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Aspergillus aculeatus pectin methylesterase: study of the inactivation by temperature and pressure and the inhibition by pectin methylesterase inhibitor

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Abstract

The thermal inactivation kinetics of purified *Aspergillus aculeatus* pectin methylesterase (PME) was investigated. In the temperature range 46–56 °C *A. aculeatus* PME inactivates following a first-order reaction. Addition of $CaCl_2$ (50 mM or 1.0 M) has no noticeable influence on the inactivation parameters. *A. aculeatus* PME is very pressure stable, at 25 °C no loss of activity was noticed after pressurization at 700 MPa for 1 h. Pressure and temperature exhibit an antagonistic effect on the enzyme stability. Furthermore *A. aculeatus* PME was unsusceptible for inhibition by PME inhibitor purified from kiwi.

Keywords: Pectin methylesterase; Inactivation; Temperature; Pressure; Pectin methyl esterase inhibitor

1. Introduction

Pectin methylesterase (pectinesterase, PME, EC 3.1.1.11) catalyzes the deesterification of pectin, a major plant cell wall polysaccharide, yielding methanol and pectin with a lower degree of esterification. The resulting pectate acts as a substrate for polygalacturonase (PG, EC 3.2.1.15), an enzyme that depolymerizes the polysaccharide chain. However, in absence of PG activity, pectate chains can cross-link via calcium bridges and form intermolecular networks [1]. In addition, pectin with a lower degree of esterification is less sensitive to non-enzymatic depolymerization (β-elimination) that can occur during heating [2]. Based on these characteristics, PME is added as exogenous enzyme, eventually together with other pectinases or calcium salts, in fruit and vegetable processing for various applications. It is used to increase the yield during extraction, to clarify and concentrate fruit juices [3,4], for gelation of fruit [5] and to modify texture and rheology of fruit and vegetable based products [6–8]. PME is produced as well by plants as by micro-organisms. In the former case it plays a role in the ripening process and the cell wall extension. In the latter case the enzyme is important for plant pathogenesis [1]. In the past, in food technological applications, plant PME was the major source of PME, to be added by use of plant extracts. Recent evolutions in biotechnology made it possible to produce preparations of pectinmethylesterase from microbial sources, free from other pectinases. Nowadays fungal PME is frequently used in fruit processing, in particular PME from *Aspergillus* species [4,9]. A possible limitation for the use of exogenous PME is the occurrence of a PME inhibitor (PMEI) in some plants. The presence of PME inhibitor has already been reported for kiwi [10], banana [11], potato [12] and figs [13].

For efficient application of exogenous enzymes in food processing, knowledge on their processing stability is necessary. Data on thermal and high pressure inactivation of different plant PME are available in literature [14–27]. So far no detailed information on processing stability of microbial PME has been published.

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In this work, the processing stability of a recombinant *Aspergillus aculeatus* PME, purified from a commercial preparation, has been studied. The production and the characterization of recombinant *A. aculeatus* PME have been described by Christgau et al. [28]. Lissau et al. [29] concluded after toxicological tests, that the commercial enzyme preparation is safe for use in food applications. The present work describes the thermal inactivation kinetics of *A. aculeatus* PME at different pH, calcium concentrations and pressure conditions. Also affinity of this fungal PME towards PME inhibitor purified from kiwi is examined.

2. Materials and methods

2.1. Materials

A. aculeatus PME was purified from a commercial enzyme preparation (Novoshape, Novo Nordisk, Denmark) by gelfiltration, using an Akta prime (Amersham Biosciences, Uppsala, Sweden), with a HiLoad^{TM}16/60 Superdex^{TM}75 prep grade column (Amersham Biosciences, Uppsala, Sweden) and 50 mM Na-acetate buffer pH 4.5 (0.2 M NaCl) as elution buffer. 2.0 ml crude sample (Novoshape diluted 1/10 in elution buffer) was applied on the column and eluted with a flow rate of 0.3 ml/min. Fractions containing PME activity were pooled, concentrated using Micrococon YM-10 centrifugal filters (Millipore Corporation, Bedford, USA) and diluted afterwards in 10 mM citric acid buffer with varying pH and calcium concentrations. The effect of pH on the thermal inactivation was investigated at pH 3.0, 3.8 and 4.5, pH values relevant for fruit applications. The influence of calcium ions was tested at pH 3.8 and $CaCl_2$ concentrations of 0.05 and 1.0 M.

PME inhibitor was purified from kiwi fruit by affinity chromatography as described by Fachin et al. [23]. After purification, fractions containing PME inhibitor activity were pooled, concentrated using Micrococon YM-10 centrifugal filters (Millipore Corporation, Bedford, USA) and diluted afterwards in 0.2 M Tris–HCl buffer pH 7.0.

2.2. PME activity assay

PME activity was determined by measuring the release of acid per time unit at pH 4.5 and 22 °C. The reaction mixture consisted of 250 μl sample and 30 ml of a 3.5 mg/ml apple pectin solution (70–75% esterification, Fluka, Bornem, Belgium) containing 0.117 M NaCl. During pectin hydrolysis, the pH was maintained at pH 4.5 (set-pH) by addition of 0.01N NaOH using an automatic pH-stat titrator (Metrohm, Switzerland). The activity unit (U) of PME is defined as the amount of enzyme required to release 1 μmol of acid per minute, under aforementioned assay conditions.

2.3. PMEI activity assay

PMEI activity was determined as its ability to block PME activity. 1.8 U of PME (measured at pH 4.5 as described above) was mixed with PMEI sample and 5 ml water in the reaction vessel of the pH-stat titrator at 22 °C. The pH of the mixture was adjusted to the desired value. After 15 min of incubation 25 ml pectin solution was added and the residual PME activity was determined as described above, with the set-pH adjusted to the desired value. Here, one unit (U) of PME activity is defined as the amount of enzyme required to release 1 μmol of acid per minute, at 22 °C and the pH considered. The PMEI activity is calculated as the difference between PME activity (U) of a blank sample (i.e. without PMEI) and the residual PME activity (U) after incubation with PMEI.

2.4. Gel electrophoresis

SDS-PAGE experiments were performed using a Phast-system (Amersham Biosciences, Uppsala, Sweden) with Phastgel Homogenous 20% and Phastgel SDS Buffer strips. Samples were boiled for 5 min at 100 °C in a buffer containing SDS (2.5%) and β-mercaptoethanol (5%). Gel staining was performed with silver nitrate according to Heukeshoven and Dernick [30].

2.5. Thermal treatment of purified A. aculeatus PME

Isothermal inactivation experiments of purified *A. aculeatus* PME were performed in a temperature-controlled water bath, in a temperature range from 46 to 56 °C. The enzyme solution was enclosed in capillary tubes (1.15 mm × 150 mm, Hirschmann Laborgerate, Germany) to ensure isothermal heating. After preset time intervals, the samples were withdrawn from the water bath and immediately cooled in ice water. The residual PME activity was measured within 1 h of storage. During storage in ice water no reactivation of the enzyme was observed.

2.6. High-pressure treatment of purified A. aculeatus PME

Isobaric–isothermal inactivation experiments were conducted in a laboratory scale high-pressure equipment (Resato, Roden, The Netherlands), with eight vessels, each surrounded by a thermostated mantel connected to a cryostat. The pressure medium was a glycol-oil mixture (Resato, Roden, The Netherlands). Enzyme samples in 0.4 ml flexible microtubes (Biozym, Landgraaf, The Netherlands) were enclosed in the pressure vessels, already equilibrated to a certain temperature. Pressure was built up slowly (100 MPa/min) to minimize temperature increases due to adiabatic heating. After pressure buildup an equilibration period of 2 min was taken into account to allow temperature to evolve to its desired value, at that moment the experimental time was started.