ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation and biochemical characterization of polygalacturonase isoforms from *Trichoderma spp.*

Afaf S. Fahmy[1], El-Shamaa M. El-Sayed[1], Somia S. Abdel-Gany[1], Hala A. Salah[1], Saleh A. Mohamed[1,2,*], Amr S. Abdelhamed[3].

[1]Molecular Biology Department, National Research Centre, Cairo, Egypt.
[2]King Abdulaziz University, Department of Biochemistry, Jeddah, Saudi Arabia.
[3]Chemical Department, Cairo University, Cairo, Egypt.

ABSTRACT

In the present study, the *Trichoderma spp.* were grown on three different media contained 5% of pectin, 5% citrus peel and 5% sugar beet pulp for producing extracellular polygalacturonases (PGases). By chromatography on DEAE-Sepharose column, two isoforms of PGase were separated from Trichoderma spp. except of four and one isoforms were separated from *Trichoderm virens* and *T. v6*, respectively. The characterization of the PGase isoforms from the examined Trichoderma spp. was reported. The K_m values of PGase isoforms were ranged from 1.69 to 10.10 mg/ml. Some of PGase isoforms had high affinity toward pectins than polygalacturonic acid. Optimum pH values of PGase isoforms exhibited a range between 4.0 and 6.0. The temperature optima of PGase isoforms ranged from 30 to 50°C. The influence of metal cations on the activity of PGase isoforms was detected. Some PGase isoforms acted as endo-action and others acted as exo-action using viscometric assay. In conclusion, the properties of *Trichoderma spp.* PGase isoforms meet the prerequisites needed for fruit processing.

Keywords: Trichoderma spp., Polygalacturonase, Isoforms, Chromatography, DEAE-Sepharose

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Pectin is polymer contained galacturonic acid residues that are linked with to methylated or acetylated groups [1,2]. Polygalacturonases hydrolyzed glycosidic α-1-4 linkages in pectin. Polygalacturonases are grouped into endo and exo-types [3,4]. Endo-polygalacturonases hydrolyzed the internal α-1,4 glycosidic linkages of polygalacturonic acid to release oligogalacturonic acids. Exo-polygalacturonases cleave the α-1,4 glycosidic linkages from the non-reducing end, releasing unsaturated mono- or digalacturonic acids [5].

Fungal polygalacturonases have been isolated and the characterized. Most fungal polygalacturonases are mesophilic exhibited optimal activities at 30–55ºC for *Rhizopus stolonifer* [6], *T. aurantiacus* [3], *Bispora sp.* MEY-1 [7] and *A. niger* [8] and pH 3.5–5.5 for *Penicillium viridicatum* [9], A*spergillus niveus* [10] and *Fusarium oxysporum* [11]. Several polygalacturonases hydrolyzed esterified pectins [12-14].

Polygalacturonases are one of the upcoming enzymes of fruit and textile industries. These enzymes break down complex polysaccharides of plant tissues into simpler molecules like galacturonic acids [15,16]. *Aspergillus carbonarius* and *sojae* polygalacturonases also improved the yield of juice [17,18] and reduced the viscosity of papaya juice [19]. In this study, the isolation and the biochemical characterization of PGase isoforms from *Trichoderma spp.* were performed for meet the prerequisites needed, concerning pH and temperature, in some applications such as food processing.

MATERIALS AND METHODS

Organisms

Trichoderma harzianum, Trichoderma viride, Trichoderma reesei, *Trichoderma psudokoningii*, *Trichoderma virens* and *T. V6 were* obtained from Plant Pathology Unit, National Research Centre, Cairo, Egypt.

Medium

T. harzianum, T. viride, T. hamatum, T. reesei, T. psudokoningii, T. virens and *T. V6* were cultivated and maintained on slants of potato dextrose broth.

Cultivation of organisms

Conidia was scrapped from mycelia which was grown on slants for five days at 28°C and suspended by hand shaking in sterile distilled water. One ml aliquot of this suspension was used to inoculate the medium under aseptic conditions, 250 ml Erlenmeyer flasks each containing 100 ml of sterile medium which contained 5% of pectin or sugar beet pulp or citrus peel. The inoculated flasks were incubated at 30°C on rotary shaker at 200 rpm for five days. The culture filtrate was obtained by filtration through four layers of Guzes.

Isolation of polygalacturonase isoforms from Trichoderma spp.

Preparation of cell-free broth

The culture filtrate of *T. harzianum, T.viride, T.reesei, T. psudokoningii, T. virens* and *T. V6* were concentrated by lypholization then dialyzed against 50 mM sodium acetate buffer, pH 5.0. The dialyzate was designated as cell-free broth. The cell free broth was frozen at -20ºC for further purification steps.

DEAE-Sepharose column

The cell-free broth was loaded on a DEAE- Sepharose CL-6B column (2.0 x 7.0 cm i.d.) equilibrated with 50 mM sodium acetate buffer, pH 5.0. Polygalacturonase isoforms were eluted with a stepwise gradient from 0.0 to 0.4 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 30 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for polygalacturonase activity.

Polygalacturonase assay

Polygalacturonase activity was determined by measurement of galacturonic acid released from polygalacturonic acid according to the method of Miller [20]. The reaction mixture was incubated at 37°C for 30 min in tubes containing 1% polygalacturonic acid, 50 mM sodium acetate buffer, pH 5.0, 10 µl enzyme solution and distilled water to give a final volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml dinitrosalicylic acid reagent, followed by incubation in a boiling water bath for 10 min followed by cooling. The absorbance was recorded at 560 nm using Shimadzu Spectrophotometer. The enzymatically liberated reducing sugar was calculated from a standard curve using galacturonic acid. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as galacturonic acid per hour under the standard assay conditions.

pH and temperature optima

The pH optimum of polygalacturonase activity was determined in the pH range from 4.5 to 7.0 using 0.05 M sodium acetate buffer (pH 4.5–5.5) and sodium phosphate buffer (pH 6.0–7.0). The temperature optimum of polygalacturonase activity was determined by incubating the enzyme-substrate mixtures at various temperatures (10- 80ºC) in 0.05 M sodium acetate buffer, pH 5.0 and the liberated reducing sugars were measured.

Km

The km values were determined from Line-weaver-Burk plots by using polygalacturonic acid concentrations from 0.2-1.0 mM.

Effect of metal ions

The effects of metal cations on polygalacturonase e activity were investigated by preincubating the enzyme with 2 mM Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Hg^{2+} , Ba^{+} and Ni^{2+} for 15 min prior to substrate addition. Activity in absence of metal cations was taken as 100% activity.

Determination of change in specific viscosity (η)

Viscometric assays were done in an Ostwald viscometer containing of reaction mixture (9 ml of 0.1% polygalacturonic acid in 0.05 M sodium acetate buffer, pH 5.0 and 1 unit of enzyme). Measurements were made at room temperature in a glass tube viscometer. Loss in viscosity was determined at 30 min intervals for 180 min.

Protein determination

Protein was determined by the method of Bradford [21 2] for bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In the present study, polygalacturonases (PGases) were produced by *Trichoderma spp.* grown on three different media: 5% pectin, 5% sugar beet pulp and 5% citrus peel (Table 1). The level of the PGase activity was in the order of sugar beet pulp (3.97-6.75 units/ml) > citrus peel (0.16-1.92 units/ml) > pectin (0.12-0.70 units/ml). The highest activity of PGase (6.75 units/ml) was detected in *T. reesei* grown on sugar beet pulp. Sugar beet pulp appeared more productive for PGase activity because sugar beet pulp (19.53%) had higher concentration of pectin [22] than that for orange peel (8.15%) [23]. Therefore, the isolation and characterization of PGases produced by *Trichoderma spp.* grown on sugar beet pulp were studied.

By chromatography on DEAE-Sepharose column, two isoforms of PGase were separated in all *Trichoderma spp.* except of four and one isoforms were separated from *T. virens* and *T. v6*, respectively. The isolation of PGase isoforms from tested *Trichoderma spp.* were summarized in Table 2. The specific activities of the PGase isoforms were increased than that detected for the cell-free broth. The highest specific activity was detected for *T. harzianum* PGase II (1093.75 units/mg protein). The highest fold of purification (18.77) was also

detected for *T. harzianum* PGase II. Similarly, by ion exchange chromatography different PGase isoforms has been reported for several fungi such as *Mucor flavus* [24], *Trichoderma harzianum* [25, 26].

The characterization of the PGase isoforms from the examined *Trichoderma spp.* were focused on kinetic parameters, substrate specificity, pH optimum, temperature optimum and the effect of metals and viscosity.The K_m values of PGase isoforms from *Trichoderma spp.* ranged from 1.73 to 10.10 mg/ml using polygalaturonic acid (Table 3) and were higher than K_m's for PGases from *F. soloni* (1.34 mg/ml) [27], *Streptomyces lydicus* (1.63 mg/ml) [28], *T. harzianum* (1.42 mg/ml) [25], *A. Sojae* (0.424 g/l) [15] and *A. niger* NRRL3 PGI (0.8 mg/ml) [29].

A variety of different pectins have been tried as substrates. The level of hydrolysis of tested pectins decreased with the degree of esterification (DE) from 26 to 93% in all PGase isoforms from *Trichoderma spp.* (Table 4). No PGase activity was detected for some forms of PGase using citrus pectin (DE 89 and 93%). The results showed that the esterfication and methylation of citrus pectins tested have higher affinity toward most PGase isoforms. This is similar to *T. harzianum* PGI [25]. In *A. niger* NRRL3, citrus pectin DE 93% had 75% relative activity higher than citrus pectin DE 89% [29].

The very important physical parameter influencing the activity of enzymes was the optimum pH. In the present study, the pH profile for the PGase isoforms from the examined *Trichoderma spp.* was performed. The optimum pH values for all examined PGase isoforms exhibited a range between 4.0 and 6.0 (Table 5). These similar to pH optima for PGases reported from some fungi such as *Kluyveromyces marxianus* (pH 4.0) [30], *A. tubingensis* (pH 4.2) [31], *Thermoasus aurantiacus* (pH 5.5) [32], *T. reseei* (pH's 4.5 and 4.2) [14], *A Steptomyces lydicus* (pH 6.0) [28].

The effect of temperature on PGase isoforms from the examined *Trichoderma spp.* was examined. The temperature optima of PGase isoforms ranged from 30 to 50˚C (Table 5). The similar temperature optima were detected for PGases from *A. japonicus* (30˚C) [33], *Mucor rouxii* NRRL1894 (35˚C) [34], *A. niger* NRRL3 (40˚C) [29] and *T. reesei* (40 and 50˚C) [14].

The influence of metal cations on the activity of PGase isoforms was detected (Table 6). Some metals such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+} and Ni^{2+} had a stimulation effect on the activity of some PGase isoforms and also caused partial and complete inhibition on the activity of other PGases. Hg^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+} had partial and complete inhibition on the activity of all PGase isoforms. However, in *T. harzianum* cultured on citrus peel, all examined metals cations at 1mM caused partial inhibitory effects on PGase except for Hg^{2+} which had a complete inhibitory effect [25]. In *T. harzianum* cultured on synthetic medium and *A. niger* NRRL3 all the examined metals cations had the same effect on PGII [29, 26].

Table 1. Screening of polygalacturonase activity in cell–free broth of some *Trichoderma spp.* grown on three different media.

Trichoderma species	PGase activity (units /ml)		
	Pectin	Citrus peel	Sugar beet pulp
T. harzianum	0.70 ± 0.04	0.94 ± 0.05	5.29 ± 0.30
T. v6	0.50 ± 0.03	1.92 ± 0.09	5.46 ± 0.20
T. reesei	0.25 ± 0.02	0.46 ± 0.03	6.75 ± 0.40
T. psudokoningii	0.32 ± 0.03	1.51 ± 0.26	5.33 ± 0.15
T. virens	0.17 ± 0.002	0.16 ± 0.01	3.97 ± 0.18
T. viride	0.12 ± 0.01	0.50 ± 0.03	6.13 ± 0.40

- Each value represents the mean of 3 experiments ± S.E.
- The culture medium contained 5% each of pectin, citrus peel and sugar beet pulp.

ISSN: 0975-8585

Table 2. Isolation of polygalacturonase isoenzymes from some *Trichoderma spp*.

Purification steps	Total units	Total protein (mg)	Specific activity (unit/mg protein)	Fold purification	Recovery (%)
Cell–free broth of *Trichoderma spp*. cultured on sugar beet pulp					
T. harzianum	110.73	1.90	58.27	1.00	100.00
T. viride	69.35	1.82	38.21	1.00	100.00
T. reesei	98.13	2.04	48.10	1.00	100.00
T. psudokoningii.	45.00	0.93	48.23	1.00	100.00
T. virens	50.92	0.86	59.21	1.00	100.00
T. hamatum	60.13	1.59	37.81	1.00	100.00
*T. hamatum**	23.61	0.15	158.46	1.00	100.00
T. V6	131.73	1.39	94.76	1.00	100.00
DEAE-Sepharose					
T. harzianum					
PGase I	34.61	0.21	176.86	3.04	31.26
PGase II	87.50	0.08	1093.75	18.77	79.02
T. viride					
PGase I	16.25	0.32	50.78	1.33	23.43
PGase II	10.68	0.18	59.33	1.55	15.40
T. reesei					
PGase I	75.87	0.16	474.19	9.86	77.32
PGase II	26.84	0.14	141.71	3.99	27.35
T. psudokoningii					
PGase I	24.56	0.04	614.00	12.77	54.58
PGase II	19.12	0.06	318.67	6.61	42.49
T. virens					
PGase I	7.5	0.15	50.00	0.84	14.73
PGase II	4.1	0.02	205.00	3.46	8.05
PGase III	13.7	0.05	274.00	4.63	26.90
PGase IV	7.6	0.02	380.00	6.42	14.93
T. V6					
PG	62.71	0.18	348.38	3.68	47.60

ISSN: 0975-8585

Table 3. K_m and V_{max} of polygalacturonase isoenzymes from some *Trichoderma spp*. K_m: the concentration of substrate at half maximum velocity; V_{max} : maximum velocity.

Trichoderma spp.	*T. harzianum*		*T. viride*		*T. reesei*		*T. psudokoningii*		*T. virens*				*T. V6*
	PGI	PGII	PGI	PGII	PGI	PGII	PGI	PGII	PGI	PGII	PGIII	PGIV	PG
K_m	7.69	10.10	5.00	9.09	1.85	6.25	2.44	2.04	3.39	1.73	2.30	10.00	8.33
V_{max}	7.14	43.48	0.90	1.09	1.15	14.49	1.88	1.97	2.77	3.13	20.00	4.00	15.87
V_{max}/ K_m	0.93	4.30	0.18	0.199	0.62	2.32	0.77	0.97	0.82	1.81	8.69	0.40	1.91

ISSN: 0975-8585

Table 4. Relative activities of polygalacturonase isoenzymes from some *Trichoderma spp*. toward different pectins.

Relative activity %													
Pectin	*T. harzianum*		*T. viride*		*T. reesei*		*T. psudokoningii*		*T. virens*				*T. V6*
	PG I	PGII	PG I	PGII	PG I	PGII	PG I	PGII	PG I	PGII	PGIII	PGIV	PG
PGA	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Pectin apple (methyl 7.8%)	152.67	58.53	651.12	391.00	148.11	251.00	232.58	557.08	6.09	57.17	88.47	1205.00	81.42
Pectin citrus (methyl 8%)	140.12	60.19	231.57	267.00	124.33	266.00	208.98	336.84	30.49	89.88	47.33	1735.00	81.55
Pectin citrus (methyl 8.9%)	126.12	107.84	36.09	35.00	103.67	305.00	141.57	102.43	37.69	16.53	152.26	405.00	79.13
Pectin citrus (DE 26%)	87.04	181.04	130.07	12.00	190.00	113.16	129.21	16.66	142.07	23.27	107.08	335.00	117.15
Pectin citrus (DE 67%)	67.28	161.61	76.69	16.00	79.49	57.66	90.44	12.55	74.33	20.71	76.23	275.00	68.91
Pectin citrus (DE 89%)	49.18	144.07	42.28	31.00	58.74	34.16	70.22	0.00	38.03	0.00	62.35	100.00	63.28
Pectin citrus (DE 93%)	36.21	58.29	0.00	6.50	40.50	0.00	40.44	0.00	22.62	0.00	60.93	0.00	42.53

ISSN: 0975-8585

Table 5. pH and temperature optima of polygalacturonase isoenzymes from some *Trichoderma spp*.

Relative activity %													
	T. harzianum		*T. viride*		*T. reesei*		*T.psudokoningii*		*T. virens*				*T.V6*
	PG I	PG II	PGI	PGII	PGI	PGII	PGI	PGII	PGI	PGII	PGIII	PGIV	PG
pH	4.0	5.0	6.0	6.0	5.0	5.0	5.5	6.0	5.0	5.0	5.5	5.5	5.0
Temperature ºC	40	50	40	40	40	40	30	40	40	40	50	60	50

ISSN: 0975-8585

Table 6. Effect of metal cations on polygalacturonase isoenzymes from some *Trichoderma spp*.

Relative activity %													
Metal cations	*T. harzianum*		*T. viride*		*T. reesei*		*T.psudokoningii*		*T. virens*				*T.V6*
	PG I	PG II	PGI	PGII	PGI	PGII	PGI	PGII	PGI	PGII	PGIII	PGIV	PG
Ca^{2+}	82.10	40.17	51.77	0.00	82.91	0.66	57.79	23.64	31.75	97.24	122.38	40.05	38.56
Ba^{2+}	63.42	79.35	0.00	0.00	107.68	11.37	109.01	22.07	83.75	0.00	74.78	41.25	85.61
Hg^{2+}	0.00	18.81	0.00	0.00	0.00	0.00	46.75	10.86	1.36	19.33	16.43	0.00	12.47
Zn^{2+}	0.00	1.098	180.20	18.51	94.83	1.98	134.41	75.58	132.36	86.80	18.13	0.00	86.65
Mg^{2+}	136.84	129.14	0.00	28.57	114.14	151.85	214.93	127.52	86.66	225.76	461.75	100.59	103.12
Ni^{2+}	11.84	14.73	0.00	217.46	18.37	0.00	17.53	26.62	15.49	163.49	295.75	42.72	18.52
Co^{2+}	55.79	34.11	0.00	22.75	73.44	16.66	23.37	48.83	37.13	86.81	92.35	91.69	11.10
Fe^{2+}	64.47	6.74	86.80	0.00	18.44	0.00	92.21	21.76	2.26	4.91	54.11	24.63	10.23
Cu^{2+}	41.05	2.49	0.00	0.00	0.00	0.00	78.57	41.08	9.20	54.60	17.56	17.80	18.05

ISSN: 0975-8585

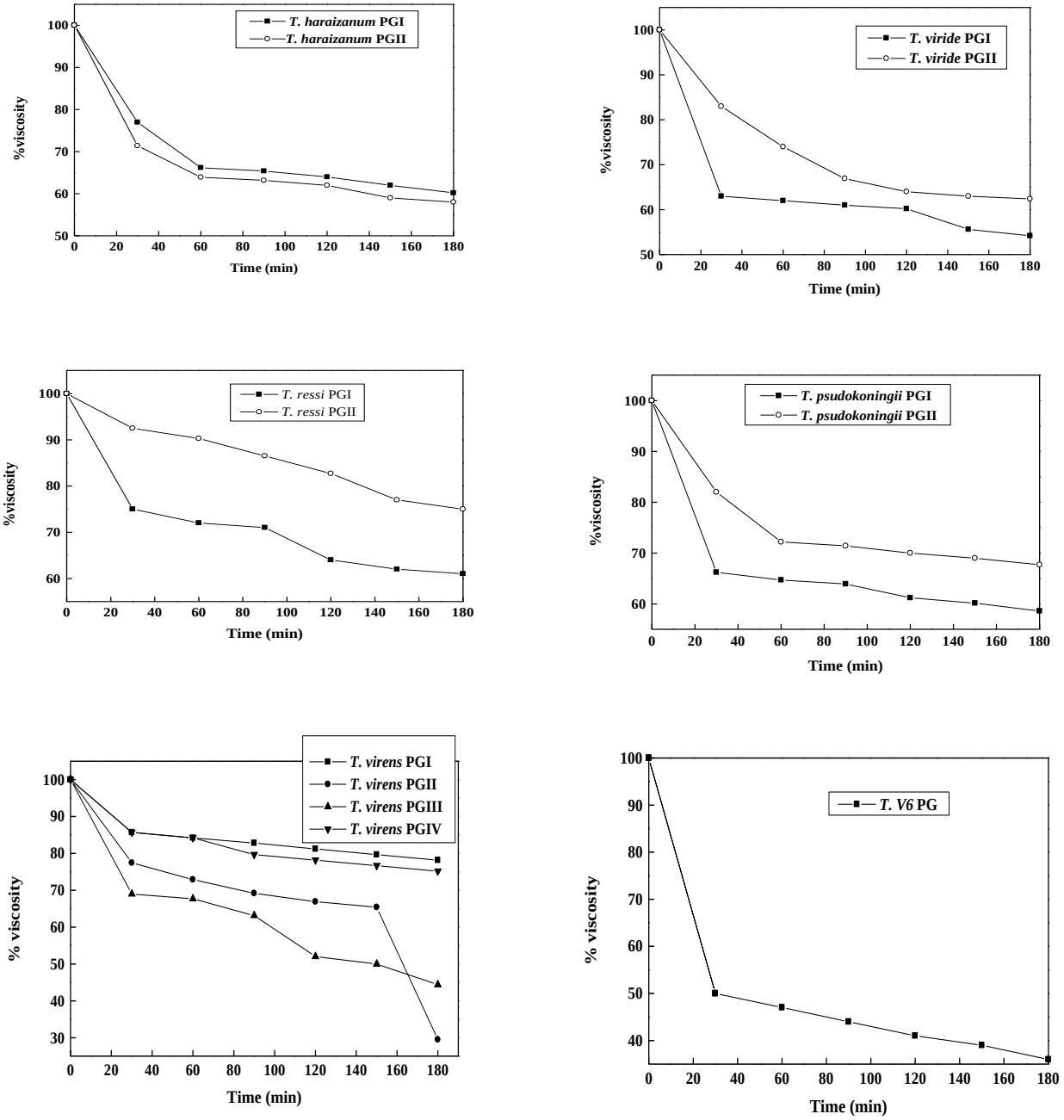

Fig. 1. Determination of viscosity of polygalacturonic acid by polygalacturonase isoenzymes from some *Trichoderma spp.*

The mode of action of the enzyme (exo PGase or endo PGase) was determined by using viscometeric assays. The results showed that some PGase isoforms from the examined *Trichoderma spp.* acted as endo-action (reduction of more than 50% viscosity) and others acted as exo-action (reduction of less than 50% viscosity) (Fig. 1). Most of PGase isoforms acted as exo-action. PGI from *Armillaria sp*. was reduced the

viscosity of PGA to 50% after 15 min of incubation with a corresponding cleavage of about 17.5% of the glycosidic bonds suggesting that PGI is an endo-action enzyme [35].

CONCLUSIONS

The properties of *Trichoderma spp.* PGase isoforms meet the prerequisites needed for fruit processing especially their broad pH optimum, temperature optimum, and high affinity toward some esterified pectins, where the most fruits contained esterified pectins.

REFERENCES

[1] Lerouxel O, Cavalier DM, Liepman AH, Keegstra K. Curr Opin Plant Biol 2006; 9: 621–630.
[2] Soumpourou E, Iakovidis M, Chartrain L, Lyall V, Thomas, Theor CM. Appl Genet 2007; 115: 1127–1136.
[3] Yuan P, Meng K, Wang Y, Luo H, Shi P, Huang H., et al, Bioresour Technol 2012; 123 171–176.
[4] Angelova MB. Microb Biotechnol Horticult 2008; 3: 101–179.
[5] Manachini P, Fortina M, Parini C. Biotechnol Lett 1987; 9: 219–224.
[6] Martins EDS, Leite RS, da Silva R, Gomes E. Enzyme Res 2013; 438645.
[7] Yang J, Luo H, Li J, Wang K, Cheng H, Bai Y, et al, Process Biochem 2011; 46: 272–277.
[8] Kant S, A. Vohra S, Gupta R. Protein Express Purif 2013; 87: 11–16.
[9] Gomes E, Leite RSR, da Silva R, Silva D. Int J Microbiol 2009; 1: 1 1–8.
[10] Maller A, da Silva TM, Damasio AR, Hirata IY, Jorge JA, Terenzi HF et al, Folia Microbiol (Praha), 2013;58: 615–621.
[11] Di Pietro A, Roncero MI. FEMS Microbiol Lett 1996; 145: 295–299.
[12] Contreras-Esquivel JC, Voget CE. J Biotechnol 2004; 11: 21–28.
[13] Kobayashi T, Higaki N, Suzumatsu A, Sawada K, Hagihara H, Kawai S, Ito S., Enzyme Microb Technol 2001; 29: 70–75.
[14] Mohamed SA, Christensen TMIE, Mikkelsen JD. Carbohyd Res 2003; 338: 515–524.
[15] Tapre A R, Jain RK. Int Food Res J 2014; 21: 447-453.
[16] Tari C, Gogus N, Tokatli F. Enzyme Microb Technol 2007; 4:0 1108–1116.
[17] Nakkeeran E, Umesh-Kumar S, Subramanian R. Bioresour Technol 2011; 102: 3293–3297.
[18] Tari C, Dogan N, Gogus N. Food Chem 2008;111: 824–829.
[19] Tu T, Meng K, Bai Y, Shi P, Luo H, Wang Y, et al, Food Chem 2013; 141: 2974–2981.
[20] Miller GL. Anal Chem 1959; 31: 426-429.
[21] Bradford MM. Anal Biochem 1976; 72: 248-254.
[22] Phatak L, Chang KC, Brown G. J Food Sci 1988; 53: 830-833.
[23] Rous AH, Crandall PG. Proc Fla State Hort Soc 1976; 89: 166-168.
[24] Gadre RV, Driessche GV, Beeumen JV, Bhat MK. Enzyme Microb Technol 2003; 32: 321–330.
[25] Mohamed SA, Al-Malki AL, Kumosani TA. Aust J Basic Appl Sci 2009; 3: 2770-2777.
[26] Mohamed SA, Farid NM, Hossiny EN, Bassuiny RI. J Biotechnol 2006; 127: 54–64.
[27] Zhang J, Bruton BD, Biles GL. Physiol Molec Plant Pathol 1999; 54: 171-186.
[28] Jacob N, Poorna CA, Prema P. Bioresour Technol 2008; 99: 6697–6701.
[29] Fahmy AS, El-beih FM, Mohamed SA, Abdel-Gany SS, Abd-Elbaky EA. Appl Biochem Biotechnol 2008; 149: 205–217.
[30] Serrat M, Bermudez RC, Villa TG. Appl Biochem Biotechnol 2002; 97: 193–208.
[31] Gewali MB, Maharjan J, Thapa S, Shrestha JK. Sci World 2007; 5: 19-22.
[32] Martins ES, Silva D, Leite RS, Gomes E. Antonie Van Leeuwenhoek 2007; 91: 291– 299.
[33] Hasunuma T, Fukusaki EI, Kobayashi A. J Biotechnol 2003;106: 45–52.
[34] Saad N, Briand M, Gardarin C, Briand Y, Michaud P. Enzyme Microb Technol 2007; 41: 800–805.
[35] Mwenje E, Ride JP. Physiol Molec Plant Pathol 1999; 55: 131-139.