Characterization of pectinases from *Acrophialophora nainiana* and *Trichoderma harzianum* strain T6

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Abstract
Two pectinase preparations have been isolated from liquid-state cultures of *Acrophialophora nainiana* and *Trichoderma harzianum* strain T6 containing pectin as the carbon source. Pectin lyase and pectin methylesterase activities were only detected in the enzyme preparations from *A. nainiana*. The exo-polymethylgalacturonase from *A. nainiana* was most active at pH 7.0 and 60ºC, while exo-polymethylgalacturonase optimal activity from *T. harzianum* strain T6 was obtained at pH 4.3 and 40ºC. Their stability at 40, 50 and 65ºC differed, being the exo-polymethylgalacturonase activity from *A. nainiana* more stable. Both enzyme preparations showed good stability at acidic pH. The crude extracts were partially purified by ultrafiltration, and gel filtration chromatography on Sephacryl S-100. Kinetic parameter studies showed that the lowest K_m values were obtained with exo-polymethylgalacturonase from *T. harzianum* strain T6. The treatment of fruit juices with pectinase samples from *A. nainiana* and *T. harzianum* strain T6 resulted in a decrease of viscosity. Pectinase from *T. harzianum* strain T6 appeared to be more effective in the reduction of turbidity from apple juice. Only pectinase from *T. harzianum* strain T6 was able to extract juice from banana fruit.

Keywords: pectin; fungus; enzyme characterization.

INTRODUCTION

Pectins are the major group of polysaccharides present in the primary cell wall and the middle lamella of higher plants, accounting for as much as 30% of the dry weight of plant tissue (COLMER; RIED; MOUNT, 1988; KASHYAP; CHOPRA; TEWARI, 2001; SOARES et al., 2001; STRASSER; AMADO, 2001). Pectic substances are a complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by a(1-4) linkages and, depending on their origin, may be substituted with side chains of L-rhamnose, arabinose, galactose and xylose. (COLMER; RIED; MOUNT, 1988; STRASSER; AMADO, 2001; GUMMADI; PANDA, 2003)

Pectinases are produced by a wide range of microorganisms, including plant pathogenic fungi and bacteria. Enzymes which breakdown pectin are classified according to their substrate preference, reaction mechanism, and action pattern on the galacturonan backbone of the polymer (COLMER; RIED; MOUNT, 1988; SOARES et al., 2001; BONNIN et al., 2003).

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They show different preferences for methylated and unmethylated forms of pectin, and cleave either glycosidic linkages internally at random or in the terminal non-reducing end of the pectin chain. (REID; RICARD, 2002)

Pectinases are one of the most important groups of enzymes used in the fruit and vegetable industry, and have been employed to improve the cloud stability of fruit nectars, in the clarification of fruit juices and wines, coffee and tea processing, maceration of vegetable tissue and papermaking (KASHYAP; CHOPRA; TEWARI, 2001; SOARES et al., 2001; REID; RICARD, 2002; GUMMADI; PANDA, 2003;). Due to their biotechnological potential, extensive study has been directed to the production, isolation and characterization of the pectinase systems from microorganisms. (SHANLEY et al., 1993)

Early studies have demonstrated that the fungi *Acrophialophora nainiana* and *Trichoderma harzianum* strain T produce high pectinase activity when grown on liquid-state cultures containing banana plant residue (MEDEIROS et al., 2000). In this present work, we report the production, characterization and some applications of pectinase activities from *A. nainiana* and *T. harzianum* strain T6.

MATERIALS AND METHODS

Chemicals

Pectin from citrus fruits and Sephacryl S-100 were purchased from Sigma (St. Louis, MO, U.S.A.) and Amersham Biosciences do Brasil (São Paulo, SP, Brasil.), respectively.

Microorganisms and enzyme production

For production of pectinolytic enzymes, the fungi have been cultured in 1-liter Erlenmeyer flasks containing 300 ml of liquid-state medium at 28ºC for 5 days (*T. harzianum* strain T6) and 40ºC for 9 days (*A. nainiana*) with rotary shaking (100 rpm). The composition of the medium (w/v) was as follows: 0.5 % pectin from citrus fruits, 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.06% yeast extract. Flasks were inoculated with spore suspensions of 1 x 10^6 and 1 x 10^4 ml^{-1} from routine agar cultures of *T. harzianum* strain T6 and *A. nainiana*, respectively. The contents of each flask were filtered through Whatman filter paper No. 1. The supernatants, hereafter called crude extract, were frozen and stored for subsequent use as sources of pectinase activity.

Enzyme assays

Exo-Polymethylgalacturonase activities from *T. harzianum* strain T6 and *A. nainiana* were routinely determined by mixing 50 ml of enzyme solution with 100 ml of pectin from citrus fruits (1%, w/v), previously prepared in 50 mM sodium acetate buffer, pH 4.3 at 40ºC and 50 mM sodium phosphate buffer, pH 7.0 at 50ºC, respectively for 30 min. The release of reducing sugar was measured using the dinitrosalicylic reagent method (MILLER, 1959). Galacturonic acid was used as the standard. The enzyme activity was expressed as mmol of reducing sugar formed min^{-1}, i.e., as IU. Pectin lyase and pectin methylesterase activities were measured as described by Soares and others (2001), and Spagna, Barbagallo and Ingalinera (2003), respectively. Each assay described above was repeated at least three times with standard deviation less ±20% of the mean.

Enzyme partial purification

All purification steps were carried out at 4ºC unless otherwise specified. The crude extract samples from *A. nainiana* and *T. harzianum* strain T6 were concentrated about 10-fold by ultrafiltration using an Amicon system with a 10 kDa cut-off point PM 10 membrane.The concentrated crude extracts from *A. nainiana* and *T. harzianum* strain T6 were applied to a Sephacryl S-100 column (2.4 x 44 cm) and eluted with 50 mM sodium phosphate buffer, pH 7.0. Fractions of 4 ml were collected at a flow rate of 24 ml/h.. For both enzyme samples, fractions containing the highest exo-polymethylgalacturonase activity were pooled, concentrated by ultrafiltration and stored for later use at 4ºC.

Enzyme characterization

The determination of optimum temperature of crude exo-polymethylgalacturonase samples was carried out in the temperature range of 30 to 80°C in pH 5.0. Optimum pH values were determined by measuring the enzyme activity at 40 and 60°C for exo-polymethylgalacturonases from T. *harzianum* strain T6 and A. *nainiana*, respectively at pH values from 3.0 to 8.0. The following buffers were used: 50 mM sodium acetate buffer (3.0-5.5), 50 mM sodium phosphate buffer (6.0-8.0). The buffers, regardless of pH, were adjusted to the same ionic strength with NaCl. The temperature stability of exo-polymethylgalacturonase samples was determined by pre-incubating the enzyme at 40, 50 and 65°C and pH 7.0. To study the pH stability, exo-polymethylgalacturonase samples of T. *harzianum* strain T6 and A. *nainiana* were incubated in 50 mM glycine-HCl buffer at pH values of 2,2 and 2,5 and temperatures of 40 and 50°C, respectively. At various time periods, aliquots were withdrawn and the residual activity was measured under standard conditions. For the kinetic experiments, pectin from citrus fruits was used as substrate in a concentration range of 1.0-30.0 mg/mL K_m and V_{max} values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program (LEATHERBARROW, 1987). The effect of enzyme treatment on viscosity and turbidity of fruit juices was determined as follows: The enzyme activities from T. *harzianum* strain T6 and A. *nainiana* were assayed viscometrically at 40 and 50°C, respectively. The reaction mixtures composing of 1 ml of enzyme sample plus 30 ml of lemon or orange juices were incubated at 100 rpm. In the control, enzyme solution was replaced by distilled water.The rate of viscosity reduction was measured every 3 min in an Ostwald viscometer and calculated using the equation:

$$\mu_{juice} = (\mu_{water} \times T_{juice} \times \rho_{juice})/(T_{water} \times \rho_{water}).$$

Where m is the viscosity, T is the flow time and r is the density. Pectinolytic enzymes from *T. harzianum* strain T6 and *A. nainiana* were also submitted to turbidimetric assay containing apple juice as the substrate at the same conditions as reported above for the viscosimetric assay.The suspension was centrifuged at 3,951 g for 5 min at 4°C. Turbidities of the supernatant samples were subsequently measured in a Hanna LP 2000 turbidimeter (UK). Turbidity was expressed as nephelometric turbidity unit (NTU). A peeling banana fruit (30 g), previously cut and macerated, was incubated with 1 ml of concentrated crude extract samples from *T. harzianum* strain T6 and *A. nainiana* for 30 min at 40 and 50°C, respectively in a reciprocating shaking water bath. Later the samples were filtered by vaccum through filter paper and the juice volume was determined (SOARES et al., 2001). Appropriate controls were used in all experiments. Each experiment described above was repeated at least three times with standard deviation less ±20% of the mean.

RESULTS AND DISCUSSION

Enzyme characterization

The ability of *A. nainiana* and *T. harzianum* strain T6 to utilize pectin as the sole carbon source in liquid-state media resulted in the production of high extracellular pectinase activity. Pectin lyase and pectin methylesterase activities of 0.045 and 0.008 IU were only detected in the enzyme preparations from *A. nainiana*, respectively

The crude exo-polymethylgalacturonase activities from *T. harzianum* strain T6 and *A. nainiana* were optimal at 40 and 60°C, respectively. The optimum pH profile showed that the crude exo-polymethylgalacturonase from *A. nainiana* was more alkaline resistant than that of *T. harzianum* strain T6, with pH values of 7.0 and 4.3, respectively. The polygalacturonase from *Sporotrichum thermophile* was also reported to have an optimal activity at pH 7.0 (KAUR; KUMAR; SATYANARAYANA, 2004). Alkaline pectinases are used

mainly in the retting and degumming of fiber crops and treatment of pectic wastewater (KASHYAP; CHOPRA; TEWARI, 2001). The polymethylgalacturonase from *Aspergillus niger* was optimally active over a pH range of 6.5-7.0 (GUMMADI; PANDA, 2003), while a maximum activity was obtained at pH 9.0 for pectinase from *Bacillus macerans* (MYAZAKI, 1991). The optimal pH value for exo-polymethylgalacturonase activity from *T. harzianum* strain T6 was in the acidic range reported for some fungal pectinases (KOBAYASHI et al., 2001). An endopoly-galacturonase from *Aspergillus kawachii* had maximum activity in the pH range 2.0-3.0 (CONTRERAS; VOGET, 2004). The polygalacturonase from *Mucor flavus* was most active at 45°C and pH range of 3.5-5.6. (GADRE et al., 2003)

The stability under different processing conditions (pH and temperature) was determined for both crude exo-polymethylgalacturonase preparations. Thermal and pH stabilities are considered to be important parameters to industrial application of a given pectinase. An improved knowledge of the properties of microbial pectinases is important in commercialization of industrial production and to apply these enzymes in various potential fields (GUMMADI; PANDA, 2003). While the enzyme from *T. harzianum* strain T6 had half-life of approx. 2 min at 65°C, the exo-polymethylgalacturonase from *A. nainiana* had half-life of 60 min. The half-lives of endopolygalacturonase activities from *Phanerochaete chrysosporium* (SHANLEY et al., 1993) and *Saccharomyces cerevisae* (BLANCO et al., 1994) were 14.5 and 20 min, respectively at 60°C. The polygalacturonase from *Mucor flavus* retained its activity in the pH range between 2.6-6.0 and was denatured at and above 50°C (GADRE et al., 2003). At 40 and 50°C, the crude enzyme preparation from *A. nainiana* was also remarkably more stable than the enzyme preparation from *T. harzianum* strain T6. It retained 100% of the activity after six days of incubation, while the enzyme from *T. harzianum* strain T6 had half-lives of six days and 7 min, respectively. The pH stability of exo-polymethylgalacturonases from *A. nainiana* and *T. harzianum* strain T6 showed half-lives of 70 and 113 h at pH values of 2.5 and 2.2, respectively. The acidic tolerant property of the above exo-polymethylgalacturonases, especially from *T. harzianum* strain T6, demonstrate potential to be used in the fruit industry. The pH and temperature profiles of the exo-polymethylgalacturonases from *A. nainiana* and *T. harzianum* strain T6 is in the same range for those reported for pectinolytic preparations from *Aspergillus niger* (BEHERE; SATYANARAYAN; PADWAL-DESAI, 1995), *Saccharomyces cerevisae* (BLANCO et al., 1994) and *Bacillus* sp. strain KSM-P576. (KOBAYASHI et al., 2001)

Kinetics of exo-polymethylgalacturonases over pectin from citrus fruit were according to the Michaelis-Menten model. The K_m values of crude and partially purified exo-polymethylgalacturonase from *T. harzianum* strain T6 were lower than that observed for exo-polymethylgalacturonase samples from *A. nainiana* (TABLE 1). PG II showed higher affinity for pectin than pectinases from *Neurospora crassa* and *Paenibacillus amylolyticus.* (LOURDES et al., 1991; SAKIYAMA et al., 2001)

Table 1 - Kinetic parameters of exo-polymethyl-galacturonase from A. nainiana and T. harzianum strain T6

Enzyme Sample	K_m (mg/ml)	V_{Max} (IU)
Crude enzyme (1)	19.24	0.171
PG I	13.33	0.133
Crude enzyme (2)	8.53	0.0094
PG II	4.32	0.0058

(1) A. nainiana
(2) T. harzianum strain T6

Enzyme partial purification

Ultrafiltration of crude extract samples from both fungi showed that exo-polymethylgalacturonase activity was completely retained by the membrane. In the context of the purification, it should be noted

that the ultrafiltration procedure gave a recovery of 100% activity. Besides, the ultrafiltration step avoided the centrifugation and desalting procedures associated with ammonium sulphate and removed most of the coloured pigments (GADRE et al., 2003). After the ultrafiltration procedure, the concentrated crude extract samples from *A. nainiana* and *T. harzianum* strain T6, hereinafter called the retentate, were partially purified by gel filtration chromatography on Sephacryl S-100 colunm (FIGURE 1; FIGURE 2). The elution profile of the retentate sample from *A. nainiana* on Sephacryl S-100 displayed only one peak of exo-polymethylgalacturonase activity (PG I). Pectin lyase activity was also detected at the. same activity peak (result not shown). Partial purification of exo-polymethylgalacturonase activity led to almost 100% recovery and approx. 5-fold purification. A major peak of exo-polymethylgalacturonase activity (PG II) of *T. harzianum* strain T6, corresponding to a fraction range of 22 to 28, was resolved by gel filtration.

Enzyme application

The action of pectinases from *A. nainiana* and *T. harzianum* strain T6 on fruit juices was studied (TABLE 2; TABLE 3). Because of juice viscosity is primary caused by pectin, pectinases have attracted considerable attention as an alternative approach in the reduction of fruit

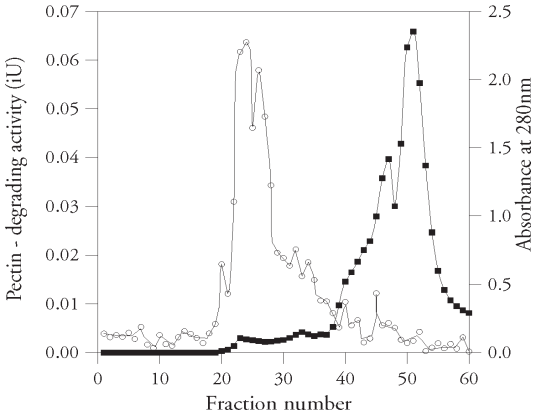

Figure 1 - Fractionation on Sephacryl S-100 of *T. harzianum* strain T6 crude extract.
Symbols: O- exo-polimethylgalacturonase activity; g- Absorbance at 280 nm.

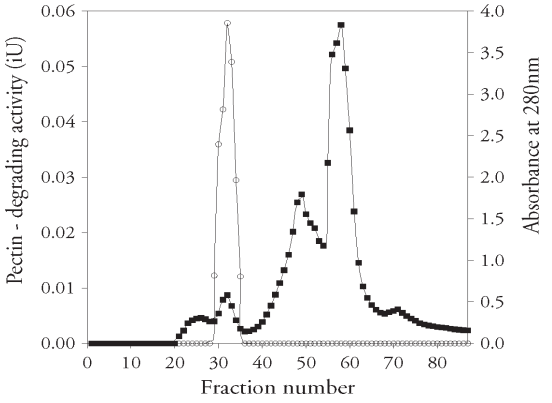

Figure 2 - Fractionation on Sephacryl S-100 of *A. nainiana* crude extract.
Symbols: O- exo-polimethylgalacturonase activity; g - Absorbance at 280 nm.

Table 2 - Effect of enzyme treatment on viscosity of orange and lemon juices

Enzyme Sample	Viscosity (cP)	Viscosity (% reduction)
Crude Pectinolytic enzyme from *T. harzianum* (1)	1.37	26
Retentate from *T. harzianum* (1)	1.08	31
Retentate from *T. harzianum* (2)	1.06	43
Crude Pectinolytic enzyme from *T. harzianum* (2)	1.04	31
PG II (1)	1.51	29
Retentate from *A. nainiana* (1)	1.38	05
PG I (1)	1.28	12

(1) Lemon Juice
(2) Orange Juice

Table 3 - Effect of enzyme treatment on turbidity of apple juice

Enzyme Sample	Turbidity (NTU)	Turbidity (% reduction)
Crude Pectinase from *T. harzianum*	165	44.4
Retentate from *T. harzianum*	278	6.4
PG II	163	45.1
Retentate from *A. nainiana*	413	115.1
PG I	196	2.1

viscosity and turbidity (KASHYAP; CHOPRA; TEWARI, 2001; KOBAYASHI et al., 2001; KAUR; KUMAR; SATYANARAYANA, 2004;). As can be seen from the tables, the treatment of orange and lemon juices with crude pectinases samples from *T. harzianum* strain T6 caused higher reduction in the fruit viscosity than those obtained for enzyme samples from *A. nainiana*. This behavior suggest an endo-splitting mechanism for both enzyme preparations (BLANCO et al., 1994). The best drop in viscosity was obtained by a pectinase from *T. harzianum* strain T6 concentrated by ultrafiltration. All pectinase preparations from *T. harzianum* strain T6 were able to reduce the turbidity of apple juice, being the preparation from gel filtration on Sephacryl S-100 more efficient. On the other hand, only the gel filtration sample from *A. nainiana* was able to decrease, in a lesser extent, the turbidity of apple juice. Preliminary results showed that the pectinase from *T. harzianum* strain T6 extracted 4 ml of juice from banana fruit. Banana fruit is described to have a high content of soluble pectin in its structure (SOARES et al., 2001). An increase in the yield of banana juice was observed after pulp tretament with pectinase from *S. thermophile* (KAUR; KUMAR; SATYANARAYANA, 2004). The enzyme from *A. nainiana* did not perform the fruit extraction.

Conclusion

In conclusion, *T. harzianum* strain T6 and *A. nainiana* produce exo-polymethylgalacturonases with good activity at acidic and neutral pH, respectively, being the enzyme from *A. nainiana* more thermostable. Pectinase preparation from *T. harzianum* strain T6 was more effective in the reduction of fruit viscosity.

Caracterização de pectinases de Acrophialophora nainiana e Trichoderma harzianum linhagem T6

Resumo

Duas preparações de pectinases foram isoladas de culturas em estado líquido de Acrophialophora nainiana e Trichoderma harzianum linhagem T6, contendo pectina como a fonte de carbono. As atividades de pectina liase e pectina metilesterase foram somente detectadas nas preparações enzimáticas de A. nainiana. A exo-polymethylgalacturonase de A. nainiana foi mais ativa a pH 7,0 e 60ºC, enquanto que a atividade ótima da exo-polymethylgalacturonase de T. harzianum linhagem T6 foi obtida a pH 4,3 e 40ºC. As suas estabilidades a 40, 50 e 65ºC diferiram, sendo que a atividade de exo-polymethylgalacturonase de A. nainiana apresentou maior estabilidade. Ambas preparações enzimáticas mostraram boa estabilidade em pH ácido. Os extratos brutos foram parcialmente parcialmente purificados por ultrafiltração e cromatografia de filtração em gel em coluna de Sephacryl S-100. Estudos de parâmetros cinéticos mostraram que os valores de K_m mais baixos foram obtidos com exo-polimetilgalacturonase de T. harzianum linhagem T6. O tratamento de sucos de frutas com amostras de pectinase de A. nainiana e T. harzianum linhagem T6 resultou em um decréscimo na viscosidade. A pectinase de T. harzianum linhagem T6 parece ser mais eficiente na redução da turbidez de suco de maça. Somente a pectinase de T. harzianum linhagem T6 foi capaz de extrair suco da banana.

***Palavras-chave**: pectina; fungos; enzima-caracterização.*

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