). Extracellular polygalacturonase enzyme complex from *Penicillium citrinum.* Thom. Associated with internal mouldiness of cocoa (Theobroma cacao) beans Acta phytopathological Academiae Scientiarum Hungaricae 17: 239-247.

[16] Prescott LM, Harly JP, Klein DA (2003). McGraw Hill Higher Education, New York, pp. 221-248.

[17] Robert KM, Daryl KG, Peter AM, Victor WR (2003). Harper's illustrated Biochemistry twenty sixth edition. 2003 by the McGraw-Hill companies Inc. Printed in London. pp. 12-21.

[18] Sakar A, Upadhyay SN (1994). Purification and properties of cellulase from *Micrococcus roseus.* World J. Microbiol. Biotechnol. 10: 1081-1086.

[19] Schmidt O, Angermann, Frommhold TI, Hope K (1995). Experimental and theoretical investigations of submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus niger.* Appl. Microbiol. Biotechnol. 43: 420-430.

[20] Seymour GB, Taylor TE, Tucker GA (1993). Biochemistry of fruitripening 1: 405-440.

[21] Shewfelt RL (1986). Postharvest treatment for extending the shelf-life of fruits and vegetables. Food Technol. 40 (5): 70-89.