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Purification and partial characterisation of pectin methylesterase produced by *Fusarium asiaticum*

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ABSTRACT

Fusarium asiaticum is the predominant causal agent of Fusarium head blight (FHB) in China. When grown in liquid cultures containing potato tuber extract as the sole carbon source, *F. asiaticum* (strain 7071) from wheat (China) produced pectin methylesterase (PME), polygalacturonase (PG), and pectin lyase (PNL). The activity of these pectolytic enzymes was detected by a gel diffusion assay. Three forms of PME were identified in a culture filtrate of *F. asiaticum*. Two forms of PME with molecular weights of 31 kDa and 42.5 kDa, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were purified using a combination of chromatographic techniques. These PMEs did not bind to Concanavalin A (Con A), which was confirmed by rechromatography using a Con A agarose column. The 31 kDa purified PME was thermostable in a temperature range of 25–55 °C. The optimal pH for the PME of *F. asiaticum* was 6.5. This research provides the basis for future investigations of pectolytic enzymes from *F. asiaticum*.

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Introduction

Fusarium asiaticum is the predominant causal agent of Fusarium head blight (FHB) in China (Qu *et al*. 2008). Generally, Fusarium pathogens cause diseases in cereal crops, such as wheat, barley, and other small grains (Kikot *et al*. 2009). Infection of wheat with FHB leads to worldwide yield losses of grain and to food spoilage by its mycotoxins (Legzdina & Buersmayr 2004). Natural resistance of wheat against FHB is inadequate, and new strategies for controlling the disease are required (Li *et al*. 2008; Xu *et al*. 2010). Therefore, understanding of the mechanisms leading to fungal pathogenicity is a prerequisite to consider new control strategies. In most studied cases, there is a correlation among the presence of pectic enzymes, disease symptoms, and virulence, which is also the decisive point for development of the infection process (Kikot *et al*. 2009). For example, it has been postulated that *Fusarium graminearum* penetrates and invades its hosts with the help of cell wall degrading enzymes (CWDE) (Wanjiru *et al*. 2002; Wanyoike *et al*. 2002; Jenczmionka & Schafer 2005). Pectic substances constitute the main components of the middle lamella and also make up a large portion of the primary cell wall of young plant cells in which they form an amorphous gel filling the spaces between the cellulose microfibrils. Due to its complex structure, pectin modification and breakdown are catalysed by a variety of pectolytic enzymes. Fungal pectin

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methylesterase (PME) has a key role in the propagation of plant pathogens (Duvetter *et al.* 2006). PME (EC 3.1.1.11) catalyses the hydrolysis of the methyl ester bonds altering the degree and pattern of methyl esterification. PME activity is assumed to allow the subsequent action of depolymerising enzymes, such as polygalacturonase (PG) cleaving glycosidic bonds by hydrolysis and pectate lyases breaking polygalacturonic acid into oligogalacturonides by β-elimination (Reignault *et al.* 2008). PMEs are found in phytopathogenic fungi, such as *Aspergillus* (Baron *et al.* 1980; Lyutskanov *et al.* 1988; Christgau *et al.* 1996), *Botrytis* (Reignault *et al.* 1994), and *Fusarium* (Miller & Macmillan 1971; Phalip *et al.* 2005). Only a limited number of PMEs have been purified and characterised in detail (Van Alebeek *et al.* 2003). PMEs are medium sized enzymes (25–50 kDa). For example, the following PMEs have medium sized molecular weights: *Aspergillus aculeatus* PME (36.2 kDa) (Christgau *et al.* 1996), *Aspergillus niger* PME (isolate RH5344; 43 kDa) (Khanh *et al.* 1991), *A. niger* PME (38 kDa and 40 kDa) (Warren *et al.* 2002), *Aspergillus oryzae* PME (isolate KBN616; 38.5 kDa) (Kitamoto *et al.* 1999), *Aspergillus japonicus* PME (46 kDa and 47 kDa) (Semenova *et al.* 2003), *Fusarium oxysporum f.sp. vasinfectum* PME (35 kDa) (Miller & Macmillan 1971), and *Botrytis cinerea* (37 kDa) (Valette-Collet *et al.* 2003). The pectinesterase-I complex of *Phytophthora infestans* consists of two protein bands with molecular weights between 45 kDa and 48 kDa and one protein band between 35 kDa and 40 kDa (Forster & Rasched 1985). In general, most fungal PMEs have optimal pH values between 4 and 6. PMEs randomly attack the methyl groups on the pectin molecules resulting in a random distribution of the nonmethylated GalpA (D-galacturonic acid) residues (Dongowski & Bock 1984; Kester *et al.* 2000; Benen *et al.* 2002).

The investigation of PME is significant for understanding the plant–fungal interactions, development of new antifungal control strategies, and understanding the process of pectin modification. Some enzymes are extensively used in industry to produce fermented foods and secondary metabolites. The most important classes of industrial pectolytic enzymes are PMEs (Willats *et al.* 2006).

Fusarium asiaticum and *F. graminearum* are two species responsible for FHB of wheat with the former being the predominant causal agent in China. *Fusarium graminearum* has been shown to produce of PME and other pectolytic enzymes, but *F. asiaticum* has not been studied in this context.

The present study describes the purification and partial characterisation of PME from recently described *F. asiaticum*.

Materials and methods

Fungal isolate and culture conditions

Fusarium asiaticum (strain 7071) from wheat in China (Qu *et al.* 2008) was grown on modified liquid synthetic culture medium (Grossman 1968) containing the following components: 0.5 g of $CaSO_4 \cdot 2H_2O$, 0.375 g of $(CaNO_3)_2 \cdot 4H_2O$, 75 mg of $FeCl_3 \cdot 6H_2O$, 31 mg of $MgSO_4 \cdot 7H_2O$, 0.2 mg of $ZnSO_4$, 1.5 mg of H_3BO_3, 15.25 mg of cysteine-HCl, 6 mg of thiamine hydrochloride, and 4 mg of riboflavin per litre of 0.15 M Na–K phosphate buffer (pH 5.8) with addition of potato tuber extract as the sole carbon source (125 ml potato tuber extract per litre of culture medium). Potato tuber extract was prepared as follows: (1) unpeeled potato tubers were autoclaved for 15 min at 121 °C; (2) after peeling, 90 g of potato tubers were ground in a mortar in 250 ml H_2O and then autoclaved for 15 min at 121 °C; and (3) the potato tubers were filtrated using a sterile aseptic system (Millipore, Billerica, MA, USA) through a 0.45 μm pore-size Durapore® membrane filter (Millipore, Ireland). *Fusarium asiaticum* was cultivated at 25 °C with shaking at 120 rpm for 14 d.

Preparation of crude extract

Fusarium asiaticum was cultivated for 14 d as described above followed by the separation of the culture medium from the mycelium by filtration through two layers of Miracloth. The supernatant was filtered using a sterile aseptic system (Millipore Corporation, Billerica, MA, USA) with 0.45 μm Durapore® membrane filters. The obtained supernatant was precipitated by $(NH_4)_2SO_4$ at 90 % saturation overnight at 4 °C. The precipitate was collected by centrifugation at 15,000*g* for 30 min, dissolved in 0.01 M K–Na phosphate buffers (pH 6.5), and dialysed against the same buffer overnight. The dialysed culture filtrate was passed through a 100 kDa cut-off Amicon Centricon® Plus-70 centrifugal filter device (Millipore, Billerica, MA, USA) and then concentrated using a 10 kDa cut-off Amicon Centricon® Plus-70 centrifugal filter device (Millipore, Billerica, MA, USA). We obtained a sample of crude extract with proteins in a molecular mass range of 10 –100 kDa.

Enzyme assays

PME activity measurement based on the decrease in pH

Quantification of PME activity was carried out according to the method described by Forster & Rasched (1985) with some modification. The reaction mixture for the PME assay contained 0.3 ml of 2 % (w/v) apple pectin, 0.3 ml 10 mM of K–Na phosphate buffer (pH 6.5), 10 mM EDTA, and enzyme sample. The total volume was 0.7 ml. The enzyme activity was assayed by measuring the pH decrease within 30 min at 22 °C. One unit of enzyme activity was defined as the amount of enzyme which caused a decrease in pH of the reaction mixture of 0.1 in 30 min.

Gel diffusion assay for detection of PME activity and quantification of PME activity

A gel diffusion assay based on a cup-plate method as described by Dingle *et al.* (1953) was used for identification of PME activity in samples. PME activity was quantified by the gel diffusion assay as described by Downie *et al.* (1998) with some modifications. The medium contained 1 % agar, 0.05 % apple pectin with a 70–75 % degree of esterification (Sigma, Switzerland), 0.03 M K–Na phosphate buffer (pH 6.5), and 10 mM EDTA. Wells with a diameter of 4 mm were made in agar plates, and the protein samples were loaded in each well. Plates were incubated at 30 °C for 16 h. The gels were stained with 0.05 % (w/v) ruthenium red for 45 min and destained with water. The PME activity appeared as dark red areas against a light red background. The radius of the red stained areas resulting from the hydrolysis of esterified pectin in the gel was measured. PME activity was expressed in relative units (RU; 1RU = 1 mm of dark red areas).

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