Chapter

Characterisation of Endo-Polygalacturonases Activities of Rice (*Oryza sativa*) Fungal Pathogens in Nigeria, West Africa

Adekunle Odunayo Adejuwon, Marina Donova, Victoria Anatolyivna Tsygankova and Olubunmi Obayemi

Abstract

Rice (*Oryza sativa*) is cultivated in swampy geographical locations of tropical Nigeria, West Africa. Here it is infected by a host of fungal pathogens on the field or contaminated at postharvest. This has led to its loss and reduction in its production in both the national and global market. *Lasiodiplodia theobromae* and *Rhizoctonia solani* have recently been identified as the major fungal phytopathogens causing the deterioration of this grain on the field and at postharvest and affecting its production in Nigeria leading to gross capital loss. Hence the need to determine physiological control measures for the eradication of both phytopathogens on the field and at postharvest. In this study, tropical strains of *Lasiodiplodia theobromae* and *Rhizoctonia solani* obtained from deteriorated rice (*Oryza sativa*) were grown in a growth nutrient medium composed of $MgSo_4.7H_20$, K_2HPO_4, $FeSO_4.7H_20$, potassium nitrate and pectin at 30°C. Endo-Polygalacturonase activities were produced by the fungal isolates in the growth medium within ten days. The endo-polygalacturonases from both fungi were purified by a combination of ammonium sulphate precipitation, dialysis, gel filtration (on Sephadex G-100 column) and ion-exchange chromatography (on CM-Sephadex C-50 and CM-Sephadex C-25 columns). The molecular weight of endo-polygalacturonase from the *Lasiodiplodia theobromae* using Sephadex G-100 was estimated as 124,000 Daltons while that of the *Rhizoctonia solani* was estimated as 92,000 Daltons. The purified endo-polygalcuronase from the *Lasiodiplodia theobromae* exhibited optimum activity at 30°C and at pH 4.5 while that from the *Rhizoctonia solani* exhibited optimum activity at 32°C and at pH 5.0. The purified endo-polygalacturonases from both fungi exhibited optimum activities at 0.2% pectin concentration. They were stimulated by Ca^{2+} but inhibited by ethlylenediamine tetracetic acid (EDTA) and 2,4-dinitrophenol. The purified endo-polygalacturonase from the *Lasiodiplodia theobromae* lost 80% of its activity within 20 minutes of heat at 80°C. While the purified endo-polygalacturonase from the *Rhizoctonia solani* lost 82% of its activity within 20 minutes of heat at 80°C. Potassium nitrate as nitrogen source in the defined growth medium with pectin as carbon source supported highest activity of endo-polygalacturonase by the *Lasiodiplodia theobromae* while ammonium chloride as nitrogen source in the defined growth medium with

pectin as carbon source supported highest activity of endo-polygalacturonase by the *Rhizoctonia solani*. In conclusion, the conditions inhibiting endo-polygalacturonases from *Lasiodiplodia theobromae* and *Rhizoctonia solani* capable of degrading the pectin portion of rice (*Oryza sativa*) can be adapted as feasible control measures limiting the infection and contamination of rice (*Oryza sativa*) by these phytopathogens on the field and at postharvest. Temperature and pH extreme from 30°C and pH 4.5 will be feasible inhibitory control measures for the growth of *Lasiodiplodia theobromae* on rice (*Oryza sativa*) in Nigeria while temperature and pH extreme from 32°C and pH 5.0 will inhibit growth of *Rhizoctonia solani* on the grain. These physiological conditions will preserve pectin in rice (*Oryza sativa*) from degradation by these two fungal phytopathogens.

Keywords: rice (*Oryza sativa*), phytopathogen, fungi, microbial enzymes, polygalacturonase, endo-polygalacturonase, purification, characterisation

1. Introduction

Oryza sativa (Asian rice) and *Oryza glaberrima* (African rice) are species of rice grown all over the world [1]. Rice is the species of the seed of grass. It is a cereal grain consumed mostly in Asia and Africa [2]. It is an agricultural grain which has the third-highest worldwide production only after sugarcane and maize [3, 4] and the world's most consumed staple food [4]. It is rich in starch, protein, minerals and vitamins but low in calories and fats [5]. Rice is grown in all the geographical zones of Nigeria, West Africa depending on the variety [6]. The area of land used for rice cultivation in Nigeria, West Africa is about 2 million hectares. Nigeria however has the potentials of cultivating about 5 million hectares [7, 8].

Rice is affected by a host of fungal pathogens which include: *Magnaporthe grisea* which causes Rice blast; *Rhizoctonia solani* which causes Rice sheath blight; *Cochliobolus miyabeanus* (an Ascomycete) which causes brown spot disease in rice [1]. In Nigeria, the major fungal pathogens of rice include: *Fusarium moniliforme* Sheldon which causes Bakanae foot rot disease; *Cercospora oryzae* Miyake which causes Narrow brown leaf spot; *Rhynchosporium oryzae* Hashioka and *Rhynchosporium oryzae* Yokogi which cause Leaf scald; *Rhizoctonia solani* Kühn which causes Basal sheath rot; *Pyricularia oryzae* Cav. which causes Rice blast; *Cochliobolus miyabeanus* Ito Dreschler ex Dastur and *Cochliobolus miyabeanus* Kuribayashi Dreschler ex Dastur which cause Rice brown spot; *Lasiodiplodia theobromae* which causes Root rot disease complex in rice [9, 10].

Endo-Polygalacturonase (EC: 3.2.1.15) also known as Pectin depolymerase, PG, Pectolase, Pectin hydrolase, and Poly-alpha-1,4-galacturonide glycanohydrolase, is an enzyme that hydrolyzes the alpha-1,4 glycosidic bonds between galacturonic acid residues. It degrades pectin by hydrolyzing the O-glycosyl bonds yielding alpha-1,4-polygalacturonic residues [11–14]. This enzyme has multiple parallel beta sheets which form a helical shape that is called a beta helix. This highly stable structure has numerous hydrogen bonds and disulfide bonds between strands common to all pectin degrading enzymes. The interior of the beta helix is hydrophobic [14, 15]. Exo-Polygalacturonases and Endo-Polygalacturonases have differing hydrolytic modes of action. Endo-Polygalacturonases hydrolyze pectin in a random fashion along the polygalacturonan chain resulting in oligogalacturonides. Exo-Polygalacturonases hydrolyze pectin at the non-reducing end of the polymer resulting in monosaccharide galacturonic acid [14]. Fungal Polygalacturonases

are affected by a variety of factors which include: pH, substrate concentration, substrate specificity, and temperature [13]. Phytopathogenic fungi expose plant cell walls to Cell Wall Degrading Enzymes (CWDEs) such as Polygalacturonases [14]. Siddiqui [16] purified a monomeric polygalacturase with molecular weight of 32 kDa and optimum activity at 55°C and at pH 5.0 using Sephadex G-200 and Sephacryl S-100 from thermophilic *Rhizomucor pusillus* isolated from decomposting orange peels. Anand *et al*. [17] purified an endo-polygalacturonase using acetone precipitation and gel filtration from a strain of *Aspergillus* fumigatus. They determined the molecular weight to be 43.0 kDa, stable at a pH range of 7–10 and with optimum activity at 30°C. The polygalacturonase was stimulated by Cu^{2+} and K^{+} but inhibited by Ag^{+}, Hg^{2+} and Ca^{2+}. Doughari and Onyebarachi [18] produced polygalacturonase from *Aspergillus flavus* isolated from orange peel with maximum activity in the presence of polygalacturonic acid at 35°C and pH 4.5. Carrasco *et al*. [19] reported from their studies the expression of a polygalaturonase in *Pichia pastoris* with an optimum activity 15°C higher than its mesophilic counterpart. According to Thakur [20], *Mucor circinelloides* was able to produce an extracellular polygalacturonase in a growth medium with pectin methyl ester (1% w/v) as carbon source and a combination of casein hydrolysate (0.1% w/v) and yeast extract (0.1% w/v) as nitrogen source. Optimum polygalcturonase activity was obtained at pH 4.0.

The aim of this study was to determine the physiological conditions that will inhibit the growth of major and specific fungal phytopathogens of rice (*Oryza sativa*) in Nigeria, West Africa. The present study will establish the contributions of endo-polygalacyuronases produced by *Lasiodiplodia theobromae* and *Rhizoctonia solani* to the deterioration of rice (*Oryza sativa*) cultivated in Nigeria. Control measures which include conditions inhibiting endo-polygalacturonases from these specific fungal phytopathogens can be adapted in the cultivation (pre-harvest) and storage (post-harvest) of rice (*Oryza sativa*) globally.

2. Materials and methods

2.1 Source and identification of isolates

The tropical fungal strains of *Lasiodiplodia theobromae* and *Rhizoctonia solani* for this investigation were isolated from deteriorated rice (*Oryza sativa*) obtained from Cocoa Research Institute, Ibadan, Nigeria. The isolates were identified at the International Institute of Tropical Agriculture, Ibadan, Nigeria. The isolates were cultured on potato dextrose agar on plates and slants.

2.2 Inocula and their culture conditions

Lasiodiplodia theobromae and *Rhizoctonia solani* isolated from deteriorated rice (*Oryza sativa*) were grown in a fungal growth medium with defined specific nitrogen and carbon sources for growth. The isolates were cultured on plates and slants containing potato dextrose agar at 30°C. Ninety six-hour-old cultures of isolates were used in the investigation [21]. Isolates were cultured in a growth medium composed of $MgSO_4.7H_20$ (0.1 g), K_2HPO_4 (2 g), KH_2PO_4 (0.5 g), $FeSO_4.7H_2O$ (1 mg), KNO_3 (9.9 g) and pectin (10 g) source (Sigma-Aldrich, USA) per 1 litre of distilled water (The pH of the medium was adjusted to pH 5.0 using 0.1 N HCl and 0.1 N NaOH). Conical flasks (250 ml) containing 100 ml growth medium were inoculated with 1 ml of an aqueous spore suspension containing approximately 6 x 10^5 spores

per ml of each isolate. These were the experimental flasks. Control flasks contained un-inoculated medium. Experimental and control flasks were incubated without shaking at 30°C. Protein content was determined [22].

2.3 Enzyme extraction

Contents of flasks were carefully filtered through glass fibre filter paper (Whatman GF/A) on the tenth day of inoculation of growth medium. Protein content of the filtrates was determined [22]. The filtrates were also assayed for polygalacturonase activity [23, 24].

2.4 Precipitation using ammonium sulphate

The crude enzymes of the isolates was treated with ammonium sulphate (analytical grade, Sigma) within 40–90% saturation. Precipitation was at 4°C for 24 hours. Centrifugation was done at 4000 rpm for 30 minutes at 4°C using a high speed cold centrifuge. The precipitate was re-constituted in 0.2 M citrate phosphate buffer (pH 5.0). Protein content of the precipitated enzyme was determined [22]. Polygalacturonase activity was also determined [23, 24].

2.5 Dialysis

The ammonium sulphate precipitated enzyme of each isolate was dialyzed using acetylated dialysis tubings (Visking dialysis tubings, Sigma) [25] and a multiple dialyser (Pope Scientific Inc. Model 220, USA). The enzyme preparation was dialyzed using 0.2 M citrate phosphate buffer (pH 5.0) at 4°C for 24 hours under several changes of the buffer. The protein content of the dialyzed enzyme was afterwards determined using the Lowry *et al.* [22] method. Polygalacturonase activity of the dialyzed enzyme was also determined [23, 24].

2.6 Enzyme assay

2.6.1 Endo-polygalacturonase (PG)

Reaction mixture consisted of 1 ml of 0.1% (w/v) pectin in 0.2 M citrate phosphate buffer (pH 5.0) as substrate and 0.5 ml enzyme. Controls consisted of only 1 ml of substrate with no enzyme added. The contents of both experimental and control tubes were incubated at 35°C for 1 hr. The reaction in each tube was terminated with 3 ml of dinitrosalicylic acid reagent (Appendix 1). Thereafter, 0.5 ml of enzyme was added to the controls. The contents of tubes were then boiled for 15 minutes. Optical density readings were taken at 540 nm using a colorimeter. The total reducing sugars released in the reaction mixtures was determined by the Dinitrosalicylate (DNSA) method [23, 24]. One unit of endo-polygalacturonase activity was defined as the amount enzyme in 1 ml of reaction mixture which released reducing sugars equivalent to 100 μg galacturonic acid per minute under specified assay conditions. Specific activity was expressed as enzyme units per mg protein.

2.7 Determination of protein concentration

Using the Lowry *et al*. [22] method, protein concentration was determined at every stage of the investigation. Reaction was with copper in the presence of alkali (Appendix 1).

2.8 Fractionation of enzyme using gel filtration and ion-exchange chromatography

Endo-Polygalacturonase from *Lasiodiplodia theobromae* and *Rhizoctonia solani* were subjected to further purification by gel filtration using a Sephadex G-100 column and ion-exchange chromatogeaphy using CM-Sephadex C-25 and CM-Sephadex C-50 columns. The Sephadex G-100 column was calibrated with proteins of known molecular weight [26, 27].

2.9 Characterisation of the purified endo-polygalacturonases

The effects of temperature, pH, substrate concentrations, certain cations and specific inhibitors on the activity of the purified endo-polygalacturonases from *Lasiodiplodia theobromae* and *Rhizoctonia solani* were investigated.

2.10 Effect of temperature

The substrate used was 0.1% (w/v) pectin (Sigma-Aldrich, USA) in 0.2 M citrate phosphate buffer (pH 5.0). The reaction mixture consisted 1 ml of substrate and 0.5 ml of enzyme. Incubation was at a range of 5-70°C for 1 hr.

2.11 Heat stability test at 80°C

The effect of heat (80°C) on the stability of the purified endo-polygalacturonase at different periods, 5, 10, 15, 20 and 25 minutes was carried out. The activity of the heated endo-polygalacturonase was determined by incubating 0.5 ml of enzyme plus 1 ml of 0.1% (w/v) pectin (Sigma-Aldrich, USA) in citrate phosphate buffer (pH 5.0) substrate at 35°C for 1 hr.

2.12 Effect of pH

The substrate used was 0.1% (w/v) pectin (Sigma-Aldrich, USA) in 0.2 M citrate phosphate buffer at different pH values ranging from pH 4.0–8.5. The reaction mixture consisted 1 ml of substrate and 0.5 ml of enzyme. Incubation was performed at 35°C for 1 hr.

2.13 Effect of concentrations of substrate (pectin)

Concentrations which were 0.05–0.3% (w/v) pectin (Sigma-Aldrich, USA) constituted in 0.2 M citrate phosphate buffer (pH 5.0) were used as substrate in this investigation. The reaction mixture consisted 1 ml of substrate and 0.5 ml of enzyme, incubated at 35°C for 1 hr.

2.14 Effects of cations

The cations NaCl and $CaCl_2$ were used at different concentrations (5, 10, 15, 20 and 30 mM) for the activity of the purified endo-polygalacturonase. Each cation was constituted in 0.1% pectin in citrate phosphate buffer (pH 5.0. The reaction mixture was 1 ml of substrate and 0.5 ml of enzyme incubated at 35°C for 1 hr.

2.15 Effects of certain inhibitors

2,4-Dinitrophenol and ethylenediamine tetraacetic acid were the inhibitors used in this investigation. They were prepared at concentrations of 2, 4, 6, 8 and 10 mM

in 0.1% pectin (Sigma-Aldrich, USA) in citrate phosphate buffer at pH 5.0. These were the substrates.

2.16 Effects of specific nitrogenous compounds

The nitrogenous compounds used were potassium nitrate, ammonium sulphate and ammonium chloride. Pectin was the constant carbon source in the growth medium. Endo-polygalacturonase activity expressed by the fungal strains of *Lasiodiplodia theobromae* and *Rhizoctonia solani* were investigated. Endo-Polygalacturonase activity expressed on the tenth day of incubation at 30°C was recorded.

3. Results

When strains of *Lasiodiplodia theobromae* and *Rhizoctonia solani* isolated from deteriorated rice (*Oryza sativa*) were grown in a defined growth medium containing potassium nitrate as nitrogen source and pectin as carbon source, they expressed endo-polygalacturonase activities at 30°C within ten days. The enzymes were isolated and purified by ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. The purification steps are presented on **Tables 1** and **2**. Purification of the endo-polygalacturonase from the *Lasiodiplodia theobromae* by gel filtration using Sephadex G-100 gave four peaks of absorption designated A, B, C. Only the fractions with peak B expressed endo-polygalacturonase activity. The molecular weight estimate of endo-polyglacturonase produced by the *Lasiodiplodia theobromae* was 124,000 Daltons. Purification of the endo-polygalacturonase from *Rhizoctonia solani* by gel filtration (Sephadex G-100) gave two peaks of absorption designated D and E. Only the fractions of peak E expressed endo-polygalacturonase activity. The molecular weight estimate of endo-polygalacturonase from *Rhizoctonia solani* was 92,000 Daltons.

Fraction	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)	Purification fold
Crude extract	4120	60.2	68.4	100	1
90% $(NH_4)_2SO_4$ Precipitation	3228	40.3	80.0	78.3	1.16
Sephadex G-100 Gel filtration Chromatography					
Peak A	2139	10.6	201.79	51.9	2.95
Peak B	1023	8.3	123.25	24.8	1.80
Peak C	995	4.6	216.3	24.1	3.16
CM-Sephadex C-50 Ion-Exchange Chromatography					
Peak Ba	966	2.8	345	23.4	5.04

Table 1.
Purification of endo-polygalacturonase from Lasiodiplodia theobromae *isolated from deteriorated rice (*Oryza sativa*).*

Fraction	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)	Purification fold
Crude extract	1615	32.2	50.1	100	1
90% $(NH_4)_2SO_4$ Precipitation	1328	22.1	60.1	82.2	1.2
Sephadex G-100 Gel filtration Chromatography					
Peak D	1121	3.1	361.6	69.4	7.2
Peak E	926	7.4	125.1	57.3	2.5
CM-Sephadex C-25 Ion-Exchange Chromatography					
Peak Eb	895	6.5	137.7	55.4	2.7

Table 2.
Purification of endo-polygalacturonase from Rhizoctonia solani *isolated from deteriorated rice (*Oryza sativa*).*

The purified endo-polygalcuronase from the *Lasiodiplodia theobromae* (ion-exchange chromatographic fraction - CM-Sephadex C-50) exhibited optimum activity at 30°C and at pH 4.5 while the purified endo-polygalacturonase from the *Rhizoctonia solani* (ion-exchange chromatographic fractions - CM-Sephadex C-25) exhibited optimum activity at 32°C and at pH 5.0.

The purified endo-polygalacturonases from both the *Lasiodiplodia theobromae* and the *Rhizoctonia solani* exhibited optimum activities at 0.2% pectin concentration.

The purified endo-polygalacturonases from both fungi were stimulated by Ca^{2+} They were inhibited by ethlylenediamine tetracetic acid (EDTA) and 2,4-dinitrophenol.

The purified endo-polygalacturonase from the *Lasiodiplodia theobromae* lost 80% of its activity within 20 minutes of heat at 80°C. While the purified endo-polygalacturonase from the *Rhizoctonia solani* lost 82% of its activity within 20 minutes of heat at 80°C.

Potassium nitrate as nitrogen source in the defined growth medium with pectin as carbon source supported highest activity of endo-polygalacturonase by the *Lasiodiplodia theobromae* however, ammonium chloride as nitrogen source in the defined growth medium with pectin as carbon source supported highest activity of endo-polygalacturonase by the *Rhizoctonia solani*.

4. Discussion

Pectin is found in the tissues of rice (*Oryza sativa*) [28]. In this investigation, we observed that the fungal pathogens *Lasiodiplodia theobromae* and *Rhizoctonia solani* isolated from deteriorated rice (*Oryza sativa*) produced endo-polygalacturonases in a growth medium containing pectin as carbon source and potassium nitrate as nitrogen source of fungal growth at 30°C. In Nigeria, West Africa, *Lasiodiplodia theobromae* and *Rhizoctonia solani* are fungal pathogens of rice (*Oryza sativa*) in the field [9, 10, 29]. From the results of this study, we can therefore establish that *Lasiodiplodia theobromae* and *Rhizoctonia solani* are capable of causing the deterioration of rice (*Oryza sativa*) at 30°C by breaking down the grain's pectin portion.

In this research, we observed that the molecular weight of endo-polygalacturonase from our strain of *Lasiodiplodia theobromae* using Sephadex G-100 has an estimate of 124,000 Daltons while that of our strain of *Rhizoctonia solani* has an estimate of 92,000 Daltons. Adejuwon *et al.* [11] reported from their studies a polygalacturonase from *Penicillium funiculosum* Thom. isolated from tomato fruits with molecular weight estimate of 89,100 Daltons. Ajayi *et al*. [12] in their own investigation purified polygalacturonase from *Rhizopus arrhizus* Fisher isolated from tomato fruits with molecular weight estimates of approximately 166,000 Daltons and 60,260 Daltons. Olutiola [13] reported from his investigation, an extracellular polyglacturonase complex from *Penicillium citrinum* associated with internal mouldiness of cocoa (*Theobroma cacao*) beans having molecular weights of 30,000 and 56,000.

The purified endo-polygalcuronase from our *Lasiodiplodia theobromae* exhibited optimum activity at 30°C and at pH 4.5 while that from our *Rhizoctonia solani* exhibited optimum activity at 32°C and at pH 5.0. Olutiola [30] reported that *Penicillium sclerotigenum* Yamamoto isolated from rotten yam tuber produced a polygalacturonase with optimum activity at pH 5.0.

The purified endo-polygalacturonases from both fungi exhibited optimum activities at 0.2% pectin concentration. The purified endo-polygalacturonases from both fungi were stimulated by Ca^{2+} but inhibited by ethlylenediamine tetracetic acid (EDTA) and 2,4-dinitrophenol. Ajayi *et al.* [31] purified polygalacturonase from *Rhizopus arrhizus* Fisher with optimum activity at pH 4.5, stimulated by Ca^{2+} but inhibited by ethlylenediamine tetracetic acid (EDTA) and 2,4-dinitrophenol. The purified endo-polygalacturonase from our *Lasiodiplodia theobromae* lost 80% of its activity within 20 minutes of heat at 80°C. While the purified endo-polygalacturonase from our *Rhizoctonia solani* lost 82% of its activity within 20 minutes of heat at 80°C. Potassium nitrate as nitrogen source in the defined growth medium with pectin as carbon source supported highest activity of endo-polygalacturonase by *Lasiodiplodia theobromae* while ammonium chloride as nitrogen source in the defined growth medium with pectin as carbon source supported highest activity of endo-polygalacturonase by *Rhizoctonia solani*. According to Olutiola [32], pectin as carbon source with L-asparagine as nitrogen source of a defined growth medium supported the growth and sporulation of *Fusarium oxysporum* Schlecht. In conclusion, the conditions inhibiting endo-polygalacturonases from *Lasiodiplodia theobromae* and *Rhizoctonia solani* capable of degrading the pectin portion of rice (*Oryza sativa*) observed in this study can be adapted as feasible control measures limiting the infection and contamination of rice (*Oryza sativa*) by these phytopathogens on the field and at postharvest. Temperature and pH extreme from 30°C and pH 4.5 will be feasible inhibitory control measures for the growth of *Lasiodiplodia theobromae* on rice (*Oryza sativa*) in Nigeria and particularly the preservation of pectin in rice (*Oryza sativa*) from degradation by this particular phytopathogen. Temperature and pH extreme from 32°C and pH 5.0 will inhibit growth of *Rhizoctonia solani* on rice (*Oryza sativa*) and preserve pectin in this grain from degradation by *Rhizoctonia solani*.

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Conflict of interest

Authors declare no conflict of interest.

A. Appendix

A.1 DNSA reagents for terminating endo-polygalacturonase activity

Reagents [17]

i. Sodium hydroxide – 10 g

ii. Potassium sodium tartrate – 200 g

iii. Phenol – 2 g

iv. 3, 5-Dinitrosalicylic acid – 10 g

v. 5% Sodium sulphite – 10 ml

vi. 0.03% Glucose – 10 ml

vii. Distilled water – 1Litre

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