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A Pectin-Methylesterase-Inhibitor-Based Molecular Probe for *in Situ* Detection of Plant Pectin Methylesterase Activity

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In the quest of obtaining a molecular probe for *in situ* detection of pectin methylesterase (PME), the PME inhibitor (PMEI) was biotinylated and the biotinylated PMEI (bPMEI) was extensively characterized. Reaction conditions for single labeling of the purified PMEI with retention of its inhibitory capacity were identified. High-performance size-exclusion chromatography (HPSEC) analysis revealed that the bPMEI retained its ability to form a complex with plant PME and that it gained the capacity to strongly bind an avidin species. By means of dot-blot binding assays, the ability of the probe to recognize native and high-temperature or high-pressure denatured plant PMEs, coated on an absorptive surface, was investigated and compared to the binding characteristics of recently reported anti-PME monoclonal antibodies. Contrary to the antibodies, bPMEI only detected active PME molecules. Subsequently, both types of probes were used for PME localization in tissue-printing experiments. bPMEI proved its versatility by staining prints of carrot root, broccoli stem, and tomato fruit. Applying the tissue-printing technique on carrot roots after thermal treatment demonstrated the complementarity of bPMEI and anti-PME antibodies, with the former selectively detecting the remaining active PME and the latter staining both native and inactivated PME molecules.

KEYWORDS: Pectin methylesterase (PME); pectin methylesterase inhibitor (PMEI); biotinylation; immunolocalization; tissue printing

INTRODUCTION

Pectin methylesterase (PME, EC 3.1.1.11) is widely distributed in plants, bacteria, and fungi. The enzyme catalyzes the demethoxylation of pectin, one of the main cell wall polysaccharides contributing to cellular adhesion and firmness, elasticity, and integrity of the plant tissue. PME modifies the degree and pattern of methyl esterification of the linear homogalacturonan chains of pectin (*1*) and, by pectin remodeling, plays a key role in vegetative and reproductive plant development in addition to plant−pathogen interactions (*2*). For food technologists, endogenous PME activity is highly relevant because the amount and distribution of methyl-esters in pectin determine to a large extent its physicochemical and functional properties, impacting favorably or deleteriously on the quality of plant-derived food products. Demethoxylated pectin is prone to enzymatic depolymerization by polygalacturonase (PG, EC 3.2.1.15), inducing texture or viscosity loss (*3*, *4*). However, demethoxylation of pectin reduces its susceptibility to chemical β-eliminative depolymerization that can occur during thermal processing and is associated with heat-induced softening of fruits and vegetables (*5*, *6*). In addition, pectin with a reduced degree of methyl esterification can form intermolecular networks through divalent ion cross-links, promoting gel formation (*7*) but also compromising cloud stability in fruit and vegetable juices (*8*−*10*). Consequently, rigorous tailoring of PME-induced pectin conversions, through PME stimulation or inactivation, allows for the control of pectin-related functional properties of plant-derived food products (i.e., structure, texture, rheology, and cloud stability). In this context, traditional thermal and novel high hydrostatic pressure processing have shown to be valuable tools in PME activity control (*4*, *11*, *12*).

To gain in-depth insight in the relation between enzymatic pectin conversions and allied food functional properties (such as firmness and viscosity), identification of the locations of endogenous PME activity in plant-based, complex-structured food matrices (i.e., *in situ*) on both a macroscopic (e.g., by means of tissue printing) and microscopic (fluorescence and/or electron microscopy) level would be extremely helpful, in addition to investigating how food processing affects these locations (e.g., through enzyme inactivation or tissue damage). To date, several authors have reported on the production of (polyclonal and monoclonal) antibodies raised against plant PMEs for immunolocalization (*13*−*19*). However, these probes were not yet applied for the food technological purposes proposed here.

Besides anti-PME antibodies, an alternative strategy toward a molecular probe for PME detection could be hypothetically based on the proteinaceous PME inhibitor (PMEI), found in kiwi fruit (*Actinidia deliciosa*) (*20*). This PMEI is a glycoprotein with a molar mass of 16.3 kDa (*21*, *22*) that forms a reversible, noncovalent, but highly polar 1:1 complex with all plant PMEs

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tested (*20*, *22*, *23*) while not binding microbial PMEs (*24*−*26*). The strong enzyme−inhibitor interaction has already enabled the use of kiwi PMEI for laboratory-scale plant PME purification by affinity chromatography (*22*, *27*) and led to its postulated application in inhibiting undesired PME activity in food applications, e.g., to obtain cloud-stable juices (*28*), but it also opens perspectives for the development of an innovative technique based on labeled PMEI to localize PME *in situ*. Recent cloning of the cDNA of kiwi PMEI and its expression in the yeast *Pichia pastoris* encourages a widespread application (*29*). However, efficient use of PMEI, in either PME inhibition or detection, requires a 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). Necessary information was collected in previous studies, using inhibition capacity measurements (*30*), surface plasmon resonance interaction analysis (*31*), and size-exclusion chromatography for complex formation monitoring (*32*). With these data at hand, bridging the gap toward the actual application of PMEI can be aimed.

The present study intended for the development of a molecular probe based on the PME inhibitor and the assessment of its potential as a tool to detect PME *in situ*. First, focus was on the labeling of PMEI and characterization of the conjugate. Once the labeling was implemented, the ability of the probe to detect PME was tested in dot-blot binding assays and plant tissue printing. For the sake of comparison, blots were also probed with the recently reported anti-PME monoclonal antibodies (*16*).

MATERIALS AND METHODS

Plant Materials. Red carrots (*Daucus carota*), broccoli (*Brassica oleracea*), red ripe Roma tomatoes (*Solanum lycopersicum*), and ripe green kiwi fruits (*A. deliciosa* cv. Hayward) were purchased from a local supermarket. PME from carrot root and broccoli stem and PMEI from kiwi fruit were extracted and purified by single-step affinity chromatography methods on activated Sepharose matrices, according to the procedures outlined by Jolie et al. (*30*). Purified enzyme and inhibitor solutions were quickly frozen in liquid N_2 and stored at −80 °C until use.

PME Activity and PMEI Inhibitory Capacity Assays. PME activity was measured by the continuous titration of carboxyl groups formed during pectin de-esterification, 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 (degree of esterification, 70−75%; Fluka, Buchs, Switzerland) containing 0.117 M NaCl. One unit (U) of PME activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of methyl-ester bonds per minute under aforementioned conditions.

PMEI inhibitory capacity was determined as the ability to block PME activity in a titrimetric assay. It was calculated as the difference between the PME activity of a blank sample (i.e., without PMEI) and the residual PME activity after 15 min of pre-incubation with a PMEI sample, routinely at pH 6.5 and 22 °C. One unit of inhibition (UI) is defined as the amount of PMEI blocking 1 U of PME under the assay conditions described above.

Protein Determination. The protein content of the enzyme and inhibitor solutions was determined according to the Sigma procedure TPRO-562 using the bicinchoninic acid (BCA) kit (Sigma, Darmstadt, Germany). This method is based on the formation of a colored complex of BCA and Cu^+, after reduction of Cu^{2+} by proteins at alkaline pH. The complex was quantified spectrophotometrically at 562 nm and 25 °C. The protein concentration (mg/mL) was estimated by comparison to a standard curve of bovine serum albumin.

PMEI Biotinylation. PMEI was biotinylated by means of an EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific, Waltham, MA). Hereto, kiwi PMEI was dissolved in phosphate-buffered saline (PBS) at pH 7.2 at a 1 mg/mL concentration, mixed with a 25-fold molar excess of a water-soluble biotin derivative (sulfo-NHS-LC-biotin), and incubated for 60 min at 25 °C. Subsequently, biotinylated kiwi PMEI was separated from unbound biotin and the sulfo-NHS leaving group on a Zeba Desalt Spin column (7 kDa MWCO). The protein conjugates were stored in small aliquots at −80 °C until further use.

The degree of biotinylation was determined with the FluoReporter Biotin Quantitation Assay Kit (Invitrogen, Paisley, U.K.). This fluorometric assay is based on the displacement of a ligand tagged with a quencher dye from the biotin binding sites of Biotective Green reagent. To expose any biotin groups in a possibly multiply labeled PMEI that are sterically restricted and inaccessible to the Biotective Green reagent, the inhibitor was first digested with protease (overnight at 37 °C). Biotinylated lysine was used as a standard for the assay. The resulting fluorescence was measured in a microplate reader using excitation/emission maxima of 485/530 nm.

High-Performance Size-Exclusion Chromatography (HPSEC). Size-exclusion chromatography was carried out on an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) with ultraviolet diode array detection (UV-DAD) at 220 and 495 nm (G1315B, Agilent Technologies). Analytical HPLC was performed at 25 °C using a Superdex 75 10/300 GL column (300 × 10 mm, 13 μm average particle size, > 30 000 m^{-1} theoretical plates, GE Healthcare, Uppsala, Sweden). Elution was executed with 50 mM Na-phosphate buffer at pH 6.5 containing 150 mM NaCl at a flow rate of 0.45 mL/min for 55 min. Prior to injection (100 μL), protein samples were filtered through a syringe-driven filter unit [Millex-HV, polyvinylidene fluoride (PVDF), 0.45 μm, Millipore, Billerica, MA]. Ultrapure water (organic free, 18 MΩ cm resistance) was supplied by a Simplicity water purification system (Millipore).

Thermal and High-Pressure Treatments. Thermal treatments of PME solutions and raw plant material were performed in a temperature-controlled water bath. Enzyme samples (0.1 mg/mL in 50 mM Na-phosphate buffer at pH 6.5 containing 150 mM NaCl) were enclosed in 200 μL glass capillaries (Brand, Wertheim, Germany) and heated at 65 °C for 5 min. Carrot roots (ca. 3 cm in diameter and 10 cm in length) were vacuum-packed in a plastic bag and immersed in the water bath (85 °C for a preset time from 0 to 15 min). After treatment, samples were immediately cooled in an ice-water bath. For tissue prints (cf. infra), transverse sections were made of the middle part of the carrot root. The remainder of the plant material was cut into small pieces, quickly frozen in liquid N_2, and stored at −80 °C until PME extraction (as described below).

High-pressure treatment of PME solutions was conducted in a laboratory-scale high-pressure device (Resato, Roden, The Netherlands) with 8 mL cylindrical thermostatted reactors and propylene glycol as the pressure-transmitting medium. Flexible microtubes (0.3 mL, Carl Roth, Karlsruhe, Germany) were filled with the PME solution (0.1 mg/mL in 50 mM Na-phosphate buffer at pH 6.5 containing 150 mM NaCl) and enclosed in the pressure reactors, equilibrated to 25 °C. The pressure was built up slowly to 800 MPa, and the pressure reactors were all isolated. After 60 min, the reactors were depressurized instantaneously and samples were transferred to an ice-water bath until analysis.

Dot-Blot Assays. PME samples were spotted on prewet nitrocellulose [HyBond enhanced chemiluminescence (ECL), 0.45 μm pore size, GE Healthcare, Uppsala, Sweden] in 2 μL aliquots. After air-drying for at least 30 min, irrelevant protein binding sites on the nitrocellulose were blocked overnight at 4 °C with 5% nonfat dried milk in PBS-T [i.e., 50 mM Na-phosphate buffer at pH 6.5 containing 150 mM NaCl and 0.1% Tween-20, for the membranes to be labeled with biotinylated PMEI (bPMEI)] or TBS-T (i.e., 20 mM Tris-HCl buffer at pH 7.6 containing 137 mM NaCl and 0.1% Tween-20, for the membranes to be labeled with monoclonal antibodies). Subsequently, the primary probe in PBS-T or TBS-T, containing 1% nonfat dried milk, was added (1.5 h at room temperature). As primary probes, bPMEI (2.9 μg/mL) as well as MA-TOM2-38A11 (25 μg/mL), a mouse monoclonal antibody raised against one of the isozymes of tomato PME and with shown cross-reactivity toward carrot PME (*16*), were used. After extensive washing in PBS-T or TBS-T, blots were incubated in the secondary probe solution, i.e., ExtrAvidin-peroxidase (Sigma, diluted $^1/_{2000}$ in PBS-T with 1% milk) or goat-anti-mouse-HRP (Bio-Rad, Hercules, CA, diluted $^1/_{2000}$ in TBS-T with 1% milk), for 1 h at room temperature. After further washing with PBS-T or TBS-T, blots were developed using the ECL method with luminol enhancer and peroxide solutions from Pierce (Thermo Fisher Scientific) for chemiluminescent signal detection. All blots were performed at least in duplicate.

Tissue Printing. Tissue printing was carried out in analogy with Vandevenne et al. (*16*). Nitrocellulose membranes were first soaked for 15 min in 0.2 M Tris-HCl buffer at pH 8.0 containing 1.0 M NaCl. Subsequently, transverse sections of (raw or treated) carrot root, broccoli stem, and tomato fruit were firmly pressed on the nitrocellulose membrane (ca. 30 s). The membrane was allowed to dry for 30 min and was then blocked overnight at 4 °C with 5% nonfat dried milk in PBS-T or TBS-T. After blocking, the prints were incubated for 1.5 h at room temperature with the primary probe, i.e., bPMEI (2.9 μg/mL) in PBS-T containing 1% nonfat dried milk or MA-TOM2-38A11 (25 μg/mL) in TBS-T containing 1% nonfat dried milk. A washing step with PBS-T or TBS-T was performed, followed by incubation with the secondary probe, being ExtrAvidin-peroxidase (Sigma, diluted $^1/_{1000}$ in PBS-T with 1% milk) or goat-anti-mouse-HRP (Bio-Rad, diluted $^1/_{1000}$ in TBS-T with 1% milk), for 1 h at room temperature. Finally, the membranes were washed, and detection was carried out using the ECL method (cf. previous section). Prints developed with omission of the primary probe served as negative controls. Triplicate analyses assured reproducibility of the results.

PME Extraction. Carrot tissue was frozen in liquid N_2 prior to homogenization in a Grindomix GM 200 knife mill (Retsch, Haan, Germany). A known amount of homogenized material (wet weight) was mixed with 0.2 M Tris-HCl at pH 8.0 containing 1.0 M NaCl for 3 h at 4 °C (1:1.5, w/v) to extract cell-wall-bound PME. The crude PME extract was obtained by filtration using a cheese cloth. PME activity was measured as described above. All extractions and activity measurements were run in duplicate.

RESULTS AND DISCUSSION

Purification of Kiwi PMEI, Carrot PME, and Broccoli PME. The proteinaceous PME inhibitor was purified from ripe kiwi fruits. A single sharp peak of protein and PMEI activity was obtained. Matrix-assisted laser desorption ionization time-of-flight (MALDI−TOF) mass spectrometry confirmed the absence of contaminating proteins in the PMEI solution, as described previously (*30*) (data not shown). Cell-wall-bound PME for HPSEC and/or dot-blot analysis was extracted from carrot roots and broccoli stems and purified to apparent homogeneity by preparative affinity chromatography (*30*).

Biotinylation of Kiwi PMEI. In search of a PMEI derivative to be used as a molecular probe for PME detection, kiwi PMEI was biotinylated with a water-soluble biotin derivative (sulfo-NHS-LC-biotin), equipped with a 22.4 Å spacer arm and directed toward nonprotonated aliphatic amine groups, including the amine terminus of proteins and the ε-amino group of lysines. The reaction was performed at pH 7.2 to achieve a more specific labeling of the amine terminus (which has a lower pK_a than the lysine ε-amino group), because from the three-dimensional structure of the PME−PMEI complex it is known that the N-terminal region of the inhibitor molecule is directed away from the interaction interface and seems poorly involved in the formation of the complex (*33*). A 25-fold molar excess of the biotin derivative was selected in an attempt to obtain at least one biotin per PMEI molecule.

The FluoReporter Biotin Quantitation Assay Kit allowed for an estimation of the degree of biotinylation (DOB) by comparison to a standard curve of biotinylated lysine. The measured DOB of the PMEI solution was 1.01 ± 0.02. This result points to a 1:1 molar ratio of PMEI and biotin, suggesting that all PMEI molecules be equipped with exactly one biotin molecule.

The specific inhibition capacity toward purified carrot PME was measured before (ca. 550 UI/mg) and after the biotinylation reaction and proved not to change significantly (< 5%), although a certain loss of protein (ca. 10%) occurred when taken through the labeling reaction. It can be concluded that also after biotinylation the PMEI is able to inhibit carrot PME activity.

Characterization of bPMEI by HPSEC. To further characterize the bPMEI and to check the ability of the probe to bind to PME on the one hand and to an avidin species on the other hand, HPSEC analysis was performed, in accordance with the method described previously (*32*).

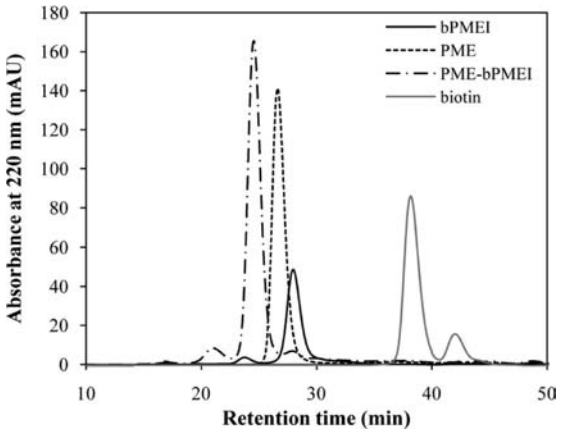

Figure 1. Size-exclusion chromatograms of purified carrot PME, bPMEI, a PME−bPMEI mixture, and the NHS-biotin reagent, all recorded at 220 nm.

In **Figure 1**, an overlay of the size-exclusion chromatograms of bPMEI, purified carrot PME, a PME−bPMEI mixture (with minor molar excess of bPMEI), and the NHS-biotin reagent, all recorded at 220 nm, is represented. The chromatogram of bPMEI showed a major, symmetrical peak at a retention time of 27.9 min and a minor peak at 23.7 min. The retention time of 27.9 min is in close agreement with the one of native kiwi PMEI (i.e., 27.8 min) as reported earlier (*32*), indicating that this peak contains single (biotinylated) PMEI molecules. The peak at 23.7 min, however, may originate from PMEI aggregates formed during the labeling reaction. The total absence of low-molecular-weight peaks, as the ones observed in the chromatogram of the NHS-biotin reagent (at 38.1 and 42.0 min), confirmed the successful removal of the unbound biotin and NHS leaving group.

After mixing bPMEI with purified carrot PME, a major PME−bPMEI complex peak (retention time = 24.5 min) was obtained. The presence of a second, minor peak at 21.0 min suggested that also the putative PMEI aggregates are capable of complex formation. A molar excess of bPMEI resulted in a small peak of free, unbound bPMEI (27.9 min), and a molar excess of PME resulted in an unresolved peak at ca. 26.6 min, corresponding to free, unbound PME (the latter not shown). These observations indicate that all PMEI after biotinylation is still available for complex formation with PME.

Furthermore, HPSEC analysis was applied to study complex formation between bPMEI and an avidin species. The ExtrAvidin-FITC conjugate (Sigma, abbreviated "ExA-FITC"), a fluorescently labeled, modified form of the egg-white avidin not exhibiting its unwanted non-specific binding, was used. **Figure 2** shows an overlay of the chromatograms of bPMEI, ExA-FITC, and a mixture of both. The elution of ExA-FITC was registered at 220 nm as well as 495 nm, the absorbance maximum of FITC. A major peak eluted at 22.5 min, and a minor peak eluted at 19.5 min. Perfect coincidence of the chromatograms at both wavelengths indicated that all ExA, be it single molecules or small aggregates, is fluorescently labeled. Upon mixing the bPMEI with the avidin species, the free bPMEI peak at 27.9 min disappeared completely and a high-molecular-weight peak at 16.6 min was observed, with the latter most likely corresponding to the bPMEI−ExA-FITC complex. In conclusion, all bPMEI can be bound by ExA-FITC if sufficient amounts of the avidin species are present.

Dot-Blot Assays. As a preliminary step to investigate the ability of the bPMEI to detect PME activity *in situ*, dot-blot binding assays with purified carrot root and broccoli stem PMEs were

performed for native as well as high-temperature or high-pressure denatured enzymes. To allow for a comparison of the PME probing qualities of bPMEI and a recently reported alternative, i.e., monoclonal anti-PME antibodies, all dot blots were carried out with both bPMEI and the mouse antibody MA-TOM2-38A11 (*16*).

The binding in dot blots of bPMEI and MA-TOM2-38A11 to native carrot and broccoli PMEs at various dilutions is depicted in **Figure 3A**. bPMEI bound to carrot PME as well as broccoli PME, with the intensity and area of the staining dependent upon the amount of PME dotted. At the highest dilution of PME (20 ng coated), hardly any of the staining was visible. In contrast, MA-TOM2-38A11 only recognized carrot PME. The lack of cross-reactivity toward broccoli PME of this anti-PME antibody as well as all other antibodies raised against tomato PMEs and described by Vandevenne et al. (*16*) was confirmed by a one-side enzyme-linked immunosorbent assay (ELISA) test (results not shown). These results illustrate a possible advantage of the PME inhibitor over the monoclonal antibodies as a probe for PME detection; the latter needs a specific epitope on the PMEs for recognition to occur, whereas the PMEI is believed to react with all plant PMEs (*25*).

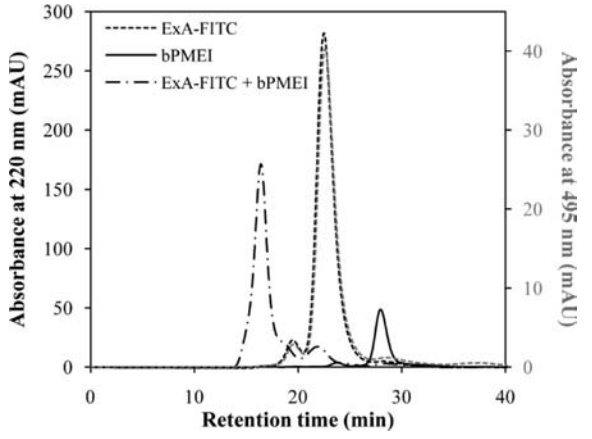

Figure 2. Size-exclusion chromatograms of bPMEI, ExA-FITC, and a mixture of bPMEI and ExA-FITC recorded at 220 and 495 nm (for ExA-FITC).

Figure 3B shows the results of the dot-blot assays of carrot and broccoli PMEs before and after a thermal or high-pressure treatment, probed with bPMEI and MA-TOM2-38A11. High-temperature and high-pressure conditions that reduced the PME activity below detectable levels, as measured by automatic titration, were selected (i.e., 5 min at 65 °C and 60 min at 25 °C and 800 MPa, respectively). In addition, blots of the treated PME samples after a centrifugation step for aggregate removal (5 min at 10000*g*, referred to as "cf") were made. In all cases, 2 μL of a 0.1 mg/mL PME starting solution was coated. The bPMEI probe bound to the untreated carrot and broccoli PMEs ("blank"), whereas high-temperature or high-pressure denatured PME samples were not recognized. Conversely, MA-TOM2-38A11 was able to bind to denatured carrot PME samples, although weakened in comparison to the untreated blank. Staining disappeared upon centrifugation of the treated samples. The antibody showed no reaction with broccoli PME, neither before nor after treatment.

The complete absence of staining of the temperature- or pressure-denatured carrot and broccoli PME samples probed with bPMEI demonstrates that a temperature- or pressure-induced denaturation of plant PME renders the binding of the bPMEI probe impossible. The finding that PMEI only binds to active PME molecules confirms earlier observations based on interaction analysis by surface plasmon resonance (*31*) and complex formation by HPSEC (*32*). The statement that the capacity of PME to bind PMEI is inextricably bound up with the enzyme activity is endorsed by the observation that PMEI covers the putative active-site cleft upon PME−PMEI complex formation, thus preventing substrate binding (*33*). In light of PMEI as a probe for PME activity, it is extremely valuable that the inhibitor, thanks to its specificity, can differentiate between active and inactive PME molecules.

It was observed that MA-TOM2-38A11 can bind to carrot PME, even after thermal or high-pressure inactivation of the enzyme. However, the signal on the blot assay is rather weak and entirely lost when PME samples are centrifuged prior to immobilization

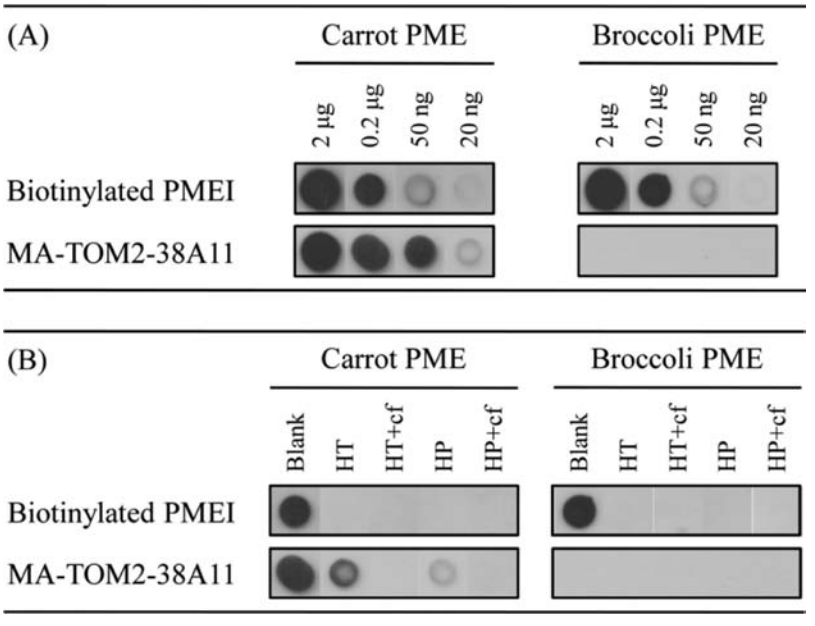

Figure 3. Dot-blot analysis of the binding of bPMEI and MA-TOM2-38A11 to PME purified from carrot root and broccoli stem. (A) Dilution series of untreated PMEs and (B) untreated ("blank"), high-temperature treated ("HT"), and high-pressure treated ("HP") PMEs, spotted before and after sample centrifugation ("cf").

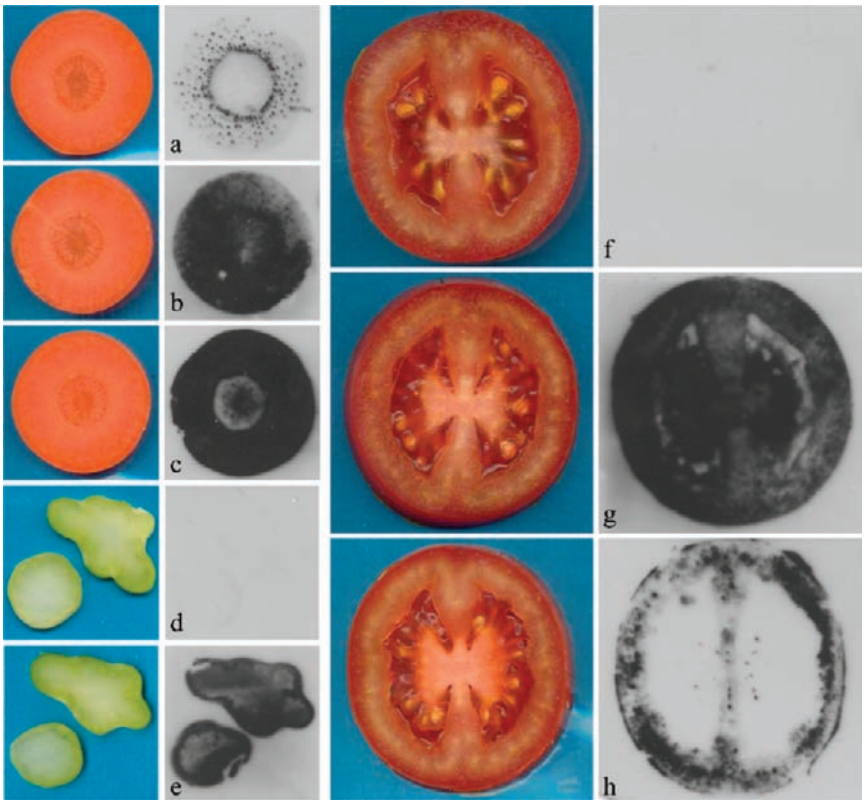

Figure 4. Tissue prints of raw carrot root, broccoli stem, and tomato fruit, probed with bPMEI (b, e, and g) and MA-TOM2-38A11 (c and h) and without primary probe (a, d, and f). Image scans of the respective cut surfaces are shown on the left of each print.

on the membrane. The latter observation indicates that antibody binding occurs at the aggregated state of the enzyme (which is removed upon centrifugation). In turn, this fact provides a possible explanation for the low reactivity toward the noncentrifuged PME samples. On the one hand, the aggregated fraction may be less evenly spread on the membrane, because of a reduced ability to migrate before drying and attachment to the nitrocellulose. On the other hand, the epitope recognized by the antibody may still be present but less abundant and/or accessible compared to the native state. Western blot analysis with MA-TOM2-38A11 already demonstrated the conservation of the affinity of the antibody toward denatured (tomato) PME (*16*). However, a comparison to the current results is hampered by the fact that, for western blotting, (thermal) denaturation is performed in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol, rendering protein aggregation unlikely.

Taken all together, it is reasonable to conclude that the monoclonal anti-PME antibody still recognizes PME in the denatured state, contrary to the PMEI probe. Therefore, an additional application of the newly developed dot-blot technique with bPMEI could be its use as a rapid, sensitive tool to selectively screen for active plant PMEs (with a detection limit around 20 ng) in certain protein fractions.

Tissue Printing for PME Localization in Various Plant Organs. Dot-blot binding assays demonstrated that bPMEI is capable of recognizing PME after coating the enzyme on a membrane. The usefulness of bPMEI as a probe for the *in situ* localization of PME was further assessed by tissue printing of carrot root, broccoli stem, and tomato fruit. In this technique, a fresh cut surface of plant material, exposing all tissues and most cell types, is pressed onto a sheet of nitrocellulose, which is then labeled with antibodies or other molecular probes. Little or no lateral mixing or diffusion occurs, and much of the spatial information regarding distribution is maintained. Hence, tissue printing can often provide a rapid overview of epitope or antigen occurrence in the context of a whole organ as well as fine details of epitope distribution. If successful, it can be a valuable starting point for an (immuno)-localization study with fluorescence light microscopy or gold electron microscopy (in which only a part of the organ, tissue, or cell can be seen), because cell wall components can be extensively developmentally regulated within organs (*34*, *35*).

In the current study, tissue printing for localization of PME was carried out on nitrocellulose membranes prewet in a high ionic strength buffer, a strategy adjusted from Tieman and Handa (*36*). This way, not only the soluble PME but also PME that is (electrostatically) bound to the cell wall were assumed to be transferred from the cut surface of the plant tissue onto the absorptive membrane, where they were then detected using bPMEI on the one hand and MA-TOM2-38A11 on the other hand. Tissue prints of raw carrot root, broccoli stem, and tomato fruit, probed with bPMEI and MA-TOM2-38A11 (not for broccoli) and without primary probe, are shown in **Figure 4**.

On prints of carrot root, bPMEI stained both the cortex and the core, whereas recognition of PME by MA-TOM2-38A11 in the central cylinder was only limited. The latter observation is in accordance with the results of Vandevenne et al. (*16*). The blot of carrot developed without the addition of primary probe showed staining concentrated at spots in the cortex and endodermis, most likely originating from endogenous carrot peroxidase activity. The restricted staining of the carrot vascular cylinder by the antibody may hypothetically point to a differential location of various isozymes of PME in carrots (some of which would then not be recognized by the antibody) or to a too low concentration of PME in the vascular tissue of ripe carrot roots to allow for antibody detection. The latter hypothesis seems rather unlikely, given the fact that bPMEI stained the cortex successfully.

The print of the broccoli stem cut surface was labeled throughout by bPMEI. As already suggested by the outcome of the dot-blot

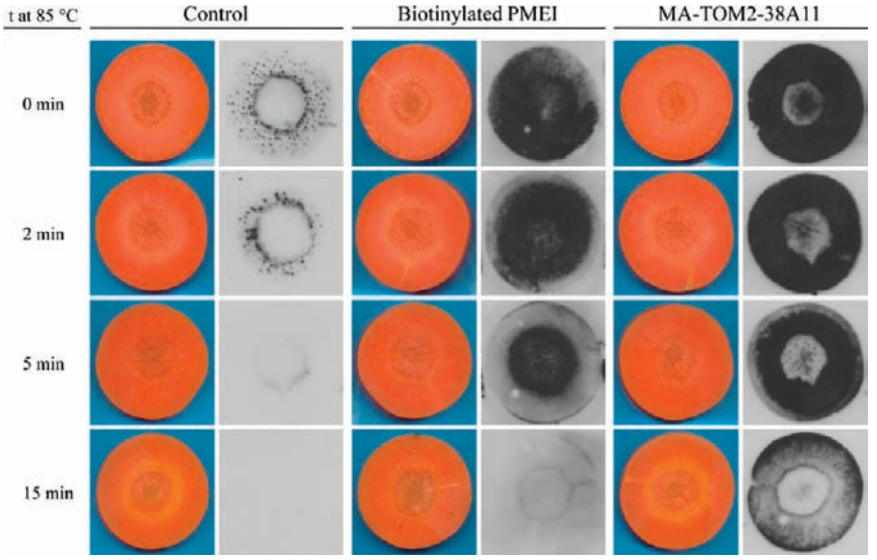

Figure 5. Tissue prints of carrot root after various treatment times at 85 °C, probed with bPMEI, MA-TOM2-38A11, and without primary probe ("control"). Image scans of the respective cut surfaces are shown on the left of each print.

binding assay (cf. previous section), MA-TOM2-38A11 (and other monoclonal antibodies raised against tomato PME) was not able to recognize PME in prints of broccoli stem tissue (results not shown). The negative control print, developed with omission of the primary probe, did not show any staining.

Both bPMEI and MA-TOM2-38A11 stained the tissue prints of ripe tomato fruit, however, with some distinct differences. In agreement with earlier results (*16*), the antibody detected PME in the pericarp and the septa, whereas only a low signal was seen in the columella and no labeling in the placenta and locular gel. With bPMEI, conversely, strong labeling was observed throughout the fruit, except for the locular cavities. The tomato print only developed with a secondary probe was free of background staining. The bPMEI print is in good accordance with the PME activity print reported by Blumer et al. (*13*), which shows that all regions of the tomato fruit (in the breaker stage) contain PME activity, including the jelly-like parenchyma, the radial pericarp, endoperiderm, and exoperiderm.

The bPMEI proves to be a versatile probe for PME, staining prints of carrot root, broccoli stem, as well as tomato fruit. In its capacity as an anti-PME probe, the inhibitor may, for instance, be employable to visualize spatial or temporal changes in PME activity between different species/varieties or ripening stages of fruits and vegetables, thus far always pursued by means of antibodies (*13*, *37*). The differences observed between bPMEI and MA-TOM2-38A11 as primary probes may indicate that the antibody, as a result of its specificity toward a defined epitope, only recognizes a distinct subset of the plant PMEs, whereas bPMEI reacts with all plant PMEs present. However, on the basis of the current results (and because of the lack of a reliable nonreactive substitute for bPMEI, as there exists for monoclonal antibodies), it cannot be excluded that bPMEI also binds to a certain (but limited) extent to plant components that are transferred to the membrane but differ from PME. A charge-mediated unspecific binding of PMEI is rather unlikely given the observation that a washing step with an elevated ionic strength buffer (i.e., 0.5 M NaCl) did not affect the blotting images (data not shown). Nevertheless, in all cases (both antibodies and bPMEI), care should be taken when drawing conclusions based on localization studies with probes.

Tissue Printing for PME Localization after Thermal Treatment. The tissue printing technique outlined in the previous section was applied to plant material after thermal treatment. **Figure 5** represents tissue prints of carrot roots after thermal treatment at 85 °C for various time intervals (0−15 min), probed with bPMEI, MA-TOM2-38A11, and without primary probe ("control"). The obtained picture was clearly dissimilar for the different probes.

On prints of untreated raw carrot, bPMEI stained the cortex as well as the core, while MA-TOM2-38A11 reacted strongly with the cortex but only weakly with the central cylinder. The control print merely showed staining concentrated at spots in the cortex and endodermis, probably corresponding to endogenous peroxidase activity.

After a thermal treatment of 15 min at 85 °C, a condition that reduced the PME activity in the carrot root to a level below the detection limit (measured by automatic titration after PME extraction with a high ionic strength buffer), hardly any labeling by bPMEI was observed at all. The antibody, however, still labeled the cortex, although somewhat weakened in comparison to the raw carrot. No background staining because of endogenous peroxidase was left.

Printing transverse sections of carrots after intermediate treatment times (i.e., 2 and 5 min at 85 °C) and probing them with MA-TOM2-38A11 gave the same picture as for the raw carrot, i.e., intense staining of the cortex and faint staining of the core. This observation contrasted sharply with the findings for the bPMEI-probed prints. In the latter case, the dark-colored area reduced progressively with the treatment time along the radius of the carrot disk. Besides the dark inner part of the carrot print, the outer part showed a light degree of background staining. When the intensely stained shape was assumed to be circular and the diameter was measured manually (with an accuracy of 1 mm), the residual staining area could be estimated. When this residual area was compared to the residual PME activity (measured per gram of fresh weight), a good accordance was observed (**Table 1**). Remarkably, the control prints revealed a similar but less pronounced image compared to the bPMEI prints, i.e., a vanishing of the signal in the radial direction with the treatment time.

To the authors' knowledge, the current study is the first one to apply the technique of tissue printing on thermally treated plant tissue. Probing the prints with two kinds of molecular probes for PME recognition, i.e., a monoclonal antibody and a biotinylated enzyme inhibitor, provides valuable insight to both the technique

Table 1. Residual PME Activity and Residual Area Stained by bPMEI on Tissue Prints of Carrot Root (cf. **Figure** 5) after Various Treatment Times at 85 °C, Both Relative to the Value of the Untreated Blank

t at 85 °C (min)	residual PME activity (%)	residual staining area (%)[a]
0	100.0	100.0
2	81.6 ± 2.3	71.3 ± 1.3
5	41.9 ± 1.0	33.9 ± 1.8
15	0.0 ± 0.0	0.0 ± 0.0

[a] The residual staining area is estimated by assuming a circular staining shape and manually measuring the diameter (with an accuracy of 1 mm).

of tissue printing and the qualitative distribution of the PME epitopes of the probes after thermal treatment.

The prints probed with MA-TOM2-38A11 retained most of the staining when increasing the duration of the thermal treatment. Together with the outcome of the dot-blot assays of raw and denatured purified PMEs, this observation proves that the antibody still recognizes PME after thermal inactivation of the enzyme. In the mean time, it allows for the conclusion that, after thermal treatment, PME is still transferred from the carrot tissue to the absorptive surface. However, the weakened staining for the most intensively treated carrot sample may indicate a (partly) reduced extractability or recognizability of the PME.

The staining of the prints of pretreated carrot tissue by bPMEI was obviously treatment-time-dependent; the longer the treatment, the smaller the labeled area. Knowing that PME is still extracted from the treated carrot tissue during printing (cf. supra) and that bPMEI only recognizes active PME molecules in dot-blot assays, it can be assumed that the stained area corresponds to the locations where active PME is found. This hypothesis is endorsed by the fairly good correspondence between the estimated residual staining area and the residual PME activity. Furthermore, the blots demonstrate that PME inactivation in an external heating medium occurs from the outside to the center along the radius of the carrot root, caused by the intruding heat. This observation is indirectly confirmed by the control blots, which also show a progressive disappearance of the signal along the radius, attributed to gradual inactivation of endogenous peroxidase. The light background color in the bPMEI prints of 2 and 5 min, however, suggests that a certain extent of unspecific binding of the bPMEI occurs, although the presence of some residual PME activity at these locations or a certain binding of bPMEI to inactivated PME cannot be excluded.

Anyhow, the whole of the data reveals that bPMEI can be used for a reliable localization of PME activity, e.g., remaining after treatment. In contrast, the anti-PME monoclonal antibodies studied here are not capable of distinguishing between native and inactivated enzyme molecules. Their applicability in the context of fruit and vegetable processing is therefore particularly interesting for the cases in which the complementarity of both types of probes can be played off.

In conclusion, a PMEI-based molecular probe could be designed that successfully labels plant PMEs in dot-blot binding assays and prints of various plant tissues. No major objections were encountered that compromise the future use of kiwi PMEI (extracted or recombinantly produced) as a probe in PME detection at the microscopic level (in fluorescence or electron microscopy), although attention should be paid concerning a certain degree of unspecific binding of PMEI to plant tissue that may possibly occur. The observation that the PMEI probe can clearly differentiate between active and (irreversibly) inactivated PME molecules is promising in the context of PME activity localization in plant tissue after different food-processing steps, with both traditional (thermal) and nonconventional (e.g., high hydrostatic pressure, microwave, and ohmic heating) processing techniques. Hence, well-considered optimization of these processes in terms of site-specific PME stimulation or inactivation can be pursued.

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