Characterization of an Apple Polygalacturonase-inhibiting Protein (PGIP) from Apple Fruits

Dong Hoon Lee[2], Sang-Gu Kang[1], In-Kyu Kang*, Yoon-Kyeong Lee[3], Cheol Choi[4] and Jae-Kyun Byun[5]

Dept. of Enviromental Horticulture, Sangju National Univ., Sangju 742-711, Korea
[1]School of Biotechnology, Institute of Biotechnology, Yeungnam Univ., Gyeongsan 712-749, Korea
[2]Citrus Research Division, National Institute of Subtropical Agriculture, RDA, Jejudo 699-803, Korea
[3]Dept. of Oriental Medicine Resources, Asia Univ., 240-3, Gyungsan 712-220, Korea
[4]Division of Plant Biosciences, Kyungpook National Univ., Daegu 702-701, Korea
[5]School of Natural Resources, Yeungnam Univ., Gyeongsan 712-749, Korea.

Received April 5, 2006 / Accepted May 30, 2006

An apple polygalacturonase-inhibiting protein (PGIP), that specifically inhibited endopolygalacturonase (PG, EC 3.2.1.15) from *Botryosphaeria dothidea*, was purified from *B. dothidea* infected apple (*Malus domestica* cv. Fuji) fruits. The apple PGIP was a mixed-type inhibitor of PG from *B. dothidea*. Optimal temperature for the maximum enzyme activity was 40℃, and optimum pH of the purified PGIP was pH 5.0. PGIP was stable up to temperature of 60°C and was completely suppressed after heating at 70℃ for 10 min, PGIP was stable at pH between 4 and 8. Inhibition of PG by PGIP was reduced by K^+, Cu^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} metal ion, sodium dodecyl sulfate (SDS) and 1,2-diaminocyclohexane tetra acetate (CDTA).

Key words – Apple, *Botryosphaeria dothidea*, polygalacturonase-inhibiting protein, polygalacturonase

Introduction

Fungal pathogens produce cell wall degrading enzymes that facilitate the invasion of higher plant tissues[4,6]. These enzymes, polygalacturonases (PG, EC 3.2.1.15), glucanases (EC. 3.2.1.6), and pectinmethylesterases (PME, EC 3.1.1.11) function by degrading structural components of the plant cell wall[5,17]. The PGs from fungal pathogens are important cell wall degrading enzymes that hydrolyze pectin and release short chains of galacturonic acids[12,20]. Lee *et al.*[14] demonstrated that the isolated PG of *B. dothidea* successfully degraded cell wall components by measuring the release of uronic acid from pectic cell wall material. These results demonstrated that PG from *B. dothidea* cleave the α-1,4 linkages between D-galacturonic acid residues in the rhamnogalacturonan, the main component of pectin, and cause the separation of cell walls from each other and the maceration of host tissue.

In higher plants, polygalacturonase-inhibiting proteins (PGIPs) that are located in the cell wall and inhibit the action of PGs[7,8,10,12,13]. The PGIPs have been detected in a number of dicotyledonous species and recently also in monocotyledonous plant species, but few PGIPs have been characterized[18,19]. In previous work, Lee *et al.*[14] purified PGIP from apples fruits and characterized the *de novo* function of the PGIP against PG on the solubilization and depolymerization of polyuronides from cell wall of apple fruits inoculated with *B. dothidea*. Those result demonstrated PGIP of plant involves one of direct defense mechanisms against the pathogen attack by the inhibition of PGs which are released from pathogens to hydrolysis of cell wall components of plants.

In this study, We describes the characterization of PGIP from apple fruit. PGIP is examined in terms of inhibition of *B. cinerea* PG kinetics of inhibition, and heat stability.

*Corresponding author
Tel : +82-54-530-5232, Fax : +82-54-530-5239
E-mail : ikkang@sangju.ac.kr

Materials and Methods

Fungal and plant materials

B. dothidea was isolated from naturally infected mature apple fruits (*Malus domestica* cv. 'Fuji'). 'Fuji' apple fruits were harvested and stored at 4°C for up to 90 days.

Preparation of fungal PGs

PG from *B. dothidea* was purified as essentially as previously described[16]. Apple fruits were washed and were inoculated to a depth of 4 mm with needle containing a

suspension of *B. dothidea* spores at a concentration of 5×10^5 spores/mL. The inoculated apples were placed in plastic boxes and incubated for 7 days at 27±1°C. Five hundred grams of decayed apple fruit were homogenized in 1 L of 10 mM sodium phosphate buffer (pH 7.0) containing 2% (w/w) polyvinylpolypyrolidone. All purification procedures were performed at 4°C. The homogenate was centrifuged at 12,000×*g* for 60 min and sufficient ammonium sulfate was added to the supernatant to achieve 85% saturation. The pellet, obtained by centrifugation as described above, was dissolved in 100 mL of deionized H_2O. The resultant solution was dialyzed against 2 L of 10 mM sodium phosphate buffer (pH 7.0) for 48 h with stirring and the dialysate was centrifuged at 12,000×*g* for 60 min. The resultant supernatant was concentrated using an Amicon Diaflo System with PM-10 membrane (Millipore, Billerica, MA). Concentrated crude extracts were loaded onto a 2.8×60 cm column of Sephadex G-100 (Sigma-Aldrich, St. Louis, MO) and were eluted with 10 mM sodium phosphate buffer (pH 6.0). The PG activity of each 5 mL fraction was measured as described below. Fractions containing PG activity were pooled and concentrated as described above. The concentrated samples were applied to a 2.8×50 cm column of DEAE-Cellulose (Sigma-Aldrich, St. Louis, MO) in 10 mM Tris-HCl buffer (pH 7.2) and protein was eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer.

Purification of apple PGIP

Apple PGIP was purified as previously described[21]. All purification steps were performed at 4°C. Diced apple fruit (1 kg) was mixed with solid 1.5% (w/w) polyvinylpolypyrolidone and 0.2% (w/w) sodium bisulfate and the resultant slurry was homogenized in 2 L of 50 mM sodium acetate buffer (pH 6.0). The homogenate was filtered through 3 layers of miracloth (Calbiochem-Behring, La Jolla, CA, USA) and the filtrate was washed three times with 1 volume of deionized water. After washing the insoluble tissue was resuspended in 1 L of 50 mM sodium acetate containing 0.2% sodium bisulfate, pH 6.0, and NaCl was added to a final concentration of 1.0 M. The resuspended sample was stirred for 30 min at 4°C and was centrifuged at 12,000×*g* for 60 min at 4°C as described above. The supernatant was dialyzed against resolubilization buffer and was concentrated using an Amicon Diaflo System with PM-10 membrane. The concentrated crude extract was loaded onto a 2.8×60 cm column of Sephadex G-100 and was eluted with 10 mM sodium phosphate buffer (pH 6.0). PGIP activity was measured in each 5 ml fraction. Fractions containing PGIP activity were pooled, concentrated using Amicon Diaflo System PM-10 membrane, and then applied to a 1.5×20 cm column of CM-Sephadex C-50 (Sigma, St. Louis, MO) in 10 mM sodium phosphate buffer (pH 6.0). Fractions containing PGIP activity were then pooled, concentrated and applied to a 1.5×20 cm column of QAE-Sephadex A-50 (Sigma, St. Louis, MO) in 10 mM Tris-HCl buffer (pH 8.0). Protein was eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer. The fractions containing PGIP activity were then pooled, concentrated, and applied onto a Sephacryl S-200 column (1.5×90 cm) and was eluted with 10 mM sodium phosphate buffer (pH 6.0). Active fractions (2 mL) were pooled and concentrated as described above. Protein was measured by using Bradford method[2].

Enzyme assay

PG activity was measured as increased of reducing sugars with 2-cyanoactamide as described by Gross[9]. The reaction mixture consisted of 100 μL of 0.4% polygalacturonic acid in 100 mM sodium acetate (pH 5.5) and 100 μL of enzyme solution. The reaction mixture was incubated for 1 h at 30°C, and then the reaction was terminated by addition of 1 mL of cold 100 mM borate buffer (pH 9.0), and 200 μL of 1% 2-cyanoactamide. The reaction mixture was incubated in a boiling water bath for 10 min. The change in absorbance at 276 nm was measured after cooling. One unit of PG activity was defined as the amount of galacturonic acid released at a rate of 1 mmol/min at 30°C. The inhibition of PG activity by PGIP was determined as rates of sugar production from polygalacturonic acid in the presence or absence of PGIP. The reaction mixture was consisted of 100 μL of 0.4% polygalacturonic acid in 100 mM sodium acetate (pH 5.5), 50 μL of PG enzyme solution and in the presence or absence of 50 μL of PGIP. After 1 h at 30°C, the reaction was terminated by the addition of 1 mL of cold 100 mM borate buffer (pH 9.0) and 200 μL of 1% 2-cyanoactamide. This mixture was incubated in a boiling water bath for 10 min. After cooling down, the absorbance was measured as described above. One unit of PGIP was defined as the amount of inhibitor required to reduce the activity of 1.5 unit of *B. dothidea* PG by 50%.

Characterization of PGIP inhibition of PG activity

Initial rate kinetics were calculated using the Michaelis-Menton equation. To determine the effects of PGIP on the V_{max} and K_m values of PG from *B. dothidea*, initial rate kinetics were performed in the presence or absence of 2 units of purified PGIP. The optimal pH for PG activity was measured using various buffer systems including, 10 mM sodium acetate (pH 4.0 and 5.0), 10 mM sodium phosphate (pH 6.0 and 7.0), 10 mM Tris-HCl (pH 8.0 and 9.0), and 10 mM glycine-NaOH (pH 10.0) and PGIP inhibition was measured as previously described. Effects of pH on stability of the PGIP was measured by keeping the enzyme in various pH adjusted buffers (10 mM) at 4°C for 18 h, and then the remaining activity was determined in enzyme assay described above. The effects of temperature on reaction mixtures of PG and PGIP were measured between 20°C and 80°C. The stability of PGIP was determined by measuring the activity remaining for aliquots of purified PGIP treated at each temperature for 10 min after cooling on ice for 5 min. Effect of various metal ions, Sodium dodecyl sulfate (SDS), and 1,2-diaminocyclohexane tetra acetat (CDTA) on the enzyme activity was investigated. Each reagent was dissolved to a final concentration of 1 mM in 10 mM sodium acetate buffer (pH 4.0). Two units of the purified PGIP and 1.5 units of the *B. dothidea* PG were added to each reaction mixture. The mixture was incubated at 30°C for 1 h, and then reactions were terminated and quantified by the addition of 1 mL of cold 100 mM borate buffer (pH 9.0) and 200 μL of 1% 2-cyanoactamide as described above.

Results and Discussion

Only one peak of PGIP was observed in the fractions obtained from Sephadex G-100 gel filtration chromatography. This was further purified by chromatography on CM-Sephadex and QAE-Sephadex ion exchange. And active fractions were pooled, desalted, and concentrated by ultrafiltration. Further Sephacryl S-200 chromatography was used to remove glycosylated proteins. After the removal step of glycosylated proteins, only one protein band of approximately 40 kDa was detected by SDS-PAGE (Fig. 1).

The effects of 'Fuji' PGIP on initial rate kinetics of *B. dothidea* PG were performed using a Lineweaver-Burk analysis (Fig. 2). The K_m and V_{max} values of PG from *B. dothidea* were 0.53 mg · mL^{-1} and 1.872 mol reducing sugar equivalents produced min · mL^{-1}, respectively. When PGIP was added to the reaction mixture the K_m value of *B. dothidea* PG increased to 2.46 mg · mL^{-1} and the V_{max} decreased to 1.273 mol min · mL^{-1} (Fig. 2). These data suggested that 'Fuji' apple PGIP was a mixed-type inhibitor of PG from *B. dothidea* (Fig. 2). This kinetic characterization of 'Fuji' apple PGIP is the same as that of 'Golden Delicious' apple PGIP[21]. Note that pear PGIP was a competitive inhibitor of PG, while bean and raspberry PGIPs were non-competitive inhibitors[1,10,12]. This difference might depend on the source of the PG rather than the source of the PGIP. We have demonstrated that PGIP inhibits PG with a mixed-type mode. This is compatible with a model where the substrate and the inhibitor bind the enzyme at the same time and the binding of the inhibitor affects the

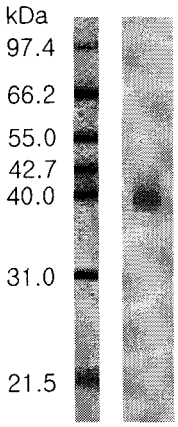

Fig. 1. SDS-PAGE of the PGIP eluted from Sephacryl S-200 gel filtration chromatography. Proteins were separated on 12.5% polyacrylamide gel and visualized by silver staining. The purified apple PGIP had showed a molecular mass of 40 kDa.

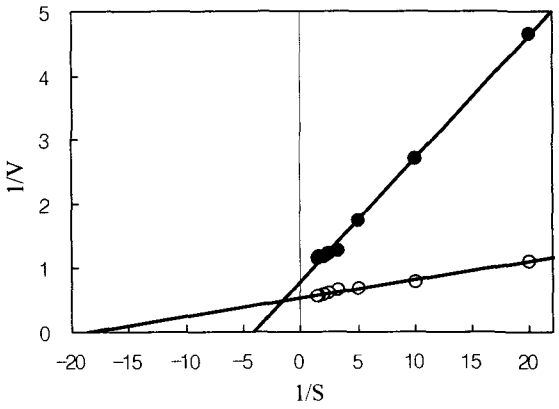

Fig. 2. Lineweaver-Burk double reciprocal plot of PG activity in the absence (- ○ -) or presence (- • -) of 2 units of purified PGIP.

dissociation constant of the substrate-enzyme complex.

Purified PGIP was effective over a broad pH range but activity showed a pH-dependent curve with increase at acidic pH and a maximum at pH 5.0 (Fig. 3).The maximal inhibitory activity against PG was observed at 40°C (Fig. 4). Heat stability of the enzyme was measured by heating the protein at various temperatures for 10 min. PGIP was stable up to temperature of 60°C, but it was completely inactivated at 70°C (Fig. 4). Plant PGIPs are relatively heat stable[1,3]. We found that the optimum temperature of 'Fuji' apple PGIP was 40°C and that the protein was stable up to 60°C. However, similar to other plants, PGIP was completely inactivated at 70°C.

For further characterization of the purified PGIP, we investigated the putative inhibitors. Various metal ions, SDS, and CDTA were added to the standard reaction buffer and the enzyme activities were measured. As shown in Table 1, the activities of PGIP were slightly reduced in the buffers containing Ca^{2+} or Zn^{2+}. However, the enzyme acti-

Table 1. Effects of various additives on activity of polygalacturonase-inhibiting protein purified from apples fruit

Additives	Concentration (mM)	Relative activity (%)
None	-	100.0
K^+	1	87.5±0.6
Cu^{2+}	1	73.1±0.3
Ca^{2+}	1	90.7±2.0
Zn^{2+}	1	95.7±1.1
Mg^{2+}	1	87.5±0.2
SDS	1	89.1±3.5
CDTA	1	82.8±0.2

*1 mM of K^+, K_2SO_4; Cu^{2+}, $CuSO_4$; Ca^{2+}, $CaCl_2$; Zn^{2+}, $ZnCl_2$; Mg^{2+}, $MgCl_2$.

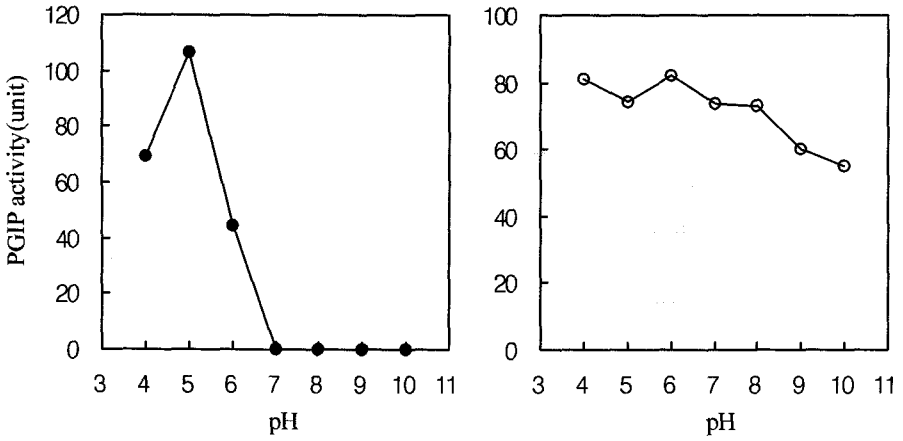

Fig. 3. Stability and optimal pH of PGIP. Effect of pH on activity (-•-) and stability (-○-) of PGIP extracted from apple fruits.

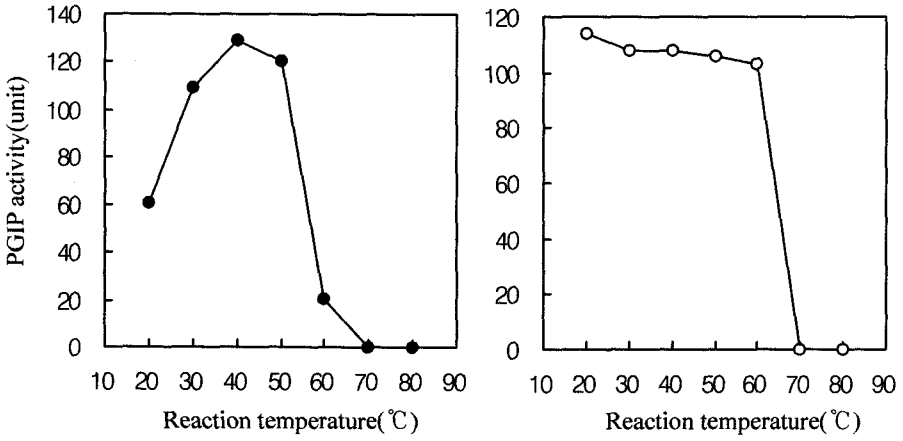

Fig. 4. Stability and optimal temperature of PGIP. Effect of temperature on activity (-•-) and stability (-○-) of PGIP extracted from apple fruits.

vities were reduced up to 20% in the buffers containing K^+, Cu^{2+}, Mg^{2+}, SDS, and CDTA. PG produced by *Rhizopus stolonifer* was inhibited Zn^{2+}, Mn^{2+}, Ca^{2+}, Hg^+[15]. The apple PGIP was also inhibited by metal ions, SDS, and CDTA. This result indicates a reduced propensity of the enzyme to interact with metal ions.

References

1. Abu-Goukh, A. A., L. C. Greve and J. M. Labavitch. 1983. Purification and partial characterization of 'Barlett' pear fruit polygalacturonase inhibitors. *Physiol. Plant Pathol.* **23,** 111-122.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.
3. Cervone, F., G. De Lorenzo, L. Degra, G. Salvi and M. Bergami. 1987. Purification characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. *Plant Physiol.* **85,** 631-637.
4. Collmer, A. and N.T. Keen. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Plant Dis.* **24,** 383-409.
5. Conway, W. S., K. C. Gross, C. D. Boyer and C. E. Sams. 1988. Inhibition of *Penicillium expansum* polygalacturonase activity by increase apple cell wall calcium. *Phytopathology* **78,** 1052-1055.
6. Cooper, R. M. 1984. The role of cell wall-degrading enzymes in infection and damage. in : Plant Diseases: Infection, Damage and Loss. R.K.S. Wood and G.J.Jellis, eds. *Blackwell Scientific Publications, Oxford,* p. 13-27.
7. Favaron, F., R. D'Ovidio, E. Porceddu and P. Alghisi. 1994. Purification and molecular characterization of soybean polygalacturonase-inhibiting protein. *Planta* **195,** 80-87.
8. Favaron, F., C. Castiglioni, R. D'Ovidio and P. Alghisi. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. *Physiol. Mol. Plant Pathol.* **50,** 403-417.
9. Gross, K. C. 1982. A rapid and sensitive method for assaying polygalacturonase using 2-cyanoacetamide. *HortScience* **17,** 933-934.
10. James, J. T. and I. A. Dubery. 2001. Inhibition of polygalacturonase from *Verticillium dahliae* by a polygalacturonase inhibiting protein from cotton. *Phytochemistry* **57,** 149-156.
11. Johnston, D. J. and B. Williamson. 1992. Purification and characterization of four polygalacturonase from *Botrytis cinerea. Mycol. Res.* **96,** 343-349.
12. Johnston, D. J., V. Ramanathan and B. Williamson. 1993. A protein from immature raspberry fruits which inhibitis endopolygalacturonases from *Botrytis cinerea* and other micro-organisms. *J. Exp. Bot.* **44,** 971-976.
13. Kemp, G., C. W. Bergmann, R. Clay, A. J. Van der Westhuizen and Z. A. Pretorius. 2003. Isolation of a polygalacturonase-inhibiting protein(PGIP) from wheat. *Mol. Plant-Microbe Interact.* **16,** 955-961.
14. Lee, D. H., H. Bae, I. K. Kang, J. K. Byun and S. G. Kang. 2006. Characterization of an Apple Polygalacturonase-inhibiting Protein(PGIP) That Specifically Inhibits an Endopolygalacturonase(PG) Purified from Apple Fruits Infected with *Botryosphaeria dothidea. J. Microbiol. Biotechnol.* (in press).
15. Manachini, P. L., M. G. Fortina and C. Parini. 1987. Purification and properties of an endopolygalacturonase produced by *Rhizopus stolonifer. Biotechnol. Lett.* **9,** 219-224.
16. Park, S. H., S. G. Suh and C. U. Lee. 1997. Purification and N-termina amino acid sequence of polygalacturonase produced by *Botryosphaeria dothidea. Korean J. Plant Pathol.* **13,** 402-407.
17. Peretto, R., F. Favaron, V. Bettini, G. De Lorenzo, S. Marini, P. Alghisi, F. Corvone and P. Bonfante. 1992. Expression and localization of polygalacturonase during the outgrowth of lateral roots in *Allium porrum* L. *Planta* **188,** 164-172.
18. Stotz, H. U., A. L. T. Powell, S. E. Damon, L. C. Greve, A. B. Bennett and J. M. Labavitch. 1993. Molecular characterization of polygalacturonase inhibitor from *Pyrus communis* L. cv. Barlett. *Plant Physiol.* **102,** 133-138.
19. Toubart, P., A. Desideri, G. Salvi, F. Cervone, L. Daroda, G. De Lorenzo, C. Bergmann, A. G. Darvill and P. Albersheim. 1992. Cloning and characterization of the endo-polygalacturonase-inhibiting protein(PGIP) of *Phaseolus vulgaris* L. *Plant J.* **2,** 367-373.
20. Van der Cruyssen, G., E. De Meester and O. Kamoen. 1994. Expression of polygalacturonase of *Botrytis cinereain* in vitro and in vivo. *Meded. Fac. Landbouwk. Toegep. Biolog. Wetensch. Univ. Gent.* **59,** 895-905.
21. Yao, C., W. S. Conway and C. E. Sams. 1995. Purification and characterization of a polygalacturonase-inhibiting protein from apple fruit. *Phytopathology* **85,** 1373-1377.

초록 : 사과 과실로부터 분리한 polygalacturonase-inhibiting protein(PGIP)의 생화학적 특성

이동훈[2] · 강상구[1] · 강인규* · 이윤경[3] · 최철[4] · 변재균[5]
(상주대학교 환경원예학과, [1]영남대학교 생명공학부, [2]농촌진흥청 난지농업연구소 감귤과, [3]아시아대학교 한약자원학과, [4]경북대학교 식물생명과학부, [5]영남대학교 자연자원학부)

사과 겹무늬썩음병균이(*Botryosphaeria dothidea*) 생성하는 세포벽 분해효소인 endopolygalaturonase를 억제하는 polygalacturonase-inhibiting protein (PGIP)를 사과 과실로부터 분리하였다. 분리되어진 사과 PGIP는 사과 겹무늬썩음병균이 생성하는 PG에 대하여 혼합형의 저해를 나타내었다. PGIP의 반응 최적온도는 40℃이며 최적 pH는 5.0이었다. 이 효소는 60℃까지는 비교적 안정하였으나 70℃에서는 효소의 활성이 완전히 억제되었으며 pH 4.0에서 8.0까지는 안정하였다. PGIP는 K^+, Cu^{2+}, Mg^{2+}, Ca^{2+}과 Zn^{2+} 등의 금속이온과 SDS 그리고 CDTA에 의해 효소의 활성이 저해되었다.