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Purification and Characterization of an Extracellular Poly-Galacturonase from *Rhizoctonia solani* Kühn (AG2-2)

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Abstract: An extracellular poly-golacturonase (PG) (E.C. 3.2.1.15) was purified up to 116.77 fold from the culture filtrate of *Rhizoctonia solani* Kühn (AG2-2) by dialysis, precipitation with 0.7 saturation ammonium sulfate, gel filtration through Sephadex G-100 and ion-exchange chromatography on diethylaminoethyl cellulose with a yield of 72.397% and specific activity of 32.5 units/mg protein. The purified enzyme exhibited maximal activity at pH 4.5 at 30° and was stable in the pH range of 3.0 to 5.0 and at temperature up to 30° k_m of the enzyme was calculated to be 3.5 mg/ml. The molecular weight was determined by SDS-polyacrylamide gel electrophoresis to be 55 kDa. Participation of SH-groups in the catalytic sites of the enzyme is confirmed. Quantitative estimation of amino acids in the purified enzyme obtained from the culture of *R. solani* showed that it contained 17 amino acids and the proteins were rich with the aromatic amino acids, phenylalanine and tyrosine (50.59% of the total amino acids). And moderate amount of Acidic amino acids, aspartic and glutamic (23.78% of the total amino acids). And on the other hand, glycine is present in abstemious proportion (2.01% of the total amino acids).

Key words: Extracellular poly-golacturonase · *Rhizoctonia solani* · Purified enzymes · Soluble protein

INTRODUCTION

The soil-borne plant pathogen *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) occurs worldwide and cases economically important diseases on a large variety of vegetable and filed crops such as cereals, cotton, potato, sugar beet, turf grasses, ornamentals, fruits and forest trees [1] inflicting yield losses averaging up to 20% yearly in over 2000 crop worldwide. This fungus is not homogenous species, but is composed of at least twelve group [2], varying in cultural morphology and pathogenicity. A system of anastomosis grouping based on hyphal fusion is widely accepted as the basis from recognizing groups within the species complex [3]. At least 12 anastomosis groups (AGs) have been reported, AGs 1-11 and AGBI [2]. AG classification of *R. solani* isolates is a valuable approach for identifying the various groups inciting plant disease. Five of the AGs have been further divided into subgroups. *R. solani* AG2 is currently divided into AG2-1, AG2-2 and AG2-3[4], based on hyphal fusion frequency [5], thiamine requirements and pathogenicity. Isolates of AG2-1 mainly infect Cruciferae, AG2-2 isolates are mainly pathogenic to Chenopodiaceae and AG2-3 isolates are soybean pathogens. In AG2-1, host plant preference indicates the presence of two distinct ecological types: AG2-1, pathogenic to Cruciferae and AG2-t, pathogen Liliaceae as well as to Cruciferae [6, 7].

Plant pathogenic fungi can produce a number of plant cell wall-degrading enzymes. Pectolytic enzymes secreted by the pathogens have been considered to play a key role in tissue maceration [8] and also suggested to be one of the important requirements for colonization of plant tissue [9]. These enzymes include pectin esterase, pectin lyase, pectate lyase and polygalacturonase. It is relevant that production and activity of cell wall-degrading enzymes of plant pathogenic fungi on cell walls of their susceptible hosts vary among fungal species of the same genus [10], races of the same subspecies [11], under the impact of various chemical and biological stresses [12]. Among the pectic enzymes involved in cell wall-degradation, this study was focused on the polygalacturonase (PG, poly [1,4-α-D-galacturonide] glycanohydrolase). The importance of poygalacturonase

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(PG, EC 3.2.1.15) produced by many plant pathogens, have been strongly implicated in contributing to pathogenicity and virulence [12]. Moreover, the enzyme is supposed to act before other pectic enzymes [13] and is the first pectolytic enzyme secreted by pathogenic fungi when cultured on isolated cell walls [14]. Polygalacturonases are able to activate the plant defense response by decreasing cell wall fragments which induce the synthesis of phytoalexins [15]. PGs are also able to interact with particular cell wall proteins suggesting a possible role in the establishment of basic compatibility between a fungus and its host plant [16] and are believed to take part in the onset of plant responses to infection [17]. Enhanced PG production has been correlated to pathogen aggressiveness on plant tissue [12].

Polygalacturonases (PGs) are hydrolases that act preferentially on pectate rather than on pectin, giving rise to mono-or oligosaccharides of different sizes. Fungal PGs show a great variation in physical and chemical properties as well as in the mode of substrate hydrolysis and in the regulation of their production [18]. PG is a highly polymorphic enzyme and exhibits either an exo-or an endo-mode of action (exo-PGs and endo-PGs, respectively). It has been suggested that each enzymes would have a different role during pathogenesis. Endo-PGs would be more effective in tissue maceration and cell death [9] and would generate oligogalacturonides that could act as elicitors of plant defense responses [19]. Exo-PGs release soluble low molecular weight oligogalacturonides from highly polymeric substrates which can enter the cell where they are catabolized and act as inducers of other pectic enzymes [12]. Several fungal polygalacturonases have been purified and biochemically characterized including those from *Verticillium albo-atrum* [20], *Rhizoctonia fragariae*, *Rhizoctonia solani* [21], *Botrytis cinerea* [22], *Fusarium oxysporum* f.sp. *radicis lycopersici* [23], *Cochliobolus sativus* [24], *Sclerotinia borealis* [25], *Monilinia fructigena* [26], *Alternaria alternata* [27], *Sclerotium cepivorum* [28], *Venturia inaequalis* [29], *Phomopsis cucurbitae* [30], seven species of *Fusarium* [31], *Colletotrichum acutatum* [32] and *Sclerotium rolfsii* Sacc [18]. Literature data concerning purification and characterization of exo-polygalacturonase from *Rhizoctonia solani* kühn (AG2-2) is very rare, this work was concerned to isolate and study the general properties of the purified exopolygalacturonase from *R. solani* kühn (AG2-2) as an effort to bridge this gap.

MATERIALS AND METHODS

Micro-Organism and Growth Medium: *Rhizoctonia solani* kühn (AG2-2) used throughout this work was previously isolated from diseased sugar beet roots by Eweis [33]. The fungus was routinely grown on potato dextrose agar (PDA) medium, maintained at 4°C and sub cultured at intervals.

Culture Medium: The experimental organism *R. solani* was inoculated in the modified medium [34] containing the following ingredients (g/100 ml) sodium polypectate, 1, $NaNO_3$, 1, $MgSO_4.7H_2O$, 0.181, K_2HPO_4, 0.697, KCl, 0.149, Thiamine-HCl, 0.001 and 2 ml of stock solution containing (mg/ml): $ZnSO_4.\ 7H_2O$, 2.85, $MnSO_4.\ 5H_2O$, 3.1, $FeCl_3.\ 2H_2O$, 8.65. The initial pH of the medium was adjusted to 6.8 and autoclaved. The sterilized flasks were inoculated with 6mm mycelium discs cut from the margin of 7 days-old colonies and incubated at 28°C for 7 days statically. Samples were taken after time intervals of inoculation, pH and other parameters were measured.

Analytical Method

The Amount of Soluble Protein: was estimated by the use of Folin Ciocalteau's reagent according to the method described by Lowry *et al*. [35] using bovine serum albumin as standard protein.

Exo-Polygalacturonase Assay: Exo-polygalacturonase was assayed by measuring the release of reducing groups from the substrate according to Naguib [36]. Reaction mixture contained 0.5 ml of 1% (w/v) sodium polypectate as substrate (Sigma, St. Louis, MO, USA) in 1 ml 0.1 M acetate buffer (pH 4.5) and 1ml of the enzyme preparation. The reaction was carried out at 30°C for 30 min. The control values, obtained by stopping, the reaction mixture by boiling for 10 min. immediately after addition of sample and were subtracted to each datum. Enzyme catalyzed cleavage of reducing groups was measured at 700 nm using Spectronic 20 spectrophotometer. Results are expressed as µg reducing sugar per ml filtrate per hour. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugars in 1min [37]. Specific activity was defined as unit of activity/mg of total extracellular protein.

Enzyme Purification: Cell-free culture liquid was dialyzed overnight at 4°C against 0.05 M Sodium acetate buffer (pH 4.5). Solid Ammonium sulfate, were slowly added to

the cell-free dialysate in a trial to precipitate the enzyme according to the method suggested by Marcus *et al*. [21]. Two and half liters sample of the filtered culture were collected and used as crude enzyme. Proteins which precipitated by 0.7 saturation of ammonium sulfate after standing overnight at 4°C contained most of the enzyme activity. This precipitate was collected by centrifugation and re-suspended in 0.05 M acetate buffer (pH 4.5). The crude enzyme protein solution was loaded on Sephadex G-100 column as described by Foldin [38].Further purification of polygalacturonase was achieved by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. DEAE-cellulose was prepared according to the procedure of Peterson and Sober [39]. The enzyme was eluted by linearly increased polarity (0.0-0.5) of NaCl solution dissolved in the mentioned buffer using the gradient mixer of LKB 2070 Ultrorac Fraction Collector. Elution was performed at a rate of 15 ml/h as controlled by the peristaltic pump. Elutes (5 ml fractions) were collected and assayed for its protein content at 254 nm in a Shimadzu UV-visible recording Spectrophotometer UV-240. The obtained fractions (5 ml each) with the highest activity were pooled and desalted by dialysis after which the enzyme solution was lyophilized, stored at 0°C and used throughout this work.

Determination of the Enzyme the Molecular Weight: The purity of the enzyme and the molecular weight was confirmed by sodium dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [40].

Amino Acid Analysis: was determined according to the method of Steven *et al*. [41]. Amino acid present in the purified exopolygalacturonase obtained from *R. solani* kühn were determined by high performance liquid chromatographic analysis (HPLC) using a modification of the PICO-TAG method. Also, the separated PTC (Phenyl Thio Carbamyle) amino acids were detected at 269 nm wave length. Before injecting the protein purified sample of *R. solani*, the instrument was calibrated by the injections of the standards.

Effect of pH on the Purified Enzyme Activity: The relative activity of the enzyme was measured at various pH values was measured the buffer used were, 0.05 M citrate (pH 3.0-3.5), 0.2 M acetate (pH 4.0-5.5), 0.1 M phosphate (pH 6.0-7.0), or 0.2 M Tris-HCL buffer (pH 7.0-8.0) for 15and 60 min.

Effect of Temperature on the Enzyme Activity: The activity of the enzyme was tested after pre-incubation of the enzyme at different temperature (25-35°C). The enzyme preparation was held at the incubated temperatures 15 or 60 minutes, substrates was then added and reaction mixture was allowed to settle for 30 min at 30°C. and then the enzyme solution subjected to assay.

The Effect of Substrate Concentration on the Enzyme Activity: The activity of the enzyme was tested after pre-incubation of the enzyme at different temperature Polypectate concentration was tried from (0–10 mg/ml). Reaction time mixture 30 min. at the indicated temperature, pH 4.5, reaction time 30 min at 30°C.

The effect of Inhibitors the Enzyme Activity: Reaction mixture contained 10 mg of sodium polypectate and the metal, ions or inhibitor in 0.2 M acetate buffer (pH 4.5) and enzyme in the total volume of 2 ml. The activity was measured after reaction time 30 min at 30°C.

RESULTS AND DISCUSSION

Extracellular polygalacturonase enzyme, precipitated from the cell-free dialysate with 0.7 ammonium sulfate saturation, was purified by gel filtration through Sephadex G-100, followed by ion-exchange chromatography on DEAE-cellulose as indicated in Table 1. The enzyme

Table 1: Summary of treatments used for the purification of extracellular polygalacturonase of *R. solani*

Treatments	Total activity (Units)	Total Protein (mg)	Specific activity (Units/mg protein)	Recovered Protein (%)	Yield (%)	Purification
Cell-Free culture	41.3	148.6	0.278	100.0	100.0	1.00
Cell-free dialysate	38.6	146.3	0.264	89.45	93.462	0.949
$(NH_4)SO_4$ precipitation (0.7 saturation)	37.4	35.6	1.052	23.96	90.557	3.780
Gel filtration (Sephadex G-100)	35.6	13.1	2.718	8.82	86.199	9.773
Ion exchange chromatography (DEAE-cellulose)	29.9	0.92	32.5	0.62	72.397	116.77

purification procedure resulted in a 116.77 fold purified exopolygalacturonase from *R. solani* with a specific activity of 32.5 units/mg proteins. The purification procedure was also used by Isshiki *et al*. [27] for *Alternaria alternata* extracellular polygalacturonase and resulted in 53- fold purification. In this connection, Isshiki *et al*. [27] purified endopolygalacturonase from the culture filtrate of *Alternaria alternata* of 25-day-old cultures. The majority of polygalacturonase activity in the culture filtrates was precipitated by (30-60%) ammonium sulphate. With these procedures, polygalacturonase was purified about 31-fold from the filtrates with a 39% yield and the specific activity of the isolated polygalacturonase was about 12 units /mg. The polygalacturonase bound to CM-Sepharose but was eluted in fractions 26-27 by salt buffer. The eluted materials were purified by cation exchange HPLC. A peak of polygalacturonase activity in fractions 18 and 19 was collected and further subjected to HPLC gel filtration. There was a major protein peak in fraction 15 and only this peak possessed polygalacturonase activity. With these procedures, polygalacturonase was purified about 31-fold from the filtrates with a 39% yield and the specific activity of the isolated polygalacturonase was about 12 units/mg. The protein concentration of the final isolated polygalacturonase from 2.5 liters of the culture filtrates was 0.23 mg. Al-Rajhi [18] found that an extracellular polygalacturonase was purified up to 123.22 fold from the culture filtrate of *Sclerotium rolfsii* Sacc. by dialysis, precipitation with 0.7 saturation ammonium sulfate, gel filtration through Sepadex G-75 and ion-exchange chromatography on diethylaminoethyl cellulose with a yield of 57.79% and specific activity of 32.903 units/mg protein.

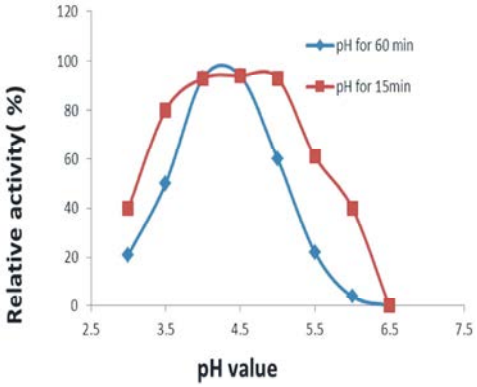

Fig. 1: Effect of pH value upon extra-cellular poly-galacturonase stability.
*The enzyme preparation was held at the indicated pH for 15 or 60 minutes. The assay conditions were those described in the legend to Fig. 1.

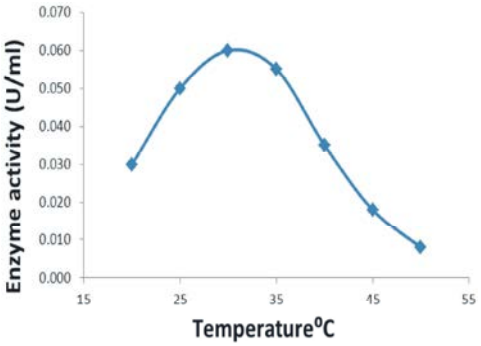

Fig. 2: Effect of temperature on extra-cellular poly-galacturonase activity.
*The reaction mixture contained 10 mg of sodium polypectate in 0.2 M acetate buffer (pH 4.5) and enzyme in the total volume of 2 ml. Reaction time mixture 30 min. at the indicated temperatures.

The effect of pH on (PG) Activity: The effect of pH values on polygalacturonase activity of *R. solani* kühn (AG2-2), presented in Fig. 1, show that maximal activity was recorded at pH 4.5, above which rapid decline occurred and complete inactivation was recorded at pH 6.5. Incubation of enzyme preparations at different pH values for either 15 or 60 min demonstrated that the enzyme is more stable at pH range with almost complete stability at pH 4.0-4.5 (Fig. 2). High inactivation of enzyme activity was recorded on either side of the pH range 3.0 to 5.0. This can be attributed to the decreasing affinity of enzyme to its substrate and/or due to an irreversible destruction of the enzyme protein. In this connection, Barmore and Brown [42] found that the pH 4.5 optimum of the purified exopolygalacturonase from *Penicillium italicum* infected orange fruit. The minimum activity was observed above pH 7.0. Pardo *et al*. [43] found that the optimal pH for the activity of exopolygalacturonase (exo-PGase) from *Geotrichum lactis* was 5.0. The activity of exo-PGase was relatively stable in the range of pH values 3.5-7.0 and decreased significantly beyond these limits. Rao *et al*. [44] found that the optimal pH for activity of the purified endopolygalacturonase from *Aspergillus ustus* was 4.6. Kollar [29] observed that the best pH for highest endopolygalacturonase activity of *Venturia inaequalis* was at pH 4.0 and decreased with increasing pH. Zhang *et al*. [30] evidenced that polygalacturonase isozyme from *Phomopsis cucurbitae* was active in low pH-range from 2.0 to 6.0 and was optimal at pH 5.0.

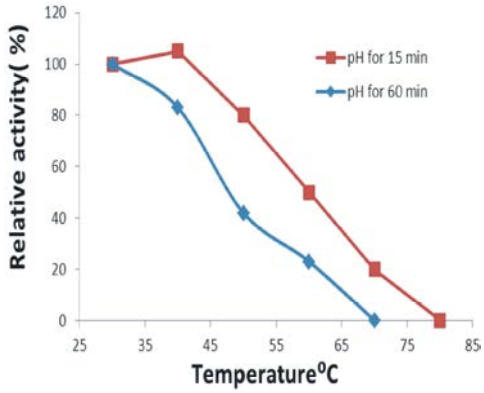

Fig. 3: Effect of temperature on extracellular polygalacturonase stability.
*The enzyme preparation was held at the incubated temperatures 15 or 60 minutes. The assay conditions were those described in the legend to Fig.3.

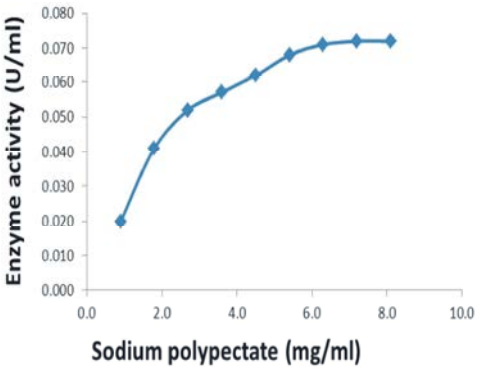

Fig. 4: Effect of sodium polypectate concentration on extracellular polygalacturonase activity.
*The reaction mixture contained the indicated concentration of sodium polypectate in 0.2 M acetate buffer (pH 4.5) and the enzyme in the total volume of 2 ml. Reaction time 30 min at 30°C.

Al-Rajhi [18] found that the purified extracellular polygalacturonase from *Sclerotium rolfsii* Sacc exhibited maximal activity at pH 4.5 at 30°C and, was stable in the pH range of 3.5 to 5.0 and at temperature up to 30°C.

Effect of Temperature on the Enzyme Activity: The enzyme preparation was held at the indicated pH for 15 or 60 minutes. Extra cellular polygalacturonase of *R. solani* (AG2-2) achieved a high hydrolyzing effect at 25-35°C (Fig. 2) with an optimum temperature at 30°C in comparison with 45°C reported for extracellular polygalacturonase obtained from *Sclerotinia sclerotiorum* [45].

Table 2: Effect of some metal ions and enzyme inhibitors on the purified extra-cellular poly-galacturonase obtained from *R. solani* kühn (AG 2-2)

	Relative enzyme	
Enzyme inhibitor or [ ] Metal ion	1 mM	10mM
0.0	100	100
Sodium arsenate	58.3	58.3
Sodium arasenite	88.2	71.3
Sodium fluoride	94.7	80.5
Sodium azide	96.9	83.7
Iodoacetate	37.4	12.6
1,4-dinitrophenol	71.2	60.5
EDTA	82.5	72.1
K^+	100.0	100.0
Na^+	100.0	100.0
Ca^{2+}	110.0	100.0
Cu^{2+}	100.0	100.0
Co^{2+}	96.4	82.3
Mg^{2+}	114.1	103.2
Mn^{2+}	135.2	122.7
Zn^{2+}	100.0	100.0
Hg^{2+}	0.0	10.0
Fe^{3+}	100.0	100.0

Although 40°C is the optimum when the enzyme was incubated for one hour, it was unable to retain complete activity results of experiments of the thermal stability of the enzyme shown in Fig. 3 indicated that the enzyme loses complete activity after incubation for 1 hr at 70°C.

Activity of *R. solani* kühn (AG2-2) exop-olygalacturonase as affected by some metal ions and enzyme inhibitors is shown in Table 2. It shows that the enzyme activity was promoted in the presence of Mn^{2+}, whereas low stimulation was recorded in the presence of 1 mM of Mg^{2+} or Co^{2+}

Effect of Substrate Concentration on Enzyme Activity: The effect of substrate concentration on enzyme activity is shown in Fig. 4. From the line weaver- Burke plot of the reciprocal of the initial velocity and sodium polypectate concentrations, the K_m value using sodium polypectate as substrate was calculated to be 3.5 mg/ml (Fig. 5) [18]. The results obtained in this study are supported by those obtained by Riou *et al*. [45], who calculated the K_m value for *Sclerotinia sclerotiorum* endopolygalacturonase as 0.03 mg/ml sodium polypectate. The results are also in accordance with other fungal PGase K_m values that range between 0.1 and 4.0 mg/ml [18].

The (PG) Enzyme Molecular Weight: It is of interest to state the enzyme was found stable for 3 and 7 days at 4 and 15°C, respectively, when kept in 0.2 M acetate

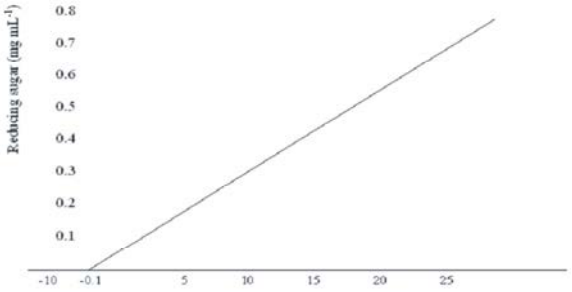

Fig. 5: Line weaver-Burke plot for determination of K_m value of *R. solani* Kühn (AG2-2) purified extracellular
*The substrate was sodium polypectate at various concentrations in acetate buffer at pH 4.5. Enzyme concentration was 0.1 g 100 m/l.

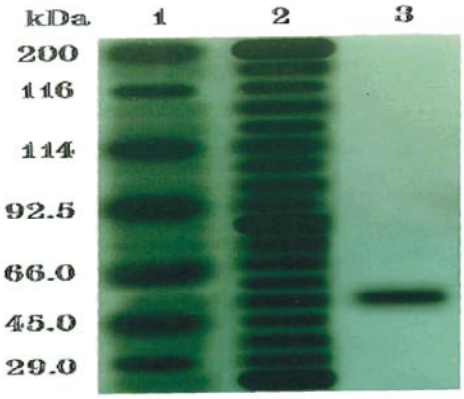

Fig. 6: SDS-PAGE for the purified extra-cellular polygalacturonase obtained from *R. solani* kühn (AG 2-2).
Lane 1 : Molecular mass standards (kDa) (myosin, 200, β-glactosidase, 116, α-glactosidase, 114, phosphorylase b, 92.5, bovine serum albumin, 66, ovalbumin, 45 and carobonic anhydrase, 29).
Lane 2: Culture filtrate of *R. solani*.
Lane 3: Purified *R. solani* extracellular polygalacturonase enzyme protein.

buffer (pH 4.5). The half-life ($T^1/_2$) of this enzyme was three weeks at both temperatures. The molecular weight of the enzyme was estimated at 55 kDa. The presence of a single protein band in SDS-PAGE indicates that the purified enzyme is homogeneous as shown in Fig 6. The characteristics of the purified *R. solani* kühn (AG 2-2) polygalacturonase were compared theoretically with other fungal polygalacturonases. Fungal polygalacturonases (PGases) are typically single subunit proteins of 30-85 kDa [18].

Table 3: Amino acid composition of the purified extra-cellular polygalacturonase obtained from *R. solani* kühn (AG 2-2)

Amino acid	Amount (mg/100 gm enzyme)
Alanine	3.01
Arginine	2.57
Aspartic acid	1.94
Cysteine	1.42
Glutamic acid	18.3
Glycine	1.71
Histidine	2.29
Isoleucine	0.54
Leucine	1.57
Lysine	3.21
Methionine	0.61
Phenylalanine	1.79
Proline	0.84
Serine	1.88
Threonine	1.52
Tyrosine	41.27
Valine	0.64
Total	85.11

The (PG) Enzyme Amino Acids Composition: Table 3 shows amino acids composition of the purified extracellular polygalacturonase of *R. solani* (AG 2-2). The enzyme contained 17 amino acids consistently rich in the aromatic amino acids especially tyrosine and phenylalanine which both comprised 50.59% of the total amino acid of the purified enzyme for *R. solani*. The enzyme was also rich in the acidic amino acids aspartic and glutamic acids which both constitute 23.78% of the total amino acids for the pathogen. The enzyme contained moderate amounts in the basic amino acids such as arginine, lysine and histidine which comprised only 9.48% of the total amino acids. Also, the polar amino acids alanine, valine, leucine and isoleucine comprised moderate portions and contributed to about 6.77% of the total amino acids. On the other hand, the enzyme of the pathogen had low content of the SH-containing amino acid cysteine, also, serine, threonine, proline, methionine and glycine. These results are similar to that obtained by Rao *et al*. [44], who reported that the pure endo-polygalacuronase enzyme from *Aspergillus ustus* contained 17 amino acids including threonine, serine, glutamic acid, tyrosine, glycine, alanine, valine, methionine, isoleucine, leucine, histidine, arginine, lysine, tryptophan, phenylalanine, proline and aspartic acid. In this respect, Reddy and Rao [46] found that glutamic acid, lysine, histidine, alanine, leucine and isoleucine were the most abundant amino acids in the hydrolysate from a virulent strain (FR) of *Rhizoctonia solani* kühn, while alanine and serine were the most abundant in a non-virulent strain (GD_2). Some amino acids cause serious

injury to plants and some increase the susceptibility of plants to fungal disease [47]. It is possible that the greater production of amino acids by the virulent strain (FR) of *R. solani* kühn may therefore be significantly related to its pathogenicity [46]. Al-Rajhi [18] revealed that for quantitative estimation of amino acids in the purified proteins obtained from the culture of *Sclerotium rolfsii* Sacc., it contained 17 amino acids.

CONCLUSION

In conclusion, *R. solani* kühn (AG2-2) extracellular polygalacturonase have been purified and partially characterized. Polygalacturonase posses some important properties worthy of industrial exploitation.

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