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*Full Length Research Paper*

# Partial purification and characterization of polygalacturonase-inhibitor proteins from pearl millet

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**Polygalacturonase-inhibitor proteins (PGIPs) are plant cell wall glycoproteins, involved in the inhibition of microbial endo-polygalacturonases (EPGs). The present study involved activity guided partial purification of pearl millet [*Pennisetum glaucum* (L.) R.Br.] protein extract by cation exchange chromatography, which resulted in two pooled protein peaks – Peak-A and Peak-B, both of which showed inhibitory activity against the *Aspergillus niger* EPG. Protein separation of the two peaks by gel electrophoresis showed prominent bands between 29 and 43 kDa, consistent with the molecular weights of the known plant PGIPs. The two PGIP peaks were further studied for their inhibitory activities with respect to three parameters viz., inhibitor concentration, pH and temperature effects. Enzyme inhibition was partial and increased with inhibitor concentration. The Peak-B was found to be the more active inhibitor of the two. The results indicate the presence of at least two isoforms of PGIP in pearl millet. This is the first such study to be undertaken in understanding the presence of the PGIPs in millets.**

**Key words:** Cation-exchange chromatography, endo-polygalacturonase, inhibitor concentration, pearl millet, polygalacturonase-inhibitor protein.

## INTRODUCTION

Plants depend on their cells walls, the mechanical barrier in warding off the constant attempts by the pathogen to access the nutrients from the host reservoir (Cuixia et al., 2006). The microbial pathogens, both bacteria and fungi, are known to employ an array of cell wall degrading enzymes targeting the various polysaccharide components constituting the wall, thus making their way into the host (Juge, 2006). Endo-polygalacturonases (EPGs) are known to be one of the first and most important virulence factors released by pathogens which degrade the homopolygalacturonate (α-1,4-linked chain of D-galacturonic acid) component of pectins, a galacturonic acid rich cell wall matrix (Karr and Albersheim, 1970). Many studies have well established the role of microbial and even insect EPGs in causing serious plant diseases (Hershonhorn et al., 1990; Hugouvieux et al., 1997; Garcia-Maceira et al., 2001; Gotesson et al., 2002; Allen and Mertens, 2008).

Polygalacturonase-inhibitor proteins (PGIPs) are cell wall glycoproteins, belonging to leucine rich repeat (LRR) super family of proteins involved in plant defense against the invading pathogens by inhibiting/ modulating the activity of EPGs (Janni et al., 2008). PGIPs inhibit fungal EPGs (De Lorenzo et al., 2001; Federici et al., 2006), but are shown to be ineffective against pectic enzymes of plant origin (Cervone et al., 1990). Pathogen infection and a number of stress-related signals have been reported to induce PGIPs (De Lorenzo et al., 2001). Bean

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**Abbreviations: CMC,** Carboxy methyl cellulose; **EPGs,** endo-polygalacturonases; **ICRISAT,** The International Crops Research Institute for the Semi-Arid Tropics; **PGIPs,** polygalacturonase-inhibitor proteins; **SDS-PAGE,** sodium dodecyl sulphate - polyacrylamide gel electrophoresis.

(*Phaseolus vulgaris*) PGIPs, PvPGIP3 and PvPGIP4 were found to be effective against the insects (D'Ovidio et al., 2004). In addition to their role in plant defense, PGIPs have also been reported to be involved in wounding responses in bean (D'Ovidio et al., 2004) and in developmental processes such as hypocotyl elongation in bean (Devoto et al., 1997) and in regulation of floral organ development in rice (Jang et al., 2003). PGIPs can inhibit non-host EPGs, for example PvPGIP2 inhibits EPG of maize pathogen, *Stenocarpella maydis*. PGIP genes within a family are differentially regulated by different signal molecules through separate signal transduction pathways (Ferrari et al., 2003). PGIP is a constitutive protein and its transcript accumulation has been observed in a number of incompatible interactions involving various plant-fungal pathogen systems (Faize et al., 2003; Favaron et al., 2000; Devoto et al., 1997). Recent biotechnological approaches such as plant transformation with *pgip* genes leading to overexpression in both dicots such as tobacco, tomato and monocot maize (Joubert et al., 2006; Manfredini et al., 2005); and anti-sense expression of *pgip* genes (Ferrari et al., 2006) have confirmed the role of PGIPs as important host–resistance factors to counter the EPGs of phytopathogenic fungi.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.], a staple food for the poor parts of Asia and Africa has a history of 4000 years. It is the fifth most important cereal crop, accounting for more than 55% of global millet production. India is the largest producer of the crop at 7.3 million tonnes with an average productivity of 780 kg/ha. Poaceous crops suffer substantial yield and quality reductions due to fungal disease as is the case with most agronomic crops, and pearl millet is no exception. Downy mildew caused by *Sclerospora graminicola* (Sacc.) Schroet is a very important disease affecting the production of pearl millet. Under favorable environmental conditions for the pathogen, the disease can spread rapidly causing as much as 40% crop loss. Although, several resistant cultivars to downy mildew pathogen have been developed, they ultimately succumb to the disease due to break down of resistance. The exact reasons for this breakdown of resistance is not known as there is a lack of clear understanding of biochemical and molecular basis of resistance in pearl millet to downy mildew. Hence continuous efforts need to be made to fill the gaps in our knowledge in this important area, which has got implications in developing newer breeding strategies for obtaining pearl millet cultivars with durable host resistance to downy mildew.

Though PGIPs have been studied in some of the monocot plants such as wheat (Kemp et al., 2003), rice (Jang et al., 2003) and more recently in oil palm (Al-Obaidi et al., 2010), there are no reports of their study in economically important millets. In that direction, the present study has been the first initiative in the isolation and characterization of the PGIPs from pearl millet. Since pearl millet, though drought-resistant is prone to various fungal and bacterial diseases leading to significant yield losses, the study of the role of PGIPs in its defense both at the biochemical and molecular level will be a significant step in devising strategies to counter this menace.

## MATERIALS AND METHODS

### Plant Material

Pearl millet seeds (IP18296) obtained from The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India were used in the study.

### Extraction of total protein from pearl millet seedlings

The total proteins were extracted from one-week-old pearl millet seedlings using a modified method of Favaron et al. (1994). All the steps were carried out at 4°C. Briefly, 250 g plant tissue was homogenized in 2 volumes of cold acetone and centrifuged at 15,000 g for 30 min. The pellet was washed twice with cold acetone under the same conditions, air-dried completely and resuspended in 2 volumes of sodium acetate buffer (20 mM, pH 5 containing 1 M NaCl). The resuspended pellet was incubated at 4°C for 72 h on a shaker to facilitate leaching out of wall bound proteins. The protein resuspension was centrifuged at 15,000 g for 30 min and the resulting supernatant was dialyzed against 20 mM sodium acetate buffer, pH 4. The dialyzed protein extract was lyophilized and appropriately reconstituted in the same buffer.

### Partial purification of PGIPs by Ion-exchange chromatography

The purification was carried out at 4°C. The cation-exchange resin, carboxy methyl cellulose (CMC) (Genei, Bangalore) was packed onto a glass column (1.5 × 30 cm) and equilibrated with 20 mM sodium acetate buffer, pH 4. One hundred milligrams of total pearl millet crude protein was loaded and the column was washed with the above mentioned buffer at a flow rate of 0.5 ml/min. The 2 ml fractions collected were monitored for protein at $A_{280nm}$. The flow-through fractions were collected and the column was washed with 5 bed volumes of buffer. Gradient elution was carried out by a step-wise increase in the salt gradient (100, 200, 300, 400, 500 and 1000 mM NaCl) in the buffer. The active protein fractions were pooled separately (9 and 5 active fractions eluted at 200 and 300 mM NaCl concentrations were pooled, respectively), dialyzed against 20 mM sodium acetate buffer, pH 4, lyophilized and appropriately reconstituted in the same buffer. Protein content of all the fractions was measured according to Lowry et al. (1951) using bovine serum albumin (Hi-Media) as standard.

### Gel electrophoresis

Fifty micrograms each of the crude, flow-through, 25 µg each of Peak-A and Peak-B fractions were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970) in a 1-mm thick, 12% separating polyacrylamide gel under reducing conditions. The standard proteins for molecular mass determination obtained from

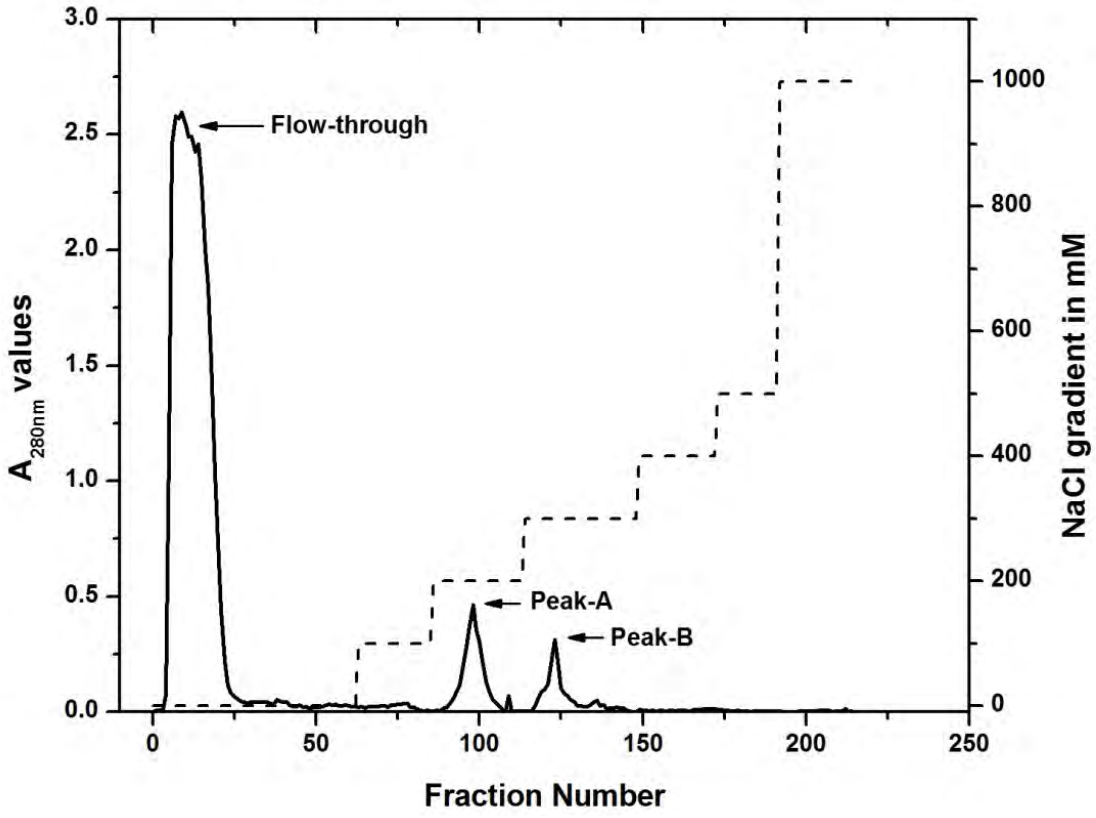


**Figure 1.** Purification profile of the pearl millet crude protein extract by carboxy methyl cellulose (CMC) column (1.5 × 30 cm).

Genei, Bangalore were used. Protein banding pattern was visualized with Coomassie blue – R 250 staining.

#### Polygalacturonase assay – Time course of hydrolysis

Pectinase from *Aspergillus niger* (Sigma) served as the source of endo-polygalacturonase. A working enzyme stock of 1 mg/ml was prepared in 20 mM sodium acetate buffer, pH 4 and a suitable aliquot of the enzyme was used for the assay. EPG activity was determined as an increase in reducing end equivalents over time. Reducing ends were measured according to the method of Wang et al. (1997) using D-glucose as the standard. The reaction mixture containing 200 µL of 2.5 mg/ml polygalacturonic acid (HiMedia), 10 ng enzyme made up to 500 µL with 20 mM sodium acetate buffer, pH 4 was incubated for 15, 30, 45 and 60 min at 30°C and assayed for the reducing equivalents. A graph with incubation time versus enzyme activity was plotted. The experiment was carried out in triplicates. The data was subjected to linear regression analysis. A time point in the linear range of the plot served as the optimal time point of incubation for the enzyme inhibition studies.

#### Enzyme inhibition studies – Assay for PGIPs

The inhibitory activity of crude, flow-through, Peak-A and Peak-B fractions was assayed as per the standard EPG assay (45 min incubation, 10 ng enzyme was used) as mentioned above. PGIP and the enzyme were pre-incubated together at 30°C for 20 min prior to assay. The effect of various parameters such as the inhibitor concentration (– at 0.5,1 and 5 µg), pH (at 4, 4.5 and 5) and temperature (at 20, 30, 40 and 50°C) was carried out. In a separate experiment, the temperature stability was studied by pre-incubating the purified peaks for 1 h at temperatures ranging from 20 to 100°C upon which its inhibition potential was assayed. Three independent experiments were performed each in triplicates. The data was subjected to Tukey's HSD test at $P < 0.05$.The percent inhibition displayed by different assayed fractions was determined in comparison to the uninhibited enzyme activity.

## RESULTS

### Partial purification of PGIPs by cation-exchange chromatography

The cation-exchanger employed in the present study in the purification of the pearl millet crude protein extract yielded two peak fractions. The first peak fraction, Peak-A eluted at a salt gradient of 200 mM NaCl whereas the second one, Peak-B eluted at 300 mM NaCl (Figure 1). The column was loaded with 100 mg crude protein and the two separately pooled peak fractions were estimated to be 3.5 and 1 mg respectively.

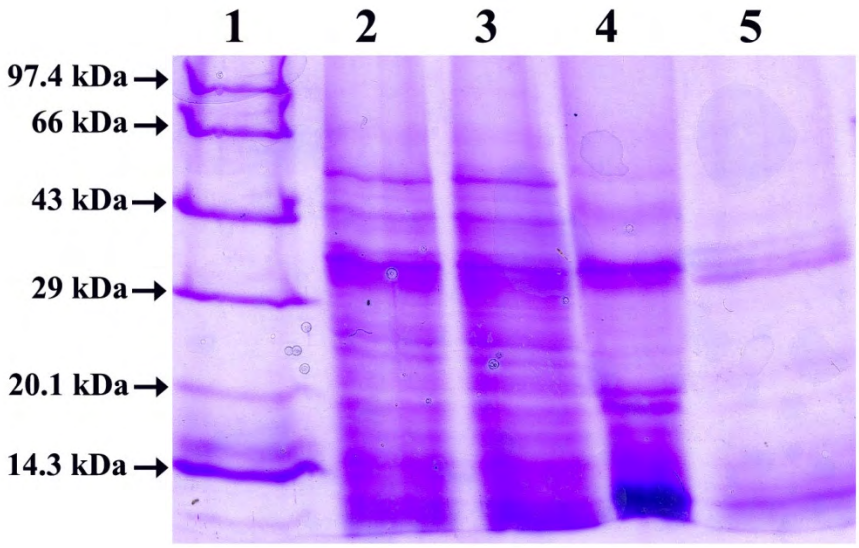


**Figure 2.** SDS-PAGE separation of proteins. Lane 1- Standard protein molecular weight markers; lane 2- pearl millet crude protein extract, lane 3- CMC column flow-through fraction; lane 4- CMC column eluted Peak-B fraction; lane 5- CMC column eluted Peak-A fraction.

**SDS-PAGE separation of partially purified protein fractions**

The analysis of peaks by SDS-PAGE, showed the presence of more number of proteins in peak B compared to peak A (Figure 2). Furthermore, silver staining of the gel did not show any additional bands. The molecular weights of the bands in Peak-A were determined to be 37 and 34 kDa, whereas Peak-B showed bands of 43, 37 and 34 kDa. In addition, few protein bands of molecular weights at and below 20 kDa and above 43 kDa were also found more predominantly in peak B.

**Endo-polygalacturonase assay optimization**

The optimal time point of incubation of the *A. niger* EPG for the enzyme inhibition studies was chosen to be 45 min based on the linearity of a plot between incubation time versus enzyme activity (Figure 3). The optimized enzyme conditions were further used for inhibition studies and percent inhibition of various protein fractions were evaluated in relation to un-inhibited enzyme activity.

**Characterization of the CMC column purified fractions - EPG inhibition studies**

**Effect of inhibitor concentration**

Inhibition studies of the crude pearl millet protein extract at three different concentrations of 0.5, 1 and 5 µg showed a very low percent inhibition of 4, 8 and 11%, respectively (Figure 4). Peak-B showed the highest percent inhibition of 18, 28 and 34% at the same concentrations tested as above. The Peak-A on the other hand showed 3, 8 and 13% inhibition, respectively which is similar to the values obtained with the crude. A general trend of increase in percent inhibition with the increase in the inhibitor concentration was observed. The CMC column flow-through showed no inhibition at any of the tested concentrations. The inhibition of all the samples was lost post boiling and upon treatment of the protein with a protease, trypsin (data not shown).

**Effect of pH and temperature**

Further characterization of the effect of physical parameters such as pH and temperature on enzyme inhibition was carried out for all the fractions at 5 µg concentration. The enzyme showed a pH optimum at pH 4. The crude extract showed inhibitory activity at all three pH units with values of 10, 12 and 12% inhibition at pH 4, 4.5 and 5, respectively. Peak-B showed the highest percent inhibition at all the three tested pH units with the percent inhibition being 36, 34 and 37%, respectively at the above mentioned pH values. In contrast, the Peak-A showed an inhibition of 12% at pH 5 and failed to show any inhibition at pH 4. At pH 4.5, slight enzyme activation was observed (Figure 5A).

The enzyme's highest activity was observed at 50°C at

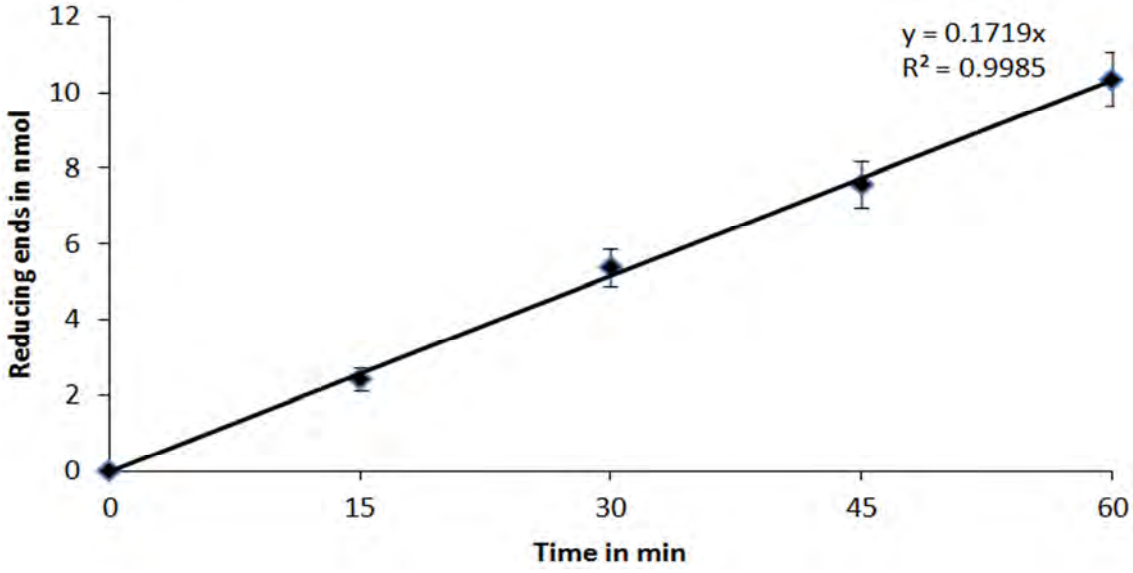


**Figure 3.** Time course of hydrolysis of polygalacturonic acid by the *A. niger* endo-polygalacturonase. The data was analyzed by linear regression analysis to determine the linear range. The data points are means of the experiment carried out in triplicates. Bars indicate ±SE.

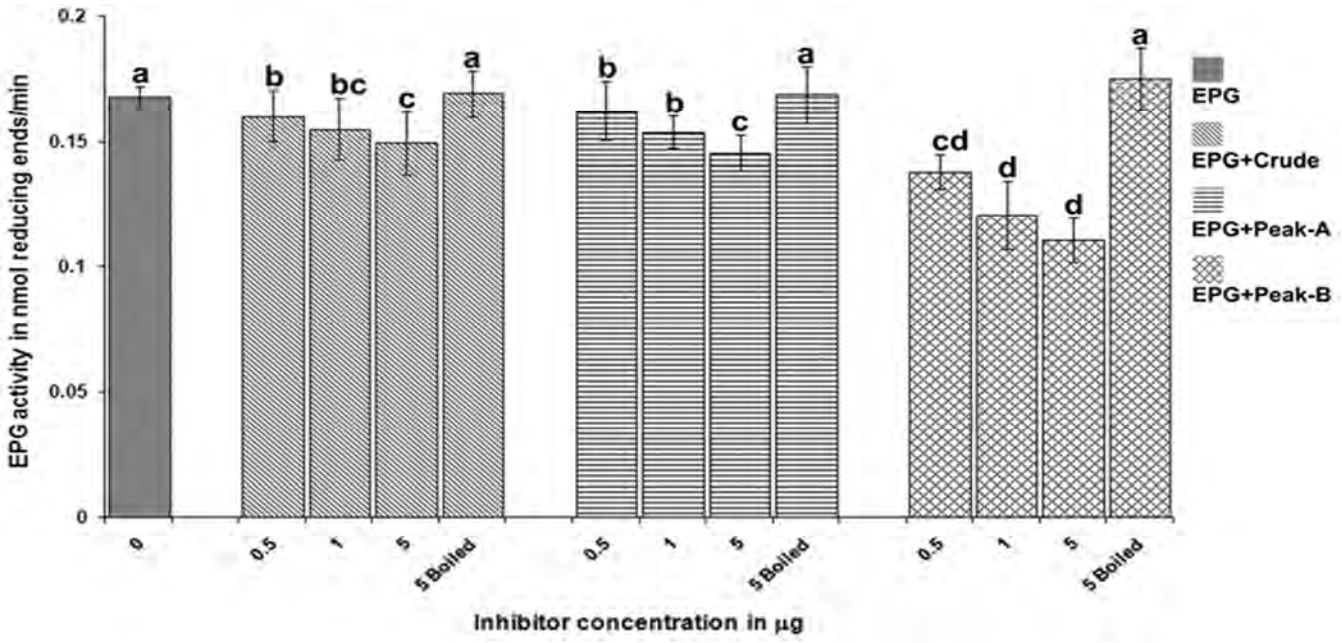


**Figure 4.** *A. niger* endo-polygalacturonase inhibition assay - Effect of inhibitor concentration. The different CMC column peaks were assayed for inhibition, each at three different concentrations (0.5, 1 and 5 µg). The data are means of three independent experiments. Bars indicate ±SE. Means designated with the same letter are not significantly different according to Tukey's HSD test at $P < 0.05$.

pH 4 with the activity values doubling at 10°C intervals. At 20°C, the crude, Peak-A and Peak-B showed 8, 15 and 35% inhibition, respectively (Figure 5B), with no inhibition being observed in case of flow-through fraction. The trend remained much the same at the other tested temperatures with inhibition reading 9, 16 and 35% at 30°C; 12, 13 and 32% at 40°C and 12, 12 and 37% respectively, at 50°C. The temperature stability studies

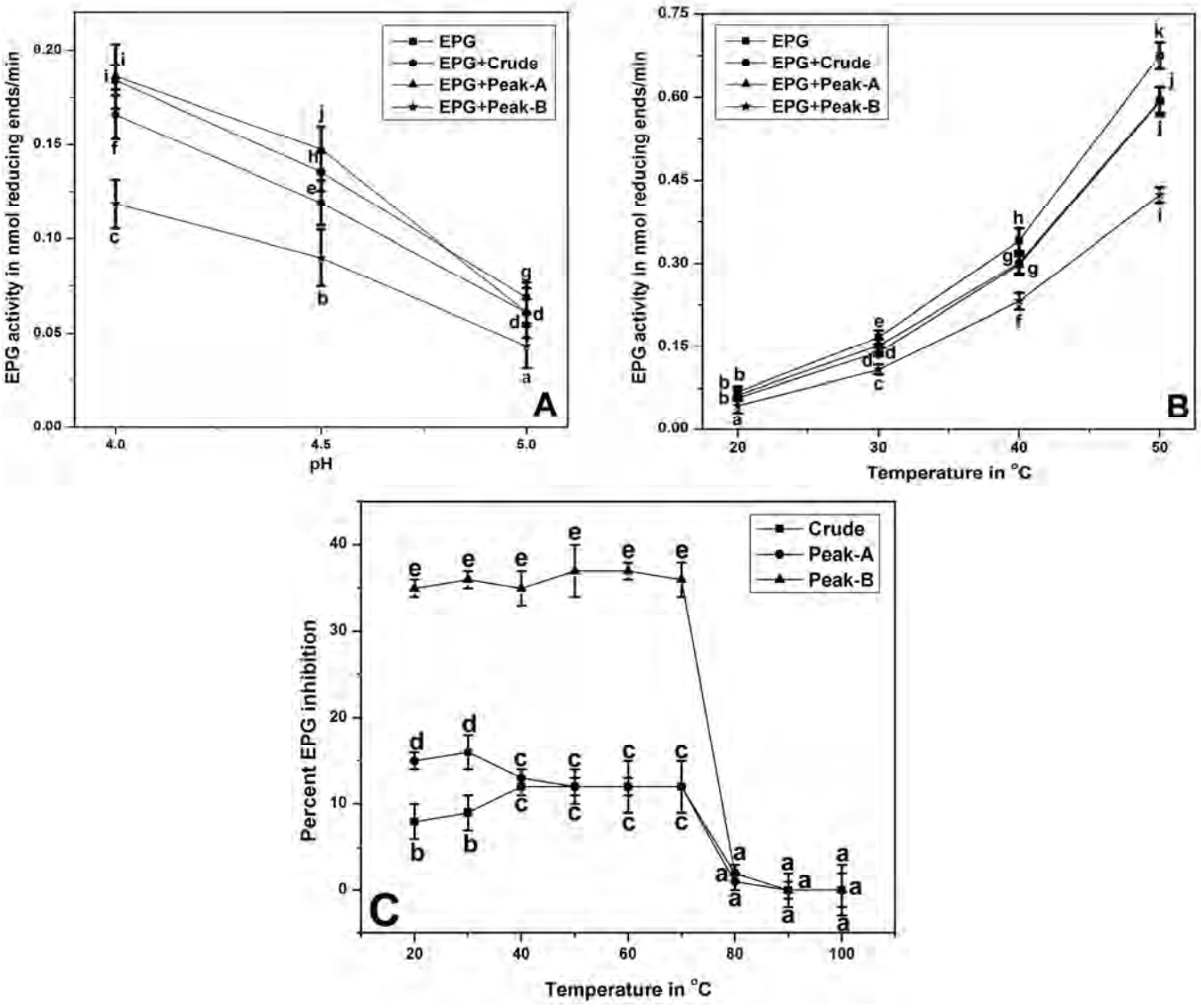


**Figure 5.** *A. niger* endo-polygalacturonase inhibition assay. (**A**) Effect of pH. The different CMC column fractions (5 µg each) were assayed for inhibition, each at three different pH units; (**B**) Effect of temperature. The different CMC column peaks (5 µg each) were assayed for inhibition, each at four different temperatures. (**C**) Thermal stability of PGIP. The different CMC column fractions (5 µg each) were assayed for inhibition, each in the range of 20 to 100°C. The data are means of three independent experiments. Bars indicate ±SE. Means designated with the same letter are not significantly different according to Tukey's HSD test at $P < 0.05$.

revealed that inhibition of both the peaks was retained even at 70°C (Figure 5C).

## DISCUSSION

Recent studies in various host-pathogen systems such as ginseng (*Rhizoctonia solani*) and other fungal pathogens (Sathiyaraj et al., 2010) and in bean-*Sclerotinia sclerotiorum* (Oliveira et al., 2010), as well as the transgenic expres-sion studies in wheat and *Arabidopsis* to successfully counter *Fusarium graminearum* (Ferrari et al., 2011) triggered our interest to explore PGIPs in pearl millet. The present study was aimed at exploring the presence of PGIPs in millets. In that direction, a partial purification of the pearl millet crude protein extract was carried out on a carboxy methyl cellulose cation-exchanger matrix. The cation-exchanger was chosen as the purification matrix as many of the already characterized PGIPs from various plant species have pI values in the range of 6.6 to 9.5 (Abu-Goukh et al., 1983; Cervone et al., 1987; Favaron et al., 1994; Stotz et al.,

1994) respectively. The proteins being positively charged 1-2 units below their pI and also the fact that PGIPs are stable and active at lower pH values prompted the use of cation-exchangers for their purification in many of the plant species. The separation of pearl millet seedling crude extract on the ion-exchanger yielded two peaks at 200 and 300 mM NaCl elution gradients which on separation by reducing gel electrophoresis, resulted in prominent protein bands distributed between the 29 and 43 kDa molecular weight protein standards. This is consistent with most of the known plant PGIPs which fall in this range with monocot wheat PGIP being 40.3 kDa (Kemp et al., 2003) and cotton PGIP is 34 kDa (James and Dubery, 2001). In addition, protein bands lower than 20 kDa and higher than 43 kDa were also observed in the 2 peak lanes which could also be putative PGIPs as occasionally some plant species have shown the presence of PGIPs with molecular weights of 15 kDa in peach (Fielding, 1981) and 91 kDa in pear (Abu-Goukh et al., 1983). Hence further purification of pearl millet PGIPs to homogeneity will be crucial in determining the actual inhibitory protein. A similar attempt to identify the presence of PGIP isoforms in *Allium porrum* L. active against *S. sclerotiorum* by partial purification resulted in soluble PGIP with two peaks of activity (P1 and P2) eluting at about 0.10 and 0.25 M NaCl, respectively and the wall-bound PGIP divided into three peaks (P3, P4 and P5), eluting at about 0.10, 0.18 M and 0.25 M NaCl (Favaron, 2001) with, multiple PGIP isoforms.

The major biotic constraint in the pearl millet production is the obligate biotrophic oomycete pathogen, *Sclerospora graminicola*. It is practically not possible to obtain good amounts of endo-polygalacturonase from the native pathogen as its axenic culture is not possible. Since plants are also known to produce polygalacturonases, it is not possible to distinguish between plant and pathogen EPGs. Crude protein extracts from *S. graminicola* infected susceptible pearl millet plants showed EPG activity, but incubation with the PGIP showed no inhibition against them, thus indicating that the observed EPG activity could be of plant origin. The *S. graminicola* zoospore extracts upon screening for EPG activity showed no activity, as biotrophs are known to produce very low amounts of the enzyme only upon infection (Simon et al., 2005). Currently, isolation of the gene encoding EPGs from the pathogen is being undertaken, which could further be expressed in suitable expression systems to obtain fusion proteins for inhibition studies. Hence the commercially available EPG from *A. niger* was used as the enzyme in the inhibition studies for screening of PGIP in the present study. The enzyme has been used earlier for screening of PGIP activity in bean (Cervone et al., 1987). The pH and temperature optimum for the enzyme was found to be 4 and 50°C, which is in correlation with the literature (Kester and Visser, 1990). A suitable aliquot of enzyme was subjected to time course of hydrolysis to determine the linear range of the enzyme activity. The enzyme activity was linear up to 1 h and for the present study 45 min was chosen as the incubation time for the inhibition experiments.

The determination of this linearity of enzyme activity is crucial for the determination of the inhibition, as any time point beyond this linearity range may not be able to detect inhibition thus leading to false negative results. The effect of inhibitor concentration on enzyme activity showed that there was only a partial inhibition and a positive correlation was observed between the inhibitor concentration and the percent inhibition. The Peak-B was the more active of the two column eluents with the maximum inhibition being 34% followed by that of Peak-A and crude with 13 and 11%, respectively at 5 µg. A similar study conducted in tomato showed that the inhibition capacity increased with increasing concentrations of PGIP. The purified tomato PGIP incubated with *A. niger* EPG (12 ng) at 0.5 µ g showed an inhibition of just below 20%, whereas at 8 µ g it was around 90%. The same PGIP at 5 µ g concentration displayed 66% inhibition against the *Stenocarpella maydis* EPG (9 µg) (Berger et al., 2000). A lack of inhibition observed in the CMC flow-through fraction is an indication that all of the PGIPs are bound onto to the column and employment of cation-exchange chromatography as an initial purification step was justified.

Many different EPG-PGIP combinations have been shown to demonstrate a range of enzyme inhibition. The study involving bean PGIP2 and the 5 PG isoforms (PGI, PGII, PGA, PGB and PGC) of *A. niger* over a pH range between 4 to 5 showed that the interaction between various EPG-PGIP combinations are pH dependent. The PGB, PGI, and PGII were inhibited by PGIP-2 over the entire tested pH range, whereas PGA and PGC isoforms were activated at pH 5.0 and inhibited at pH 4.75 and 4.2, respectively (Kemp et al., 2004). To determine if such pH dependence existed in the present system the effect of pH on the EPG-PGIP was undertaken. Interestingly, a differential inhibition pattern was observed with the Peak-B being active at all the three tested pH values whereas the Peak-A showed no inhibition at pH 4, slight enzyme activation at pH 4.5 and partial inhibition at pH 5. This means that the plant produces multiple PGIP isoforms which are active at different pH values which could be advantageous to the plant as changes in pH due to biotic or abiotic factors could be taken care of by the functional redundancy.

The pearl millet is a drought resistant crop grown in tropical conditions with temperature rising in excess of 40°C in summer. The thermal stability of the pearl millet PGIP was evaluated over a temperature range of 20 to 50°C. The results clearly demonstrate that the PGIPs are active at 50°C and the increase in temperature from 20 to 50°C had no effect on their potential of inhibition of *A. niger* EPGs. The inhibition was seen to be retained even

at 70°C. This is consistent with other such studies conducted in different plant systems. The PGIPs from orange (Barmore and Nguyen, 1985), bean (Cervone et al., 1987) and guava (Deo and Shastri, 2003) were found be active at 60°C. The peach PGIP was stable at 80°C (Fielding, 1981), whereas PGIP of chilli stable at 50°C retained some residual inhibitory activity even at 90°C (Shivshanker et al., 2010).

In conclusion, the present study was one of the first initiatives in understanding the presence of PGIPs in pearl millet. The study has been able to partially purify and characterize pearl millet PGIPs. Further purification, characterization of the pearl millet PGIPs and their encoding genes will aid in the understanding of their role in host-pathogen interaction as well as in protein-protein interaction studies. The downy mildew of pearl millet caused by *S. graminicola* is the most important disease of the host leading to significant economic losses. The use of advanced biotechnological approaches in understanding the interaction between pearl millet PGIP(s) - *S. graminicola* EPG(s) would be crucial in formulating effective crop protection strategies. Currently, the isolation and characterization of the pure protein and the gene encoding it is in progress.

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