PURIFICAREA ŞI CARACTERIZAREA POLIGALACTURONAZEI - INHIBITOR AL PROTEINEI DIN SOIURILE DE PĂR ASIATIC

PURIFICATION AND CHARACTERIZATION OF POLYGALACTURONASE - INHIBITING PROTEIN FROM ASIAN PEAR VARIETIES

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Abstract

Polygalacturonase inhibitory protein (PGIP) was extracted from Shinli pear tissue, purified and partially characterized. Extraction was carried out at 4ºC with a high ionic strength extraction buffer. After dialysis and concentration by ultrafiltration, the extract was chromatographed on size-exclusion chromatography (S-100), and its active fractions were applied on concanavalin A-Sepharose. The PGIP activity was bound by the lectin, and then eluted using 1M α-methyl mannopyranoside, resulting in a 18-fold purification of the PGIP and demonstrating its glycoprotein nature. The following ion-exchange chromatography gave a PGIP that was 360-fold purified relative to the initial tissue extract, and having a 45kDa molecular weight, as estimated by SDS-PAGE electrophoresis. PGIP inhibitory activity was tested against *A. niger*, *C. acutatum* and *B. cinerea*. The radial diffusion and reducing sugar assays showed that PGIP inhibitory to three PGs was affected by pH. In vivo tests revealed that PGIP inhibited three polygalacturonase from all three fungi. Heated for 20 min at 85ºC, the inhibitory activity of PGIP was reduced by 85-90%, and it was completely suppressed after being heated at 100ºC for 20 min.

Cuvinte cheie: interactiune gazda – pathogen, polygalacturonase, degradare
Key words: host pathogen interaction, polygalacturonase, decay

1. Introduction

Polygalacturonase (PG) (EC 3.2.1.15) is the first enzyme secreted by plant fungal pathogens when cultured on isolated cell walls (Jones et al., 1972). Degradation of plant cell wall by PG facilitates the attack of other cell wall-degrading enzymes on their substrates (Karr and Albersheim, 1970). A role for PG in pathogenicity has been proposed for soft-rot pathogens because they cause extensive degradation of plant cell walls leading to the maceration of host tissue (Bateman and Miller, 1966; Collmer and Keen, 1986; Cooper, 1984).

Polygalacturonase inhibiting proteins (PGIPs) from plant cell walls have been considered to contribute to plant defense responses against pathogens (Abu-Coukh and Labavitch,1983). These PGIPs inhibiting fungal polygalacturonases have been reported from numerous plant species (Albersheim and Anderson, 1971; Brown, 1984; Brown and Adikaram, 1982; 1983; Degra et al., 1988; Fielding, 1981; Hoffman and Turmer, 1982). The proteins isolated from bean (Cervone et al., 1987), European pear (Stotz et al., 1993), raspberry (Johnston et al., 1993), tomato (Stotz et al., 1994) and soybean (Favaron et al., 1994) have differential inhibition spectra towards a range of PGs from phytopathogenic fungi, but only a few of those proteins have been purified to homogeneity.

Biochemical characterization of PGIPs shows that they are glycoproteins (Stotz et al., 1993; Stotz et al., 1994) and relatively heat stable (Abu-Coukh and Labavitch,1983; Albersheim and Anderson, 1971; Cooper, 1984). Some PGIPs display heterogeneity in molecular mass caused by differential glycosylation of a single polypeptide (Stotz et al., 1993; Stotz et al., 1994). Kinetic studies of PGIPs revealed that some inhibit fungal PGs by a competitive-type mechanism (Abu-Coukh and Labavitch, 1983), whereas others are noncompetitive (Johnston et al., 1993; Lafitte et al., 1984). Furthermore, the inhibition of PG by PGIPs is highly specific (Yao et al., 1999; 1995). PGIP from single plant source can differentially inhibit PGs from several fungal species or PG isozymes from one fungus (Abu-Coukh and Labavitch,1983; Albersheim and Anderson, 1971; Brown, 1984; Brown and Adikaram, 1982; Johnston et al., 1993; Sharrock and Labavitch, 1994). PGIPs from different plants inhibit PG from a single fungal species to different extents (Stotz et al., 1994). For example, European pear PGIP inhibits PG from culture filtrates of *Botrytis cinerea* more strongly than does PGIP from tomato. PGIP has been shown to be a disease resistance factor against pathogen infection (Abu-Coukh and Labavitch, 1983). Ripening tomato fruit transgenic plants expressing

the European pear PGIP gene are more resistant to *B. cinerea* infection than the control fruit (Powell et al., 1994).

The loss of pear fruit in storage is substantial due to decay caused by postharvest pathogens such as *B. cinerea* and *Venturia nashicola* (Mohamed et al., 2003). Although fungicides can effectively control some of these pathogens, public concerns about health and environmental impact limit their future application. Since PGIP has proven to be a plant defense mechanism against pathogen infection, it may be suitable as an alternate method to control postharvest diseases. PGIP inhibitors of fungal PGs have been detected in European pear (*P. communis* L.) leaves and in infected and healthy fruit (Stotz et al., 1993). However, Asian pear PGIP has not been purified to homogeneity or characterized. This paper describes the purification and characterization of PGIP from mature fruit of Asian pear cv. 'Shinli' (*Pyrus bretschneideri* Reh.) and its activity against different PGs.

2. Materials and methods

Plant material

Asian pear cvs. 'Shinli' of commercial maturity were harvested from commercial orchards in Davis, California. Flowers, leaves, spurs and fruits at different ripening stages from young pear trees were collected at University of California, Davis. The samples were frozen in liquid N_2 and used immediately or stored at –20ºC until use.

PG sources

Aspergillus niger commercial pectinase (Sigma Aldrich, USA) was used as a source of PG., *Botrytis cinerea* and *Colletotrichum* sp. culture PGs were done by seeding two hundred microlitres of spore suspension ($5X10^5$/mL) were seeded in 250 mL of growing cultures in Pratt media (13.6g/L KH_2PO_4, 4.0g/L NH_4NO_3, 1.25g/L $MgSO_4$, 0.001g/L $CuSO_4$, 0.002g/L $ZnSO_4$, 0.0013g/L $(NH_4)_2MoO_4$, 0.0028g/L H_3BO_3, 0.02g/L $FeSO_4$, 0.016g/L $MnSO_4$, 1g/L yeast extract, crude cell wall extract, 3.0g/L, pH 4.5). After incubation at 20ºC with continuous shaking (100 rpm) for 10 days, fifteen grams of mycelium were extracted with buffer (50mM NaAc, pH 5.0; 1mM cysteine, 20g/L PVPP, 1M NaCl) and stirred at 4ºC for 1h. After that the suspension was filtrated through Miracloth.

PGIP extraction

Thirty grams of Shinli pear fruit having commercial maturity were processed in an Ultra turrax with 150mL of buffer (50mM NaAc, pH 5.0; 1mM cystein, 20g/L PVPP, 1M NaCl), and stirred at 4ºC for 1h. After that the suspension was filtrated through Miracloth. The extracts were dialyzed overnight against buffer (50mM NaAc pH 5.0), and then used to assay PGIP activity. Three extracts were done, and each extract was measured in triplicate.

PGIP purification

Fruit was homogenized in an equal volume of extraction buffer (1M sodium acetate, pH 5.75, 1M NaCl, 2% [w/v] PVP-40, 1mM cysteine). The homogenate was stirred on ice for 1h and then vacuum filtrated. The supernatant was saved and $(NH_4)_2SO_4$ was added to reach 50% and 100% saturation respectively. The suspension was then centrifuged at 4,000 rpm at 2ºC for 20 min, and the pellet was resuspended in 0.1M sodium acetate, pH 6.0, and extensively dialyzed at 4ºC against 50 mM sodium acetate, pH 5.0. The dialyzed fraction was mixed and concentrated by lyophilization, and dissolved in 2ml of 50 mM sodium acetate, and chromatographed by size exclusion chromatography (S-100). The active fractions were collected and lyophilized, then dissolved and mixed with an equal volume of 0.2M sodium acetate, pH 6, 2M NaCl, 2mM $CaCl_2$, 2mM $MgC1_2$, 2mM $MnC1_2$ (2X Con A buffer) and applied to a column of Con A-Sepharose 48. Chromatography was performed at 0.5ml/min. Protein bound to the column was eluted using 1M □-methyl mannopyranoside in Con A buffer. The eluent was dialyzed against 50mM sodium acetate, pH 4.5 (buffer A), and then concentrated by ultrafiltration using a pressure cell fitted with a PM-10 membrane (Amicon, Danvers, MA), or lyophilization.

Lyophilized pear fruit protein was dissolved in 2 ml of 0.1M NaH_2PO_4, pH 7.5, and dialyzed against the same buffer overnight at 4ºC, and applied to the CM ion exchange chromatography (HiPrep® 16/10 CM) with the running buffer (0.1M NaH_2PO_4, 1M NaCl, pH 7.5). The isolated fractions having more inhibitory activity were collected and stored for the application of SDS-PAGE and Western blot analysis.

PGIP activity assay

Inhibition of endo-PG activity from *Aspergillus niger* commercial pectinase (Sigma Aldrich, USA), and from the culture filtrate of *Colletotrichum sp.* and *Botrytis cinerea* was assayed by using a gel diffusion assay according to Taylor and Secor, (1988). Briefly a gel containing 1% agarose, 200 mg L^{-1} PGA (Sigma) and 100 mM NaAc buffer pH 5.75 was prepared. After gelification well cutting was done with a 4.5 mm cork-borer. Fifteen microliters of sample were loaded in each well, the cup plates were sealed with tape and incubated for 12h at 40ºC. For gel staining, freshly made ruthenium red (0.05% w/v in water) was added to cover the gel. After 30 min the gel was destained with water and PGIP activity was determined by

measuring the reduction in the destained area from the samples containing the fruit extract relative to the controls without fruit extract. To check that the inhibition was due to a heat labile compound controls containing pectinase and heat treated fruit extract were done. Measurements were done in triplicate.

Effect of pH and ionic strength on PGIP activity

Gels were prepared with buffers of 0.1M NaAc pH 3.50, 4.25, 5.00, 5.75 and 6.0 to assess the effect of pH on PGIP activity. To assess the effect of ionic strength on PGIP activity, gels containing 0, 20, 50, 100 and 200 mM KCl were prepared. Finally for heat stability assays, PGIP was incubated at 25, 40, 55, 70, 85 and 100ºC for 20 min. PGIP determination was also performed as described in *2.5*.

Effect of extraction conditions on PGIP activity

Fruit was homogenized in an equal volume of extraction buffer (1M sodium acetate, pH 5.75, 2% [w/v] PVP-40, 1 mM cysteine) containing 0, 0.25, 0.50 and 1M NaCl. The homogenate was stirred on ice for 1h and then vacuum filtrated. The supernatant was saved and used to assay PGIP activity. To evaluate the effect of the stirring time on PGIP extraction the fruit was homogenized in an equal volume of extraction buffer (1M sodium acetate, pH 5.75, 2% [w/v] PVP-40, 1mM cysteine, 1M NaCl), stirred on ice for 1, 3 or 6h, vacuum filtrated and assayed for PGIP activity.

Protein Assay

Protein was measured by the method of Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a Bio-Rad Mini-protean II cell. The gel was stained with a Bio-Rad silver stain kit according to the recommendation of the manufacturer. The molecular mass of the proteins was estimated by comparison to Sigma SDS-7 molecular weight markers (Sigma Chemical Co., St. Louis).

Kinetic properties

The characteristics of PGIP inhibition were determined by a reducing sugar assay[18] (Gross, 1982). Fruit extracts were incubated with PG from *Aspergillus niger*, *Colletotrichum sp.* and *Botrytis cinerea* at different concentrations (0.2, 0.1, 0.05 and 0.025 mg/ml) in a buffer containing 37.5mM sodium acetate, pH 5.75 and 10 mM EDTA.

Statistical analysis

Experiments were performed according to a factorial design. Data were analyzed by means of ANOVA, which were compared by the LSD test at a significance level of 0.05.

3. Results

Extraction and purification of PGIP

Most PGIP inhibitory activity of Asian pear was found in fruits, then, in spurs and flowers, the least PGIP activity was in leaves (Fig. 1). Since pear fruits present most PGIP activity, it was selected as the source for PGIP purification. The first 1 *M* NaCl-sodium acetate extract from Shinli fruits yielded a total of 2.65 x 10^6 units of PGIP activity from 5kg of fruits. Purification of the 28.5 *L* first extract started with ultrafiltration concentration to 1.14*L* giving a 5-fold relative purification, and 80.8% recovery of activity (Table 1).

The following step of purification was gel filtration, the active fractions of 134 *mL* giving a 15.8-fold relative purification and 55.1% recovery of activity. The PGIP was further purified by affinity chromatography (Con A). Activity was bound by the lectin and specifically eluted with α-methyl-mannopyranoside, giving 18.9-fold relative purification with a 51.6% recovery. No inhibitory activity was found in the column flow-through, suggesting that all of the active PGIP was a glycoprotein (Fig. 2).

Detection of PGIP protein from Shinli fruit

Western blot analysis was performed with protein extracts obtained from mature fruit of cv. Shinli using a polyclonal antibody raised against European pear PGIP. One major band (approximately 45 kDa) was detected in samples (Fig. 3). After chemical deglycosylation with TFMS, molecular mass changed to 42 kDa (Fig. 4). These results also indicate that Shinli pear PGIP is a glycoprotein.

Inhibition of PGs by PGIP

When three PGs were incubated with the same amount of inhibitor (IEC fractions of Shinli 7-8), differential inhibition activity was observed. Three PGs (*A. niger*, *C. acutatum*, *B. cinerea*) were significantly inhibited by Shinli pear PGIP (Fig. 5).

When different amount of inhibitors from different fractions of IEC were assayed against PGs, it was found that the inhibitory activity differed among the PGs. Inhibition increased as the amount of inhibitor was higher. The relationship between inhibitory activity and amount of inhibitor is shown in Fig.5. The results indicated that *B. cinerea* was more strongly inhibited by IEC-PGIP than *A. niger* or *C. acutatum.*

Heat stability of PGIP

Aliquots of purified PGIP from IEC were incubated at 0, 25, 40, 55, 70, 85 or 100 for 20 min, immediately chilled on ice, and tested spectrophotometrically for inhibitory activity. PGIP activity against A. *niger* was reduced by 30% at 55°C, and then a sharp drop occurred between 55°C and 85°C, where only a little inhibition activity (3%, 10% or 15% against A. *niger*, C. *acutatum*, B. *cinerea* respectively) remained after 20 min of treatment. No activity was found when the PGIP was boiled for 20 min (Fig. 6) .

Effect of pH on PGIP activity

Inhibitory activity of purified PGIP against *A. niger*, *C. acutatum* or *B. cinerea* was different at various pH assayed by cup plate method. *A. niger* was more susceptible than *C. acutaum* to pH, and the highest inhibitory activity against *A. niger* was found at pH 5.75 whereas PGIP inhibitory activity was increasing from pH 3.5 through pH 5.0 when it was against *B. cinerea* (Fig. 7). As to *C. acutatu*, no significant pH effect on inhibition was found.

Effect of ionic strength on PGIP activity

When differential amount of KCl was added into the gel, PGIP inhibitory activity was affected by KCl content when it was against *A. niger* its activity could be 85% at 20mM KCl, and 25% at 100mM KCl. However no effect was found when PGIP was against *C. acutatum or B. cinerea* (Fig. 8).

Effect of NaCl content on PGIP activity in extraction buffer

Since most PGIP activity was present in the sodium chloride extract, it was selected as the source for PGIP purification (Yao et al., 1995). However, in our experiment, it seems that no significant effect of NaCl on PGIP activity in extraction buffer was found when Shinli pear PGIP inhibited three PGs by cup plate assay (Fig. 9). Whereas differential inhibitory activity was found when PGIP was against different PGs, its activity was 75% and 95% when it was against *A. niger* and *B. cinerea* respectively.

Effect of extraction time on PGIP activity

When *A. niger* was introduced, inhibitory activity of PGIP from crude extract was gradually reducing as the extraction time increasing, while PGIP from 0-50AMS was increasing, and PGIP from 50-100AMS was not changed too much (Fig. 10). No significant effect on PGIP activity was found among three different extraction time (1, 3, and 6hr). No difference was detected when *C. acutatum* and *B. cinerea* was inhibited by PGIP from different extraction time.

Kinetic properties

Substrate concentration influenced the reaction rate of *A. niger.* When the same amount of inhibitor (0.1ml) added, its products were increasing as the concentration of substrate increasing, and irregularly changed as the incubation time increasing. The results indicate a competitive-type of inhibition (Fig. 11).

In vivo inhibition of PGs with PGIP

Relative infection of *B. cinerea* and *C. acutatum* was 41% and 14% respectively relative to *A. niger* (100%) and Shinli PGIP (0). PGIPs isolated from Shinli pear showed relatively higher inhibition against *A. niger* PG compared with the inhibition against *C. acutatum* or *B. cinerea* PGs (Fig. 12). Such inhibitory activity lasted until 12 days after inoculation.

4. Discussion

PGIP was isolated from Asian pear cv. 'Shinli' (*Pyrus bretschneideri* Rehd.) tissue. It showed a high degree of similarity with those previously isolated from related fruit species, such as apple (Yao et al., 1999), cherry (Zhang and Zhang, 2000), pear (Mohamed et al ., 2003) , and strawberry (Lisbeth et al., 2004).

PGIPs belong to a group of proteins with repetitive LRR sequences and that are involved in protein-protein interactions (Lisbeth et al., 2004). The consensus in the pear PGIP sequences is highly conserved all over the clones and showed very high homology to the sequences of other PGIP genes registered (Mohamed et al ., 2003). The three-dimensional structure of a PGIP from *Phaseolus vulgaris* has very recently been determined (2003) and its structure reveals a negatively charged surface on the LRR that is likely involved in binding PGs. Stotz *et al*. (2000) used site-directed mutagenesis or statistical analysis, respectively, and identified, both within and outside the solvent-exposed region of PGIPs, putative target amino acids involved in PG-PGIP interaction.

During fruit maturation the pear PGIP gene was up-regulated. It was in agreement with tomato (Stotz et al., 1994) and apple, whereas harvested apples showed elevated PGIP expression levels (Yao et al., 1999) [9]. This up-regulation of PGIP in pear could be related to factors such as oxidative stress or changes in the sugar content.

Wounding seemed to have no impact on the transcript level in strawberry (Lisbeth et al., 2004) . PGIP response 24h after *B. cinerea* inoculation has also been shown in other species, for example, in *Arabidopsis* (Ferrari et al., 2003)\, bean (Bergmann et al., 1994) , and apple (Yao et al., 1999). Fungal PG is active in the infection process at all fruit maturity stages (Van et al., 1994), and the oligogalacturonides

(OG) derived from pectin degradation from early germination of the conidia may be the source of elicitation of the PGIP induction (Albersheim and Anderson , 1971).

The cultivar show differential susceptibility towards *A. niger, C. acutatum* and *B. cinerea*. No significant differences between cultivars were observed in the induction level of PGIP following inoculation (data not shown). Apparently, the level of PGIP expression is not reflected the genetic variation. It should be also kept in mind that PGIP is not the only factor determining host resistance and that the success of the host plant in warding off the pathogen depends on the coordination of different defense strategies and the rapidity of the overall response.

Based on the present study, it is evident that PGIP expression is induced by *A. niger, C. acutatum* and *B. cinerea* with various degree of inhibition respectively. Nevertheless, the data presented here provide only indirect evidence about the impact of PGIP on *A. niger, C. acutatum* and *B. cinerea* infection in Asian pear, and the significance of PGIP in this pathosystem needs to be verified in further studies based on activity of the proteins using multiple *A. niger, C. acutatum* and *B. cinerea* isolates. In transgenic tomato fruits, over-expression of European pear PGIP resulted in an increased resistance to *B. cinerea* (Greve and Labavitch et al., 1991; Powell et al., 1994), but did not provide complete protection against this pathogen, reflecting the specificity of the PGIPs and the pathogen's ability to produce several isoforms of PG.. In the present study, the Asian pear PGIP was purified. Currently, cloning and sequence of PGIP genes isolated from several Chinese pear cultivars and strains is in progress, with the aim of also investigating their promoter regions for cis-acting elements. Furthermore, the activity of Asian pear PGIP against *A. niger, C. acutatum* and *B. cinerea* will be studied by using purified proteins and by challenging the transformed plants in vitro with the pathogen.

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Tables and figures

Table 1. Extraction and purification of polygalacturonase-inhibiting protein (PGIP) from 'Shinli' pear fruit

[x] One unit of PGIP was the amount that reduced 0.5u of PG (*A. niger*) by 50%.

[y] Five kilograms of 'Shinli' pear fruit was extracted.

Purification steps	Volume (ml)	Total protein (mg)	Total activity [x] (10^4 units)	Specific activity (10^2 units mg^{-1})	Relative purification (fold)	Recovery (%)
Salt extract [y]	28500	1507.2	265.0	14.5	1.0	100
Ultra filtration entration	1140	448.1	214.0	75.0	5.1	80.8
Gel filtration (S-100)	134	165.0	146.0	230.0	15.8	55.1
Con A	28	57.0	137.0	275.0	18.9	51.6
Ion exchange	7	0.6	10.0	5200	358.6	3.77

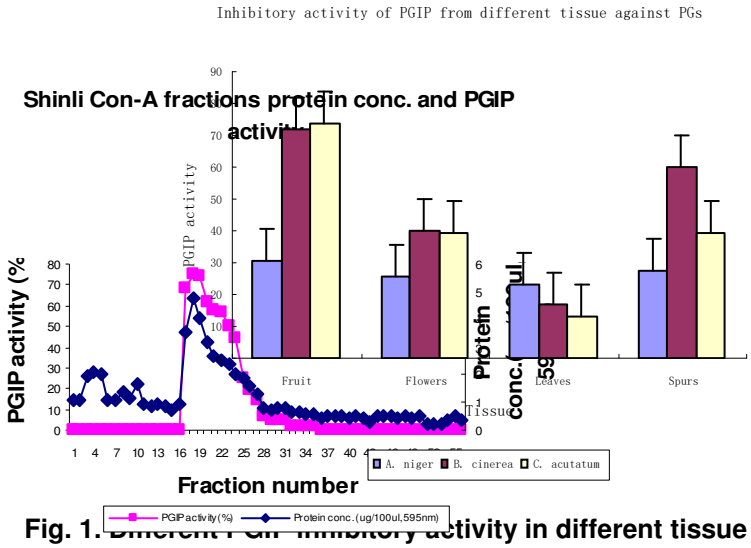

Fig. 1. Different PGIP inhibitory activity in different tissue of Asian pear

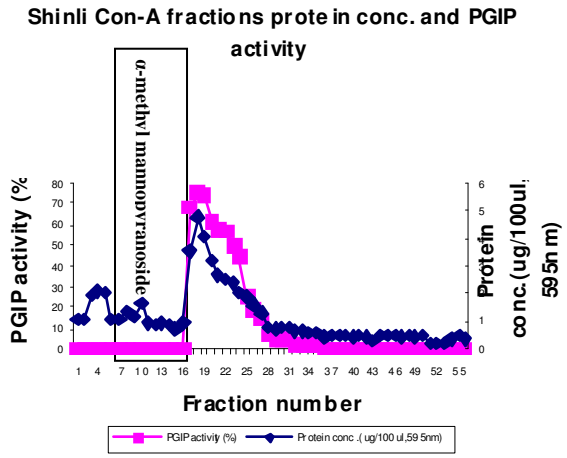

Fig. 2. Chromatography of 20 ml concentrated extract of Shinli (A) was chromatographed on size exclusion chromatography (S-100). Its active fractions of Shinli(22-35) were applied on Concanavalin A-Sepharose. After the initial elution in Con-A buffer (first 37.5 fractions), the column was eluted with Con-A buffer containing 1 *M* α-methyl mannopyranoside (B). The active fractions of Shinli (17-23) from Con-A were chromatographed on ion exchange chromatography(C). All the fractions from SEC, Con-A and IEC were assayed for PGIP activity and protein (WL 595) respectively.

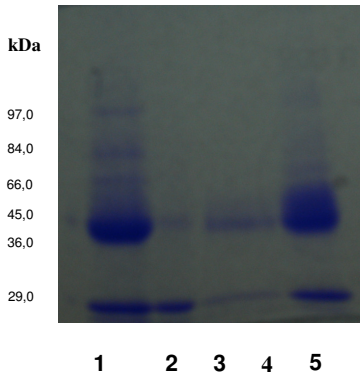

Fig. 3. Silver stained SDS-PAGE gel of proteins in fractions from the size exclusion chromatographic separations of Shinli PGIP depicted in Fig. 1, Con-A fractions of Shinli protein: lane 2, 3 (SL Con-A_{18-21} (12.05 ug/ml), lane 4 (SL Con-A_{22-25} (16.85 ug/ml), lane 1 and lane 5 (molecular weight standards)

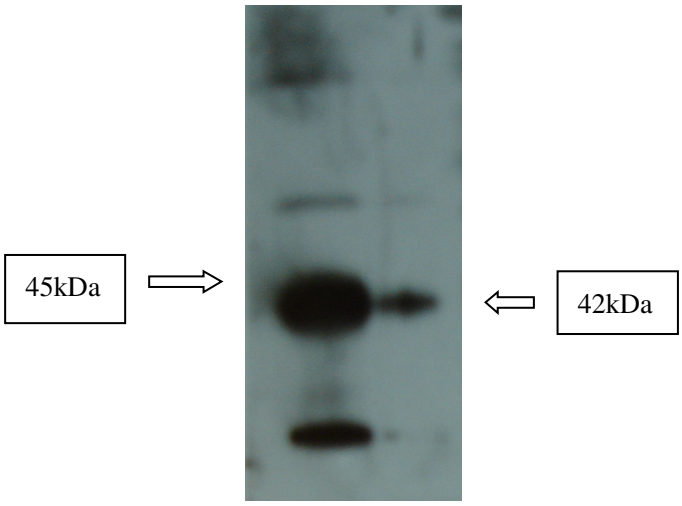

Fig. 4. PGIP detected from mature fruit of Shinli pear. Crude proteins (50ug per lane) from Shinli (lanes 1) were subjected to SDS-PAGE and detected by Western blotting with a polyclonal antibody raised against European pear PGIP. Proteins chemically deglycosylated with TFMS were shown on lane 2.

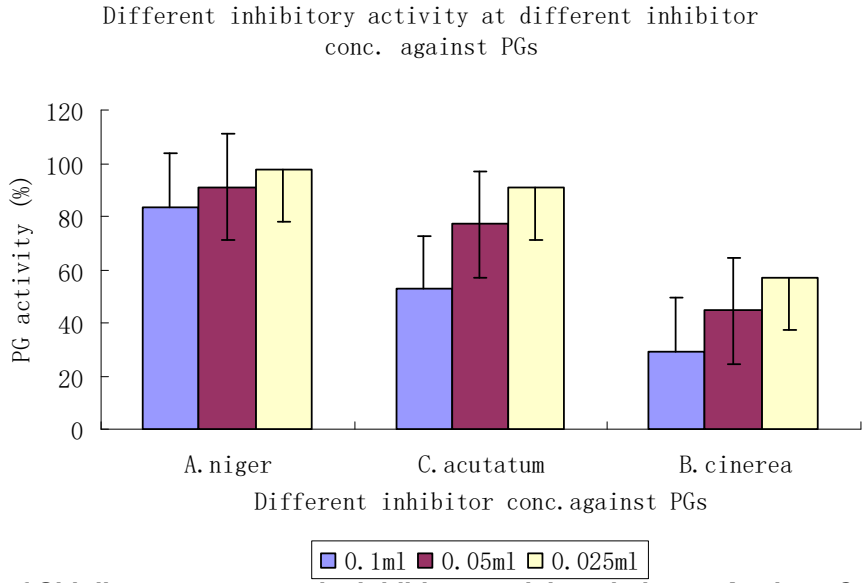

Fig. 5. IEC-PGIP of Shinli pear represent the inhibitory activity relative to *A. niger, C. acutatum* and *B. cinerea*

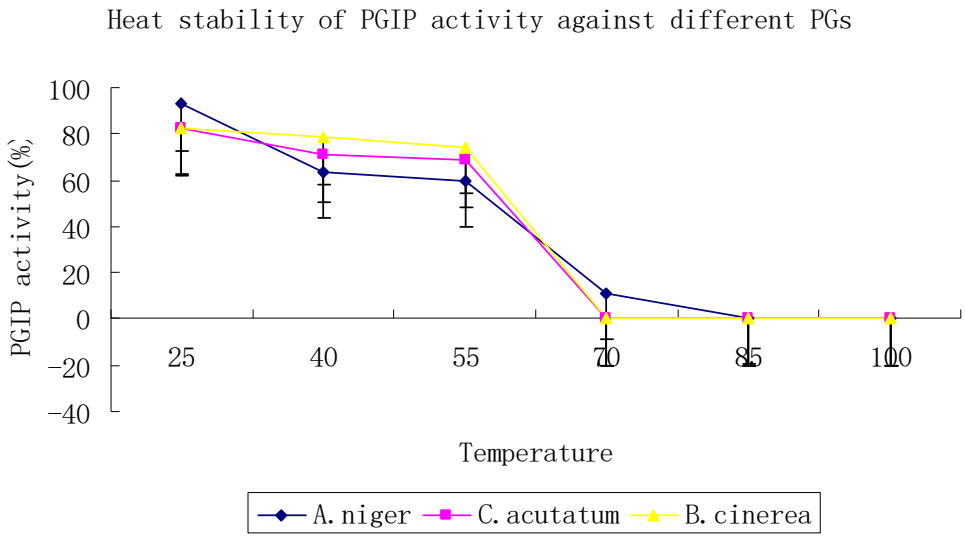

Fig. 6. Heating stability of Shinli pear PGIP, its inhibitory activity was decreasing at 55ºC, and suppressed at 100ºC

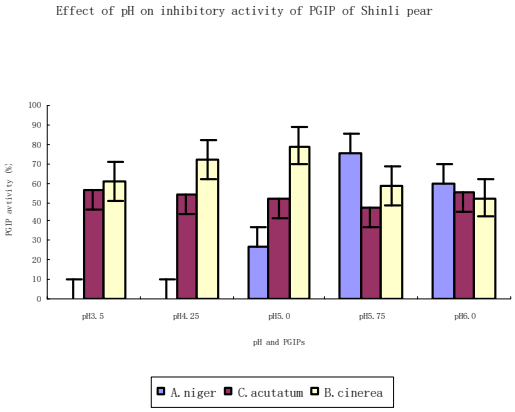

Fig. 7. Three PGs were differentially inhibited at differential pH assayed by cup plate method

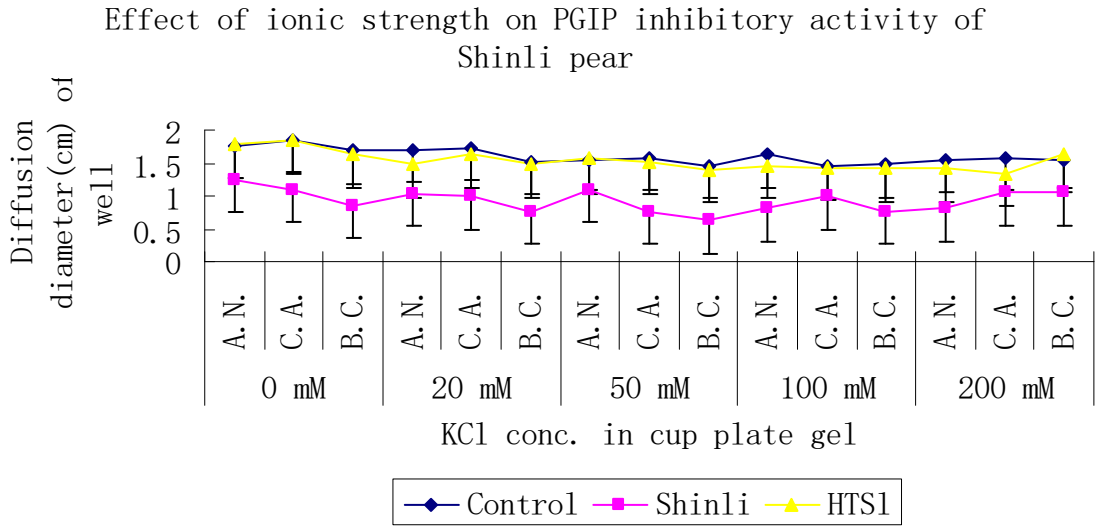

Fig. 8. Three PGs were inhibited by Asian pear PGIP at differential content of KCl in gel assayed by cup plate method

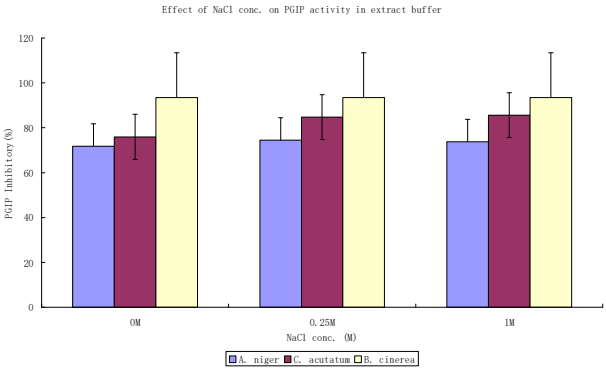

Fig. 9. Compared the effect on PGIP inhibitory, three different content of NaCl (0, 0.25 and 1 M) were added in extraction buffer. No significant effect on PGIP activity was found, whereas different inhibitory activity was found when PGIP against different PGs

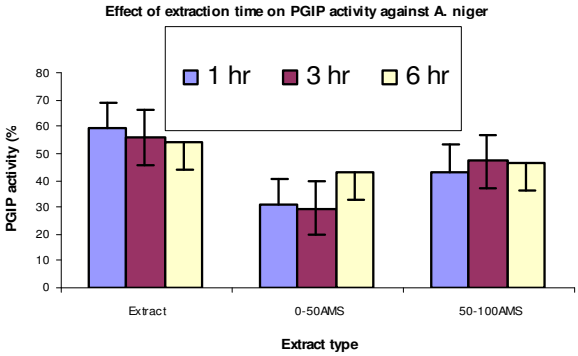

Fig. 10. The effect of extraction time on PGIP activity against different PGs

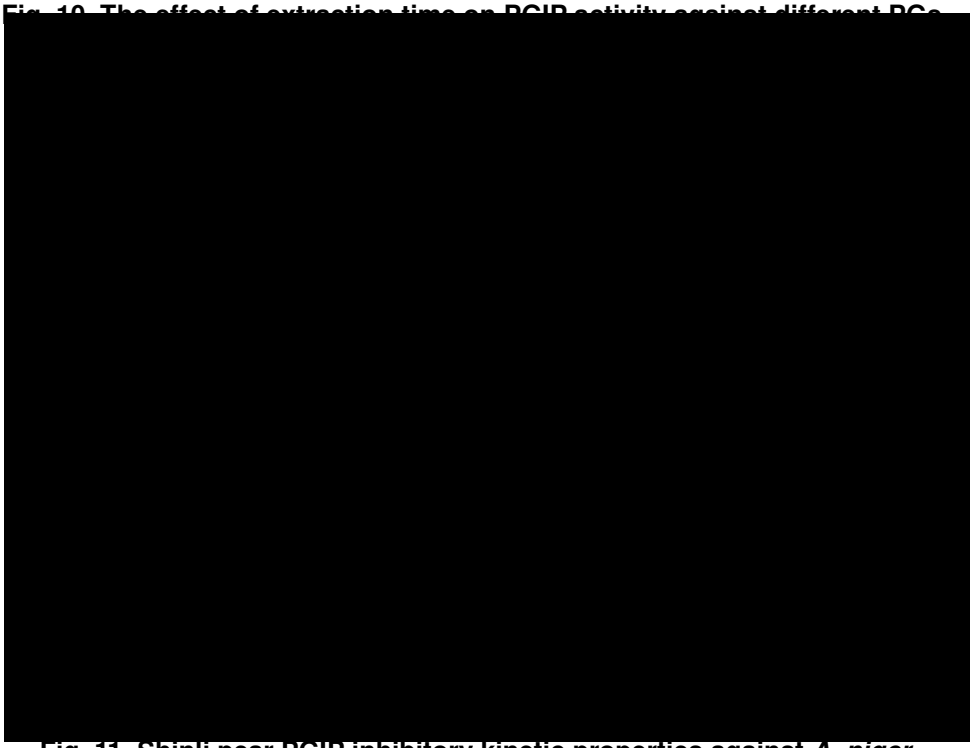

Fig. 11. Shinli pear PGIP inhibitory kinetic properties against *A. niger*

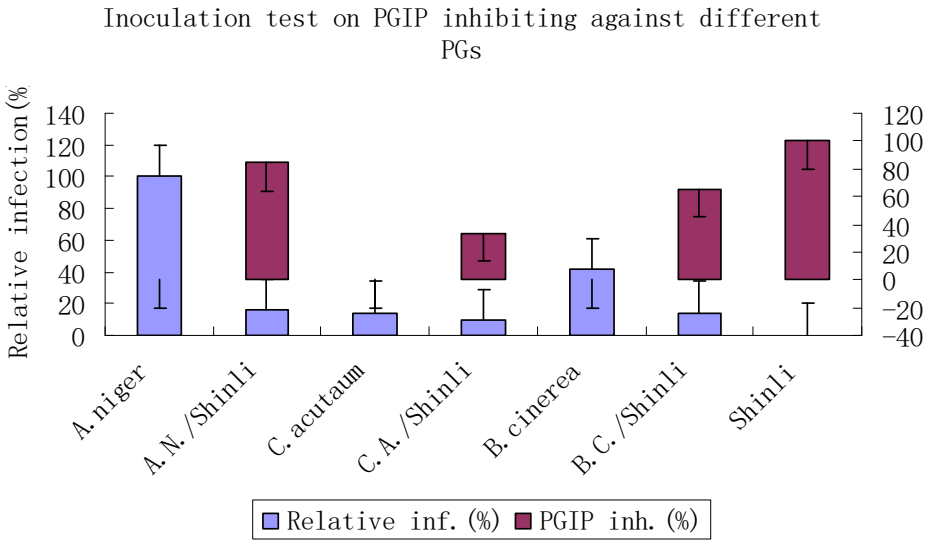

Fig.12. In vivo influence of PGs with PGIPs isolated from cvs. Shinli and Shinko. 10ul of sample was injected in fruits in depth of 2mm, and incubated for 12 days at 22ºC. The average of 30 samples was presented above.