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Analytical Methods

Plant pectin methylesterase and its inhibitor from kiwi fruit: Interaction analysis by surface plasmon resonance

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ABSTRACT

Two surface plasmon resonance (SPR)-based interaction analysis methods were successfully implemented to explore the binding between plant PME and kiwi PMEI. In a first method, plant PMEs were immobilised on a chip surface via amine coupling. This experimental setup allowed studying the effect of pH and ionic strength on the PME–PMEI interaction kinetics. Strong binding was obtained at pH < 7 and at low salt concentrations, whereas both pH ⩾ 8 and [NaCl] of ca. 1.0 M effectively caused dissociation. In a second method, kiwi PMEI was immobilised on a chip surface to which streptavidin had been covalently attached. Hereto, PMEI was biotinylated by means of a NHS-biotin reagent. With this immobilisation strategy, the effect of (partial) thermal or high pressure-induced denaturation of PME on its affinity towards PMEI was investigated. A notable degree of enzyme inactivation was required before interaction characteristics were significantly altered. Any incomplete inactivation of PME resulted in binding to the PMEI surface.

1. Introduction

Pectin is a major plant cell wall polysaccharide contributing to tissue integrity and rigidity. The main structural element of pectin, governing most of its functional properties, is a linear homopolymer of α-(1 → 4) linked D-galacturonic acids, which can be methyl-esterified (Ridley, O'Neill, & Mohnen, 2001). Changes in the pectin structure take place during fruit ripening, processing and storage, and can be both chemical (e.g. alkaline demethoxylation, β-eliminative depolymerisation) and enzymatic. Pectin methylesterase (PME, EC 3.1.1.11) is an enzyme of either plant or microbial origin that catalyses the demethoxylation of pectin, thus altering the degree and pattern of methyl-esterification and releasing methanol (Bordenave, 1996; Rexova-Benkova & Markovic, 1976). Plant PME is cell wall bound and involved in plant development and fruit ripening (Pelloux, Rusterucci, & Mellerowicz, 2007). From a food technological point of view, depending on the application, endogenous PME can have beneficial as well as detrimental effects on the quality of plant-derived foods. For instance, de-esterified pectin serves as substrate for polygalacturonase (PG, EC 3.2.1.15), a depolymerising pectinase inducing viscosity or texture loss (Rexova-Benkova & Markovic, 1976). In addition, demethoxylation of pectin reduces the susceptibility towards chemical depolymerisation by β-elimination that can occur during heating (Keijbets & Pilnik, 1974; Sajjaanantakul, Van Buren, & Downing, 1989; Van Buren, 1979). Furthermore, divalent ions can crosslink pectin with a low degree of methoxylation, inducing gel formation (Van Buren, 1979) but also causing cloud loss in fruit and vegetable juices (Krop & Pilnik, 1972; Sims, Balaban, & Matthews, 1993; Versteeg, Rombouts, Spaansen, & Pilnik, 1980). Depending on the desired intention on the product at hand, PME stimulation or inactivation can be pursued. By using processing tools like traditional thermal or novel high hydrostatic pressure treatments, PME-induced pectin conversions can be tailored, hence controlling functional properties of plant-derived food products in terms of structure, texture, rheology and cloud stability (e.g. Goodner, Braddock, Parish, & Sims, 1999; Ly-Nguyen et al., 2003; Ng & Waldron, 1997; Nienaber & Shellhammer, 2001; Sila, Smout, Vu, & Hendrickx, 2004; Verlent, Hendrickx, Verbeyst, & Van Loey, 2007).

In 1990, the discovery of a proteinaceous PME inhibitor (PMEI) in ripe kiwi fruit (*Actinidia deliciosa*) was reported (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990). This PMEI was shown to be a glycoprotein of 152 – mainly acidic – amino-acid residues, giving rise to a molar mass of 16.3 kDa and an acidic isoelectric pH (p*I*) (Camardella et al., 2000; Giovane, Balestrieri, Quagliuolo, Castaldo, & Servillo, 1995). PMEI inhibits all plant PMEs

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tested through the formation of a reversible, non-covalent 1:1 complex (Balestrieri et al., 1990; Giovane et al., 1995; Ly-Nguyen et al., 2004), but is ineffective against microbial PMEs (D'Avino, Camardella, Christensen, Giovane, & Servillo, 2003; Duvetter et al., 2005; Giovane et al., 2004). Elucidation of the three-dimensional structure of the tomato PME–kiwi PMEI complex has demonstrated that PMEI covers the putative active site cleft of the enzyme and prevents substrate binding (Di Matteo et al., 2005). Due to the strong PME–PMEI interaction, kiwi PMEI can be applied for laboratory-scale plant PME purification via affinity chromatography (Giovane et al., 1995; Ly-Nguyen et al., 2003) and for inhibiting undesirable PME activity in food applications, e.g. to obtain cloud stable juices (Castaldo et al., 1991). In addition, the discovery of this PMEI opens perspectives for the development of an innovative microscopic technique based on labelled PMEI to localise endogenous PME *in planta* and to gain further insight in the relation between enzymatic pectin conversions and related macroscopic properties (such as firmness and viscosity), in model systems as well as in complex, structured real foods. However, efficient application of PMEI for PME inhibition or detection requires profound knowledge on the PME–PMEI interaction, as influenced by intrinsic product factors (such as pH and ionic strength) and extrinsic process factors (such as high temperature and high pressure).

This work aimed at the development of a surface plasmon resonance (SPR)-based interaction analysis method to study the binding between plant PME and kiwi PMEI. SPR biosensors combined with miniaturised flow channels permit label-free and real-time monitoring of the interaction between two biomolecules (e.g. proteins), of which one is attached to a surface and the other one is free in solution. The SPR optical detection is based on the determination of changes in refractive index induced at the detector surface upon binding or dissociation (Huber & Mueller, 2006; Löfas & Johnsson, 1990). Once implemented, this method was applied to investigate the effect of pH and NaCl concentration on the PME–PMEI interaction and the effect of a temperature- or pressure-induced denaturation of PME on its affinity towards kiwi PMEI.

2. Materials and methods

2.1. Materials

Young Belgian red carrots (*Daucus carota* cv. Sirena), tomatoes (*Lycopersicon esculentum* cv. Admiro) and ripe kiwi fruits (*A. deliciosa* cv. Hayward) were purchased from a local supermarket. Apple pectin (degree of methyl-esterification: 70–75%) was obtained from Fluka (Buchs, Switzerland). The EZ-Link® Micro Sulfo-NHS-LC-Biotinylation Kit was bought from Thermo Fisher Scientific (Waltham, Massachusetts). Chemicals for the SPR-based interaction analysis experiments were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Extraction and purification of plant PMEs and kiwi PMEI

Extraction and purification of carrot PME and kiwi PMEI were based on Ly-Nguyen et al. (2003) with minor changes. Tomato PME was extracted and purified by affinity chromatography using the method described by Plaza et al. (2007). Purified enzyme and inhibitor solutions were quickly frozen in liquid N_2 and stored at −80 °C until use.

2.3. PME assay

PME activity was determined by the continuous titration of carboxyl groups formed during pectin hydrolysis, using an automatic pH-stat titrator (Metrohm, Herisau, Switzerland) with 0.01 N NaOH. Routine assays were performed at pH 6.5 and 22 °C, using 30 mL of a 0.35% (w/v) apple pectin solution containing 0.117 M NaCl. By definition, one unit (U) of PME activity is the amount of enzyme catalysing the de-esterification of 1 μmol of methyl-ester bonds per min under aforementioned conditions.

2.4. Protein content

The protein content was determined using the bicinchoninic acid (BCA) kit according to Sigma procedure TPRO-562 (Sigma, Darmstadt, Germany). The protein concentration (mg/mL) was calculated by comparison with a standard curve of bovine serum albumin.

2.5. Thermal and high pressure treatment

Purified carrot PME was thermally treated in a temperature-controlled water bath. The enzyme solution (0.07 mg/mL in 10 mM Na-phosphate buffer pH 6.5, a buffer with a small $\Delta pK_a/\Delta T$) was enclosed in 200 μL glass capillaries (Brand, Wertheim, Germany) to ensure isothermal heating. After 5 min at different temperature levels between 25 and 60 °C, samples were withdrawn from the water bath and immediately cooled in an ice water bath.

Pressure treatment of purified carrot PME was conducted in a laboratory-scale high pressure device (Resato, Roden, The Netherlands) with eight reactors, each surrounded by a thermostated mantle connected to a cryostat. The pressure medium was a glycol–oil mixture (TR 15, Resato). Flexible microtubes (0.3 mL, Carl Roth, Karlsruhe, Germany) were filled with the protein solution (0.07 mg/mL in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 6.5) and enclosed in the pressure reactors, already equilibrated to 25 °C. MES buffer was chosen because of its putative pressure stability, i.e. the ability to maintain the set pH upon pressurisation (Bruins, Matser, Janssen, & Boom, 2007). Pressure was built up slowly to 800 MPa (100 MPa/min, to minimise temperature increase due to adiabatic heating) and all pressure reactors were isolated. After preset time intervals (up to 60 min), individual reactors were depressurised instantaneously and samples were cooled in an ice water bath.

Part of each PME sample was used to measure the residual enzyme activity (within 1 h of storage in ice water). The remainder was quickly frozen with liquid N_2 and stored at −80 °C until SPR interaction analysis (as described in Section 2.7). Freezing and thawing caused only a minor PME activity loss.

2.6. Interaction analysis by surface plasmon resonance with immobilised plant PME

Real-time interaction analysis by surface plasmon resonance (SPR) with immobilised plant PME was performed on a Biacore™ 3000 analytical system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Amine coupling of both tomato and carrot PME was carried out by injecting the proteins (8 μg/mL) in 10 mM sodium acetate pH 4.5, following activation of the carboxymethylated dextran surface (CM5 sensor chip) using a 1:1 mixture of 0.05 M *N*-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), based on Löfas and Johnsson (1990). Throughout the immobilisation process, HBS-EP (0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) pH 7.4 containing 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20) was used as running buffer at 5 μL/min. After immobilisation of the PMEs, excess reactive groups on the surface were deactivated by a 7-min liquid pulse of 1.0 M ethanolamine pH 8.5.