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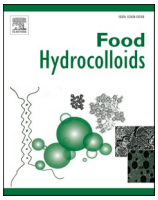

Deesterification of pectin using commercial pectin methylesterase-containing plant extracts

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ABSTRACT

A vast variety of bacterial, fungal, and plant-derived pectin methylesterases (PMEs) have been characterized in literature for their ability to deesterify pectins. However, when compared to fungal PMEs, the availability, characterisation and application of commercial enzyme preparations comprising plant PMEs is still lacking.

Here, we characterized the PME activity in commercially available crude plant extracts originating from papaya (papain), pineapple (bromelain), and kiwi (actinidin). The highest PME activity towards pectin was determined in papain preparations, which did not comprise pectin-backbone degrading side activities. The pH and temperature optimum of the salt-dependent papain PMEs ranged from 7.0 to 8.0 and 50–70 °C, respectively. Using enzymatic fingerprinting, it was shown that papain PMEs exhibited a processive mode of action towards lemon pectin. Papain PMEs had a broad substrate specificity, as 61, 83, and 58% of the methylesters were released from lemon, apple and sugar beet pectin, respectively. The release of acetic acid from sugar beet pectin indicated the presence of acetyl esterases in the papain preparations.

Both the determined processive mode of action and broad substrate specificity allows to consider papain preparations as an alternative to commercial fungal-derived PME preparations to modify the methylester distribution pattern of pectin for food applications.

1. Introduction

Hydrocolloids, like pectins, are extensively used in the food and beverage industry to modify the rheology of food systems. To obtain a purified ingredient, hydrocolloids are often chemically extracted from plants, like fruit and vegetables, and further modified to ensure their techno-functional properties in food systems. Alternative technologies to minimize the use of chemicals, such as alkali and acid treatments, to modify pectins are highly desired by the food industry, as nowadays food products are being judged by the consumer not only by their sensorial attributes, but also by the ingredients and methods employed for their production (Varela & Fiszman, 2013).

Pectin is a heteropolymer that is abundantly present in the middle lamella and primary cell wall of fruit and vegetables (Willats et al., 2001). The predominant structural elements of pectin are homogalacturonan (HG), rhamnogalacturonan I and II (RG-I & RG-II), and xylogalacturonan (Atmodjo, Hao, & Mohnen, 2013; Voragen, Coenen, Verhoef, & Schols, 2009). The proportions of those elements and their structural characteristics like degree of methylesterification, degree of branching, type and length of branches, determine important techno-functional properties of the pectin molecule (Ridley, O'Neill, & Mohnen, 2001). To better benefit from these properties, extracts comprising high concentrations of pectin rich in HG, are used in the food industry. These commercial pectin sources primarily derive from citrus fruit peels, apple pomace, and sugar beets, like lemon, apple and sugar beet pulp, respectively (Funami et al., 2011; Kravtchenko, Voragen, & Pilnik, 1992; May 1990). The HG is solely composed of α-(1 → 4)-linked galacturonic acid (GalA) units which can be methylesterified at C-6 and/or acetylated at the *O*-2/*O*-3 position (Ralet, Crépeau, Buchholt, & Thibault, 2003; Remoroza, Buchholt, Gruppen, & Schols, 2014; Wolf, Mouille, & Pelloux, 2009). In the case of lemon pectin, up to 60%, of the pectin molecule consist of HG (Voragen et al., 2009) and the DM and the distribution of methylesters along the GalA backbone of pectin depends on the source, stage of ripening, plant tissue type and location (Willats et al., 2001). Different from HG, the RG-I element is composed of alternating rhamnose and GalA dimer units (Voragen et al., 2009). The RG-I backbone chain can further be substituted at the *O*-4 and occasional *O*-3 position of rhamnose with α-L-arabinofuranosyl and/or β-D-galactopyranosyl units that form linear and/or branched side chains of

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Abbreviations

PME	Pectin methylesterase
PG	Polygalacturonase
PEL	Pectate lyase
PL	Pectin lyase
GalA	Galacturonic acid
HG	Homogalacturonan
RG-I/II	Rhamnogalacturonan-I/II
XG	Xylogalacturonan
M_w	Molecular weight
DM	Degree of methylesterification
DA	Degree of acetylation
DB	Degree of blockiness of non-methylesterified GalA units
Lemon pectin (LR DM70_DA1)	High methylesterified (randomly distributed) lemon pectin
Lemon pectin (LB DM70_DA1)	High methylesterified (block wise distributed) lemon pectin
Lemon pectin (LR DM30_DA1)	Low methylesterified (randomly distributed) lemon pectin
SBP (DM55_DA25)	Moderate methylesterified and moderate acetylated sugar beet pectin
SBP (DM35_DA13)	Low methylesterified and low acetylated sugar beet pectin
Apple Pectin (DM55_DA7)	Methylesterified apple pectin

different length (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996; Mohnen, 1999). Like reported for HG, the GalA residues of the RG-I element can be highly acetylated at the *O*-2 and/or *O*-3 position (Voragen et al., 2009).

Several methods have been established to discriminate between the diverse methylester distribution patterns of HG, including the quantification of released degradation products of the pectin digest after incubation with an *endo*-polygalacturonase (*endo*-PG) from the fungus *Kluyveromyces fragilis* to determine the degree of blockiness (DB) (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Jermendi, Beukema, van den Berg, de Vos, & Schols, 2022). Methods that allow a more detailed definition of the methylester distribution pattern of HG and methods that go beyond the DB have been established in recent years, but will not be further discussed here (Coenen, Kabel, Schols, & Voragen, 2008; Daas, Voragen, & Schols, 2000; Guillotin, Bakx, Boulenguer, Schols, & Voragen, 2007; Guillotin et al., 2005; Hocq et al., 2020; Jermendi et al., 2022; Kim et al., 2013; Ngouémazong et al., 2011; Ralet et al., 2012; Remoroza, Broxterman, Gruppen, & Schols, 2014; Remoroza, Buchholt, et al., 2014; Tanhatan-Nasseri, Crépeau, Thibault, & Ralet, 2011; Voxeur et al., 2019).

The structure of pectin isolated from plants may be altered during the extraction affecting the monosaccharide composition, such as the presence of uronic acids and neutral sugars, the molecular weight (M_w) distribution, as well as the DM and the degree of acetylation (DA) (Kaya, Sousa, Crépeau, Sørensen, & Ralet, 2014; Ralet et al., 2003). These modifications of the pectin molecule during the extraction process are a result of the specific conditions, chemicals, or enzymes employed in the process (Sénéchal, Wattier, Rustérucci, & Pelloux, 2014). Typically, the latter enzymes are pectin methylesterases (PMEs; E.C. 3.1.1.11) which are classified as Carbohydrate Esterase Family 8 (CE8) in the Carbohydrate-Active enZyme database (CAZy) and alter the methylester distribution pattern of the GalA-containing HG backbone and consequently influence the functionality of pectin (Drula et al., 2021; Osborne, 2004; Rolin, 2002; Thibault & Ralet, 2003). This demethylesterification of the GalA units by using PMEs and the resulting increase in negative charges on the surface of the GalA backbone enhances the susceptibility of the pectin molecule towards divalent metal ions, such as calcium. It was proposed that 6 - 14 adjacent non-methylesterified GalA residues are required for a calcium-mediated crosslinking of pectin and that a blockwise methylester distribution pattern is associated with an enhanced gelling behavior compared to a pectin comprising a random methylester distribution pattern (Kreiberg, Christensen, & Hyttel, 1998; Luzio, Forman, & Gerrish, 2002).

PMEs originate from microbial (i.e. bacterial and fungal) and plant-derived sources. Commercially available PME preparations that are applied for fruit and vegetable processing under acidic conditions mainly originate from *Aspergillus species* (Aids et al. & EFSA Panel on Food Contact Materials et al., 2023). The origin of the PMEs strongly affects the mode of action towards pectin. So far, three different mode of actions of PMEs have been described, including a (1) single-chain mechanism, (2) multiple-chain mechanism and (3) multiple-attack mechanism (Cameron, Luzio, Vasu, Savary, & Williams, 2011; Duvetter et al., 2006; Jolie, Duvetter, Van Loey, & Hendrickx, 2010). The majority of plant and bacterial PMEs have an alkaline pI and exhibit a processive, blockwise demethylesterification of the GalA chain. This mode of action leads to the creation of long contiguous regions of demethylesterified GalA residues and is attributed to single-chain or multiple-attack mechanisms. In contrast, most of the fungal PMEs and a few plant PMEs have an acidic pI and exhibit a mode of action that resembles a multiple-chain mechanism, leading to a randomly demethylesterified GalA chain and a random methylester distribution. A previously performed study using molecular dynamics simulations of a PME-HG-decasaccharide complex has provided valuable insights into the processivity of PMEs, contributing to a better understanding of the underlying factors of this mode of action (Mercadante, Melton, Jameson, & Williams, 2014).

In brief, microbial PMEs, like fungal PMEs having an acidic isoelectric point (pI) and acidic pH optima, were mainly reported to act following a multiple-chain mechanism which leads to a pectin comprising a random methylester distribution pattern (Duvetter, et al., 2006, 2009; Ishii, Kiho, Sugiyama, & Sugimoto, 1979; Jolie et al., 2010). One notable exception is the fungal pectin methylesterase (PME) derived from *Trichoderma reesei*, which demonstrates a processive demethylesterification (Sénéchal et al., 2014). In contrast, many of the reported plant-derived PMEs comprise a pI and pH-optimum ranging from neutral to alkaline and are believed to demethylesterify pectins exhibiting a single-chain or multiple-attack mechanism, which leads to pectins comprising a blockwise methylester distribution pattern (Cameron et al., 2011; Duvetter et al., 2009; Grasdalen, Andersen, & Larsen, 1996; Hotchkiss et al., 2002; Savary, Hotchkiss, Fishman, Cameron, & Shatters, 2003; Sénéchal, et al., 2014, 2015). A recent study elucidated the mode of action of an expressed *Arabidopsis At*PME, which displayed high (pH 8.0) to low (pH 5.0) processivity, depending on the pH (Hocq et al., 2023).

Plant-derived PMEs are ubiquitous enzymes and have been identified in leaves, stems, roots and flours of higher plants, like tomato, orange, papaya, apple, pineapple, kiwi, grapefruit, mandarin orange, carrot, and potato (Jolie et al., 2010; Kohli, Kalia, & Gupta, 2015; Sénéchal et al., 2014; Versteeg, 1979). Numerous PMEs have undergone isolation and characterization but, despite these efforts, only one commercial plant PME preparation (Collupulin®, Gist-Brocades International B.V., Charlotte, N.C., USA) has been investigated thus far for its industrial application in pectin modification (Luzio et al., 2002). However, commercial plant extracts targeting applications that are not related to pectin modification, like papain, have been shown to comprise active PMEs (Kim et al., 2013; Luzio et al., 2002; Vasu, Savary, & Cameron, 2012).

Papain-containing enzyme preparations are prepared from the latex

tapped from green papaya fruit (*Carica papaya* L.) and known to comprise a diversity of enzymes, like cysteine endopeptidases, papain, chymopapain, chitinase, an amylase or *endo*-1,3-β-glucanase (Azarkan, El Moussaoui, van Wuytswinkel, Dehon, & Looze, 2003; Azarkan et al., 1997; Messer & Ottesen, 1964; Smith, Kimmel, Brown, & Thompson, 1955). Based on their strong proteolytic activity, papain preparations are industrially relevant for meat tenderization and various other applications for the food, feed, brewing and textile industry (Fernández-Lucas, Castañeda, & Hormigo, 2017). Papain preparations are of special interest in this work, as PMEs have already been isolated and characterized, like from papaya fruit flesh and pulp (Fayyaz, Asbi, Ghazali, Che Man, & Jinap, 1994; Fayyaz, Asbi, Ghazali, Man, & Jinap, 1995; Lim & Chung, 1993; Lourenco & Catutani, 1984) and CpL-PME from a commercial papaya latex preparation (Vasu et al., 2012). Among the aforementioned PME (iso)forms derived from papaya, a comprehensive biochemical characterization of PME activity, including the respective mode of action towards pectin, has solely been described for the isolated CpL-PME (Kim et al., 2013). Moreover, Luzio et al. showed that the PME activity in a commercial papain preparations remained stable and can be applied to modify the functional properties of commercial pectin (Luzio et al., 2002).

In this study, we investigated if commercial crude plant extracts from several papaya (papain) preparation, as well as from pineapple (bromelain) and kiwi (actinidin), can be applied to modify techno-functional properties of pectin, as previously shown for isolated CpL-PME from papaya latex (Kim et al., 2013). It was hypothesized that these extracts comprise PMEs that exhibit a processive mode of action towards pectin leading to a blockwise methylester distribution pattern. The extracts were assessed for their protein content, pectin-degrading side activity, thermal and pH-dependent PME stability, pH and temperature optima, salt-dependency, substrate specificity towards pectin comprising a different DM and DA, acetyl esterase activity, and processivity. The obtained results were further discussed in the light of industrial applicability.

2. Materials and methods

2.1. Enzymes and substrates

All analytical grade chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. The following commercially available crude plant extracts (preparations) including various batches thereof were obtained: powdered PapainPME-Prep1 (Brauzyn Conc) from Prozyn (São Paulo, Brazil); three batches of powdered PapainPME-Prep2A, 2B and 2C (Enzeco® Purified Papain RS Concentrate) and powdered Bromelain PME-Prep1 (Enzeco® Bromelain Concentrate) from Enzyme Development Corporation (EDC, New York, US); powdered BromelainPME-Prep2 (Bromelain BR2400), powdered PapainPME-Prep3 (Performase® PNS1000) and liquid PapainPME-Prep4 (Xtrase® LSG200) from Enzybel International S.A. (Waterloo, Belgium); and powdered KiwiPME-Prep1 (Actinidin Active P200) from KiwiEnzyme LTD (Auckland, New Zealand). The suppliers did not disclose the production process of the plant preparations. Liquid FungalPME-Prep1 was a fungal-derived PME preparation (Rapidase® PEP) and provided by DSM Food Specialties B.V. (Delft, The Netherlands). The papain and bromelain preparations were isolated from the latex of the *Carica papaya* fruit and the stems of pineapple (*Bromeliceae* family, *Ananas comosus*), respectively. In this work, PapainPME-Prep2 refers to the use of batch 2A, unless stated otherwise.

The following pectins were used as substrates: high methylesterified (randomly and blockwise distributed) lemon pectin (LR DM70_DA1 and LB DM70_DA1), low methylesterified (randomly distributed) lemon pectin (LR DM30_DA1) were obtained from CP Kelco (Copenhagen, Denmark), as described previously (Daas, et al., 1999, 2000). Moderate methylesterified and moderate acetylated sugar beet pectin (SBP; DM55_DA25) was obtained from CP Kelco as described previously (Oosterveld, Beldman, Schols, & Voragen, 2000). Apple pectin (DM55_DA7) was obtained from Sanofi Bio Industries (France) (Kravtchenko, 1992). Low methylesterified, low acetylated SBP (DM35_DA13) was obtained from Danisco (Copenhagen, Denmark). The GalA, DM and DA are summarized in Suppl. Table S1.

2.2. Protein content and SDS-PAGE analysis

The protein content of the enzyme preparations was determined by using the BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL, USA) and bovine serum albumin (BSA) was used for calibration. In addition, the protein content was also calculated based on the nitrogen content which was determined using Dumas (FlashSmart Elemental Analyzer, Thermo Fisher Scientific, USA) using the nitrogen conversion factor 6.25. The M_w distribution of the proteins present in the plant and fungal preparations was analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, proteins were reduced with β-mercaptoethanol, heated for 10 min and loaded on 12% polyacrylamide gels (Mini-PROTEAN TGX Gels, Bio-Rad Laboratories, Hempel Hempstead, UK). In addition, a protein marker (Protein All Blue Standards, Bio-Rad Laboratories) was loaded for mass calibration. Gels were stained with the EZBlue Gel Staining Reagent (Sigma Aldrich, Steinheim, Germany). The obtained gels were washed three times using MilliQ water (MilliporeSigma, Burlington, MA, United States) to remove the remaining staining reagent.

2.3. Desalting using ultrafiltration

Papain preparations were desalted, to determine the effect of salt on the PME activity. Therefore, the preparations were dissolved in MQ water yielding to a concentration of 21 mg mL^{-1} based on the powdered and liquid preparation (w/v). The dissolved samples were filtered using Amicon® Ultra Centrifugal Filters Ultrafiltration comprising a M_w cut-off of 50 kDa (0.5 mL, Merck Millipore, Cork, Ireland) and centrifuged at 15.000×*g* and 4 °C for 30–45 min. The remaining retentates were re-diluted in MQ water and centrifuged again. The procedure was repeated again twice. The desalting of the papain preparations did not lead to a removal of proteins, based on the protein quantification in the final retentates using the BCA Protein Assay Kit and the determined PME-activity is described in section 3.2.

2.4. Determination of molecular weight (M_w) distribution using HPSEC

The presence of smaller and larger M_w molecules in the papain preparations itself and the M_w distribution of the pectic polysaccharides before and after incubation with the enzyme preparations was determined using High Performance Size Exclusion Chromatography coupled to a Refractive Index detection (HPSEC-RI) as described elsewhere (Jermendi et al., 2022). Therefore, powdered papain preparations were dissolved to yield a final concentration of 1.7 mg mL^{-1} (based on dry matter content). As PapainPME-Prep4 was liquid, a concentration of 1.6 mg mL^{-1} was used, based on the determined protein content. Pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration.

The presence of pectin-degrading activity of the preparations was determined by HPSEC and measured after incubating a lemon pectin (LR DM70_DA1) solution (50 mL, 1 mg mL^{-1}) containing 0.1 M NaCl with the papain- or fungal-derived preparations (1.6 mg mL^{-1} based on protein weight (w/v)) at 20, 40 and 50 °C for 1 h. The latter experiment was performed at pH 3.9 and 7.0. Reactions were stopped by incubating the samples at 95 °C for 10 min using a water bath. Prior to the M_w analysis, the samples were centrifuged at 18,000×*g* and 20 °C for 15 min.

2.5. Assessment of the intrinsic proteolytic activity using SDS-PAGE and pH-stat

The intrinsic proteolytic activity of the papain preparations and its effect on the protein degradation was measured using SDS-PAGE. First, each preparation was incubated with itself at the native pH (~5.0) in water at 40 and 50 °C for 1 h. The M_W distribution of the protein bands of the latter samples was compared to the distribution of the protein bands of non-incubated preparations. The intrinsic proteolytic activity and its effect on the PME activity was determined at 20 °C and pH 7.0 using pH stat (see Chapter 2.6). Therefore, preparations were incubated in water at 20 °C for 1 h and the residual PME activity of these incubated preparations towards lemon pectin (LR DM70_DA1) was compared to one of the non-incubated preparations.

2.6. PME activity analysis using pH stat

The PME activity of the preparations was measured using a thermo-controlled auto-titration system (pH-stat, 719 S Titrino, Metrohm, Herisau, Switzerland). All experiments were performed using the same titrator and a daily calibrated electrode. Checking and calibration of the system was done using a known pectin/PME mixture. To correct for the autohydrolysis/spontaneous deesterification process of lemon pectin (LR DM70_DA1) at varying pH values and temperature conditions, substrate blanks were used as a reference and the determined values were subsequently subtracted from the measured values obtained from the substrate incubated with the enzyme preparations. The PME activity determination has been performed using data points within the linear range of the reaction. With exception of data shown in Figs. 4 and 6a, analyses were performed singularly which was based on experiments conducted in duplicate comprising low standard deviations (<10%).

Lemon pectin (LR DM70_DA1) was incubated with the enzyme preparations at a fixed pH under the continuous addition of 0.01 M NaOH for 10 min. In general, 50 mL of a 1 mg mL^{-1} lemon pectin solution containing 0.1 M NaCl was used. Depending on the experimental setup, a concentration of powdered PapainPME-Prep1-3 ranging from 0.1 to 1.65 mg mL^{-1} (based on dry matter) was used, to assure an activity determination within the linear range of the reaction. As PapainPME-Prep4 was liquid, the final concentration was based on the determined protein content. The results were expressed as specific activity (U mg^{-1} protein) or relatively, as specified in each figure legend (Savary, Vasu, Cameron, McCollum, & Nuñez, 2013). The U values (μmole min^{-1}) were defined according to Bisswanger using NaOH as a titrant (Bisswanger, 2014).

Batch-to-batch variation were determined by incubating a lemon pectin (LR DM70_DA1) solution (1 mg mL^{-1}) containing 0.1 M NaCl with the papain preparations (PapainPME-Prep2A, 2B and 2C) using 10% (w/w) protein per substrate weight.

The pH and temperature optima were determined by incubating a lemon pectin (LR DM70_DA1) solution (1 mg mL^{-1}) containing 0.1 NaCl and papain preparations (0.1 mg mL^{-1} based on protein content) at varying pH (3.0–8.0) and temperature values (20–70 °C).

The temperature stability was analysed at a high solids-to-water ratio by dissolving 411 mg of the powdered papain preparation in 1 mL of MQ water (PapainPME-Prep4 was used as such). The incubation was performed at the pH (~5.0) of the preparations in solution and temperatures ranging from 20 °C to 80 °C for 1 h. Subsequently, the residual enzyme activity was analysed at pH 7.0 and 20 °C. The residual PME activity was determined by incubating a lemon pectin (LR DM70_DA1) solution (50 mL, 1 mg mL^{-1}) containing 0.1 M NaCl with the thermally treated enzyme preparations at a concentration of 0.1–0.8 mg mL^{-1} (based on dry matter). As PapainPME-Prep4 was liquid, the final concentration of 0.1–0.8 mg mL^{-1} was based on the protein content.

2.7. PME activity analysis using HS-GC-FID

To determine the PME activity, the release of MeOH from diverse types of pectins was determined using Headspace Gas Chromatography coupled to Flame Ionization Detection HS-GC-FID as described previously (Huisman, Oosterveld, & Schols, 2004).

Prior to analysis, the following experimental setup was applied: pectins were wetted with EtOH 70% (v/v) and solubilized under vigorous stirring in a McIlvaine buffer (0.15 M, pH 5.0 or 0.18 M, pH 7.0) containing 0.1 M NaCl yielding to a final pectin concentration of 5 mg mL^{-1}. The enzyme preparations were dissolved using the McIlvaine buffer (0.15 M, pH 5.0 or 0.18 M, pH 7.0). The pH of both pectin and enzyme solutions were re-adjusted. (a) For the activity screening, 0.5–1 mg mL^{-1} (based on protein content) of the enzyme preparations were added corresponding to an enzyme load of 10–20% of the initial pectin concentration. Samples were incubated at 40 °C for 1 h, followed by an inactivation step at 95 °C for 10 min. (b) The substrate specificity was determined by incubating the above-described pectin solution with 0.5 mg mL^{-1} (based on protein content) of PapainPME-Prep2. Samples were incubated at 40 °C in a water bath under agitation (100 rpm) using five timepoints from 0 to 300 min. At corresponding time points, incubated samples were directly placed in the GC autosampler followed by an incubation in the GC incubator at 50 °C for 11 min prior to HS-GC-FID analysis. The sample at timepoint zero was directly placed into the GC autosampler and incubated at 50 °C for 11 min before injection. The above-described pectin solution which was incubated without the addition of any enzyme preparation was used as a blank.

2.8. Determination of released acetic acid using HPLC

The determination of the released acetic acid was performed by HPLC using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) which was coupled to a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan) as described previously (Voragen, Schols, & Pilnik, 1986). The concentration of the SBP solutions (i.e. DM35_DA13) was 5 mg mL^{-1}. Pectin solutions were incubated with the enzyme preparations (1 mg protein mL^{-1}) at 40 °C for 1 h, followed by an inactivation step at 95 °C for 10 min.

2.9. Determination of the mode of action using enzymatic fingerprinting

The mode of action of the PapainPME-Prep1, PapainPME-Prep2 and Fungal-PME-Prep1 was determined using the following steps: 1). A 50 mL solution of lemon pectin (LR DM70_DA1) (1 mg mL^{-1}) containing 0.1 M NaCl which was partially demethylesterified using a predicted amount of PapainPME-Prep1, PapainPME-Prep2 and Fungal-PME-Prep1 that was calculated based on the measured enzyme activity data using pH stat (Chapter 2.6). The quantity of the utilized preparations varied, ranging from 82.2 mg to 246.6 mg (pH 5.0) and 41.1 mg for pH 7.0 for PapainPME-Prep1. Similarly, for PapainPME-Prep2, the amount ranged from 82.2 mg to 246.6 mg (pH 5.0) and 41.1 mg for pH 7.0. In contrast, a lower quantity of 6.8 μg was used for Fungal-PME-Prep1. The mixtures were incubated at 40 °C at pH 5.0 and 7.0. To achieve the desired DM, pH stat was used for monitoring the demethylesterification. Following the incubation, the pH was adjusted to 5.0 prior to the inactivation of the enzyme-containing pectin solution at 95 °C for 10 min. The DM and GalA content was determined after freeze drying of the incubates following previous protocols as listed below. In brief, methanol was quantified after saponification using headspace-gas chromatography (Thermo Scientific, Waltham, MA, USA) and the uronic acid content was determined after Seamen hydrolysis using an automated *m*-hydroxydiphenyl assay with addition of sodium tetraborate and an auto-analyser (Skalar Analytical BV, Breda, The Netherlands) (Blumenkrantz & Asboe-Hansen, 1973; Huisman et al., 2004; Jermendi et al., 2022; Thibault & Ralet, 2003). 2). The partially demethylesterified pectin solutions were incubated with an *endo*-polygalacturonase (*endo*-PG) (EC

3.2.1.15) from *Kluyveromyces fragilis* at 40 °C for 18 h, as described previously (Jermendi et al., 2022; Remoroza, 2014). The incubation was stopped by a heat-treatment at 95 °C for 10 min. 3). The M_w distribution of the incubates was evaluated by HPSEC-RI/UV. The released diagnostic GalA oligomers were analysed using HPAEC-PAD/UV as reported elsewhere (Broxterman & Schols, 2018; Jermendi et al., 2022). In summary, GalA standards (Sigma-Aldrich, Steinheim, Germany) were utilized for the quantification of GalA units released as mono-, di- and trimers. Due to the unavailability of standards for longer GalA units, the quantification of these units above DP3 and their unsaturated oligomers was performed by using the response factor derived from the GalA standard having a DP of 3.

3. Results and discussion

3.1. PME activity and characterization of the crude plant extracts

In total, four plant extracts from papaya (PapainPME-Prep1-4), including three batches of PapainPME-Prep2A, 2B and 2C, were analysed regarding the presence of PME activity, their protein content, M_w distribution of the proteins, proteolytic and pectin-degrading side activities. The initial selection of the crude plant extracts also included commercial preparations that originated from pineapple (BromelainPME-Prep1 and 2), kiwi (KiwiPME-Prep1) and for comparison of the mode of action, a fungal PME (FungalPME-Prep1). In this study, we did not perform statistical analyses. However, the Materials and Methods section provides comprehensive details regarding the experimental procedures, including the use of singlicates and duplicates, as well as the corresponding standard deviations.

3.1.1. PME-activity of Papain-PMEs

All four Papain-PME preparations (PapainPME-Prep1-4) contained active PMEs and the activity determined towards lemon pectin ranged from 0.07 to 0.65 U mg^{-1} protein, based on pH stat analyses and using equal amounts of proteins per preparation (Fig. 1). The liquid PapainPME-Prep4 comprised the highest PME activity (U mg^{-1} protein) compared to the powdered PapainPME-Prep1-3. Importantly, the PME activity is not prone to autohydrolysis, as the pre-incubation of the preparations at the native pH (~5.0) at 20 °C for 1 h did not lead to any reduction of the PME activity (Fig. 1). This finding is supported by the fact that liquid PapainPME-Prep4 retained its PME activity over longer storage times, which was at least 3 months after receiving this enzyme preparation. A stabile PME activity in these preparations is a prerequisite to enable a potential industrial implementation without the need for further purification. The PME activity was most pronounced in PapainPME-Prep3 and 4. The activity comparison was based on U mg^{-1} protein, and substantial differences observed in PME activity among the tested preparations can only be attributed to assumptions. Factors such as variations in the PME concentration and isoform types may arise from environmental influences during plant growth, stage of ripening, and (post)harvest conditions. Here, we carried out the majority of the experiments using PapainPME-Prep2 and partially PapainPME-Prep1, which resulted from varying timelines of the project stages and enzyme availability. *Composition of Papain-PMEs*.

Powdered PapainPME-Prep1 comprised a lower protein content (33% (w/w)) compared to PapainPME-Prep2 and 3 (90–93% (w/w), respectively). The liquid PapainPME-Prep4 contained 24% (w/w) of protein. Overall, a similar M_w distribution of the proteins was determined for the different papain preparations, as the SDS-PAGE revealed the presence of medium M_w proteins ranging from 15 to 35 kDa and a high abundance of low M_w proteins (<15 kDa) (Suppl. Fig. S1).

Papain preparations are known to comprise a diversity of enzymes in higher concentrations (>1 mM) in the laticifers, like cysteine endopeptidases, papain, chymopapain, glycyl endopeptidase and caricain (Azarkan et al., 2003). PMEs deriving from papaya fruit flesh and pulp after peeling (Fayyaz, et al., 1994, 1995; Lim & Chung, 1993; Lourenco & Catutani, 1984) and a commercial papaya latex preparation (Vasu et al., 2012) have already been isolated and characterized for their M_w, which ranged from 27 to 37 kDa.

Based on SDS-PAGE, the expected low abundance of these PMEs in the papain preparations did not allow an assignment of detected protein bands to the presence of specific PMEs, such as the previously characterized CpL-PME from papaya latex, which had a M_w of 37 kDa (Suppl. Fig. S1) (Vasu et al., 2012). Due to the high abundance of proteolytic enzymes in the papain preparations, such as endopeptidases, all preparations were pre-incubated in water at 20 °C for 1 h and the effect on the M_w distribution before and after the incubation was determined using SDS-PAGE (Suppl. Fig. S2). No shift in the M_w distribution towards smaller molecules was observed and the overall pattern of protein bands did not alter, indicating the absence of a significant auto-proteolysis and a possible degradation of pectic enzymes present. Though, upon pre-incubation of PapainPME-Prep1 and 4, a more prominent protein

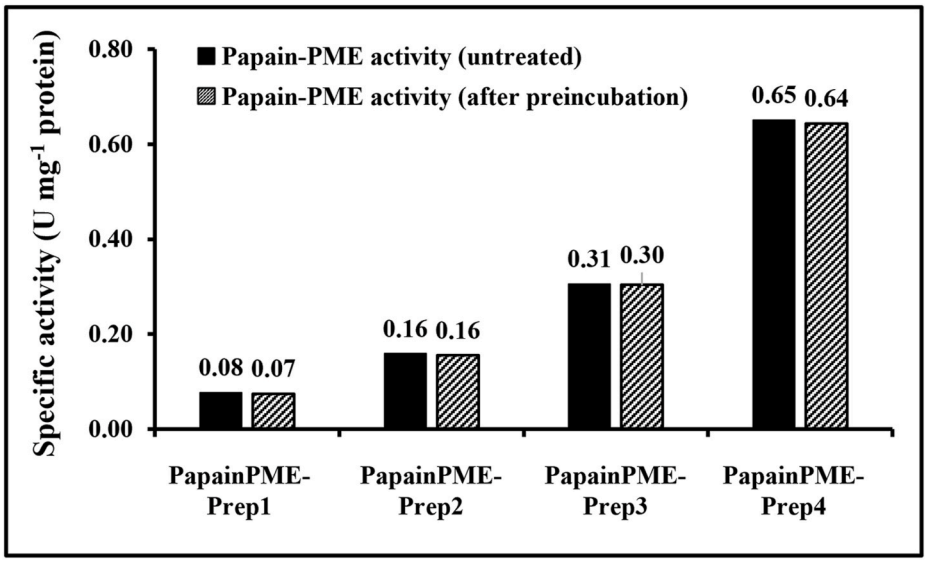

Fig. 1. PME activity of four crude papain preparations towards lemon pectin. Lemon pectin (LR DM70_DA1) was incubated with untreated (filled bar) or pre-incubated (striped pattern bar) PapainPME-Prep1-4 at 20 °C for 1 h. The PME activity (U mg^{-1} protein) was determined at 20 °C and a pH of 7.0 using pH stat. PME activity determination has been performed using data points within the linear range of the reaction. Analyses were performed singularly. See "M&M" for details.

band having a M_w of approximately 23 kDa was observed, compared to the non-pre-incubated preparations (Suppl. Fig. S2). The appearance of this protein band resembled the one of PapainPME-Prep2 and 3. The latter observation could result from an enhanced solubility of this protein fraction, a dissociation of a larger protein complex into smaller ones, or an intrinsic proteolytic activity towards a larger protein yielding to a 23 kDa protein fraction. The pH of the papain preparations in an aqueous solution was slightly acidic, ranging from 4.8 to 5.3, which was about half a unit lower compared to the pH of the papaya pulp (Hewajulige & Dhekney, 2016; Moy, 2003).

Using HPSEC, the incubation of lemon pectin (LR DM70_DA1) with the papain preparations did not lead to a change in the M_w distribution of the pectin, indicating the absence of pectin-degrading enzymes that are active towards the linear HG component, like polygalacturonase (PG), pectate lyase (PEL) and pectin lyase (PL) (Suppl. Fig. S3). Next to the presence of a stable PME activity, the absence of pectin-degrading enzymes in the papain preparations was an important finding, as this enabled the direct characterisation of PME activity present in crude plant-preparations towards pectic polysaccharides without the need for additional purification steps. Moreover, these properties enable a direct application of the crude plant extracts for the enzymatic modification and texturization of pectin and pectin-rich ingredients. The latter was the motivation for further in-depths analyses and a better understanding of the PME activity in these crude extracts towards pectin.

Based on the HPSEC-RI elution profiles, PapainPME-Prep1 contained a high amount of low M_w compounds (<1 kDa) that eluted between 14 and 15 min and likely corresponded to the presence of salt (Suppl. Fig. S4). Indeed, according to the supplier's information, NaCl was added to PapainPME-Prep1, which also explains its lower protein content (30–33 % (w/w)) compared to the protein content of the other powdered Papain-PME preparations (~90% (w/w)). As reported for the papain preparations, also the FungalPME-Prep1 preparation did not exhibit pectin-degrading activity towards lemon pectin (data not shown).

3.1.2. PME-activity and composition of Bromelain- and Kiwi-PMEs

BromelainPME-Prep1-2 and KiwiPME-Prep1 were analysed towards their protein content, which ranged from 1 to 66% (w/w), and ability to release MeOH from lemon (LR DM70_DA1), apple (DM55_DA7) and sugar beet pectin (SBP; DM35_DA13) (Suppl. Fig. S5). After these initial screenings, bromelain and kiwi preparations were excluded from a more detailed assessment due to the following reasons: The determined MeOH release from the pectic substrates by BromelainPME-Prep1-2 was 5 to 10-fold lower compared to the PME activity in PapainPME-Prep1-2 (Suppl. Fig. S5). In contrast, KiwiPME-Prep1 contained a relatively high PME activity towards lemon (LR DM70_DA1) and apple (DM55_DA7) pectin based on the protein content. Nevertheless, a potential industrial application may be challenging, as the low protein content (2% (w/w)) required a very high dosage of these preparations to demethylesterify pectins, which was several times higher than the amount of the pectin itself (Suppl. Fig. S5). To what extent the presence of proteinaceous PME inhibitors (PMEIs) in the kiwi extract, as described in detail by Balestrieri et al., affected the determined PME activity in these preparations was not further elucidated (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990; Jolie et al., 2010). Apparently, PME-activity was detected, which indicated no or only a partial inhibition of the PME activity by PMEIs in these preparations. As the aim of this study was to investigate the direct application of crude plant extracts for the modification of pectins, a separation of the PME activity from PMEIs was not further considered (Jolie et al., 2010; Sénéchal et al., 2015). Based on the high protein content and PME activity, PapainPME-Prep1-4 was further investigated for their salt, pH and temperature dependency.

3.2. Salt-dependency of PapainPME activity

To determine the salt-dependency of PapainPME-Prep1-4, all preparations were desalted against MilliQ water using ultrafiltration. As previously reported for the purified CpL-PME, we also did not determine any PME activity if lemon pectin (LR DM70_DA1) was incubated with the desalted preparations (Fig. 2a) (Vasu et al., 2012). Addition of 0.1 M NaCl to the incubation of lemon pectin with the desalted preparations led to a re-activation of the PME activity. All four untreated papain preparations exhibited PME activity towards lemon pectin, which indicated the presence of sufficient amount of NaCl or other ions in these preparations (Fig. 2a and b). The presence of salt-dependent PMEs for all four papain preparations is in agreement with the activity of previously characterized PMEs from papaya (Fayyaz et al., 1995; Lim & Chung, 1993; Lourenco & Catutani, 1984; Vasu et al., 2012). In these studies, the reported NaCl optima of papaya-derived PMEs ranged from 0.1 to 0.3 M NaCl. Notably, PMEs that are active towards pectins in the absence of salt do also exist among the plant kingdom, but are uncommon, as only a limited number of these PMEs have been reported so far, like a tomato or *Valencia* orange PME (Phan, Bo, West, Lycett, & Tucker, 2007; Savary, 2001; Savary et al., 2003). To further determine the temperature dependence of the PME activity in the presence of various NaCl concentrations (0–0.1 M), lemon pectin was incubated with PapainPME-Prep2 at various temperatures. An increase in the NaCl

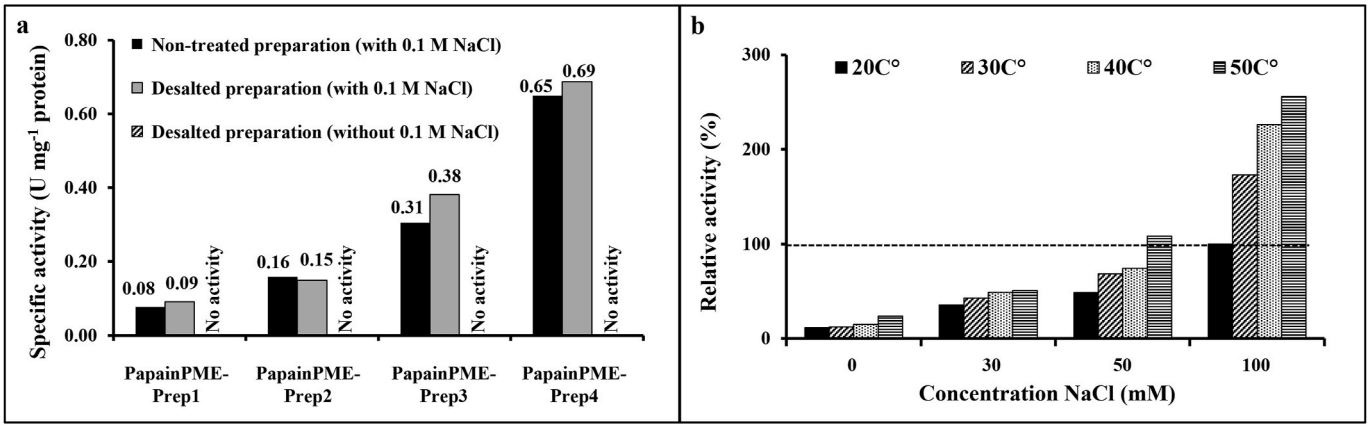

Fig. 2. **a** PME activity of PapainPME-Prep1-4 before and after desalting towards lemon pectin (LR DM70_DA1) in the absence and presence of NaCl (0.1 M). The PME activity (U mg^{-1} protein) was determined at 20 °C and pH 7.0 using pH stat. **b** Determined PME activity of the PapainPME-Prep2 preparation towards lemon pectin (LR DM70_DA1) in the presence of four NaCl concentrations ranging from 0 to 100 mM at 20, 30, 40 and 50 °C. The PME activity is presented relatively to the determined PME activity of lemon pectin incubated with PapainPME-Prep2 in the presence of 100 mM NaCl at 20 °C. PME activity determination has been performed using data points within the linear range of the reaction. Analyses were performed singularly. See "M&M" for details.

concentration led to a steady increase in the PME activity, independent of the applied temperature. Compared to incubations performed in the presence of 0 and 30 mM, a higher NaCl concentrations (50 and 100 mM) led to an approximately 2-fold increase in PME activity at 50 °C compared to the incubation performed at 20 °C (Fig. 2b). Higher NaCl concentrations were not determined in this study, as NaCl concentrations above 0.1 M seem less industrially feasible, based on nutritional aspects and taste perception.

3.3. Temperature and pH-dependency of PapainPME activity

The temperature- and pH-dependent activity of PapainPME-Prep1-4 was assessed at incubation temperatures ranging from 20 to 60 °C (at pH 5.0) and pH values ranging from 3.0 to 8.0 at 20 °C in the presence of 0.1 M NaCl (Fig. 3a and b). All incubations were performed including an appropriate pectin blank to monitor the occurrence of spontaneous demethylesterification. The dosing of the preparations was based on equal amounts of protein rather than intrinsic PME activity, thereby potentially limiting a direct comparison of the absolute PME activity between these preparations.

Consistent with the aforementioned data, the maximum PME activity over the entire pH and temperature range was highest for PapainPME-Prep3 and 4 compared to the lower one of PapainPME-Prep1 and 2, based on protein content. The elevation of the incubation temperature from 20 to 60 °C led to an about 2 to 5-fold increase in PME activity towards lemon pectin (LR DM70_DA1). In contrast, an enhancement of the pH from 5.0 to 8.0 yielded to a much stronger increase (5–38-fold) in the determined PME activity (Fig. 3b). The strong increase in the PME activity at rising pH values was also determined for previously characterized PMEs which were isolated from papaya fruit pulp and latex (Fayyaz, et al., 1994, 1995; Lourenco & Catutani, 1984; Vasu et al., 2012). Based on the activity measured, the temperature- and pH-dependent increase in PME activity was strongest for PapainPME-Prep4 and PapainPME-Prep3, followed by PapainPME-Prep2, whereas the mild increase in activity determined for PapainPME-Prep1 indicated a lower temperature- and pH-dependency.

PapainPME-Prep3-4 comprised a strong pH-dependent PME activity which was close to the one reported by Fayyaz and colleagues and Lourenco and Catutani (Fayyaz et al., 1995; Lourenco & Catutani, 1984). In comparison, the PME activity of PapainPME-Prep1-2 was less pH-dependent, like reported for CpL-PME from papaya (Vasu et al., 2012). This difference in pH dependencies indicated that different ratios of various PMEs isozymes could be present in these four preparations, which was so far not shown for isolated papaya latex PMEs (Vasu et al., 2012). Notably, buffer salts affected the PME activity, as the PME activity (U mg^{-1} protein) of lemon pectin incubated with the PapainPME-Prep1-4 at pH 5.0 and 20 °C using a McIlvaine buffer (Fig. 3b) was lower compared to the PME activity of the same setup using NaOH for pH equilibration (Fig. 3a).

Admittedly, the determination of the temperature and pH dependent PME activity of the four papain preparations at one pH or one temperature value limited the ability to draw more comprehensive conclusions.

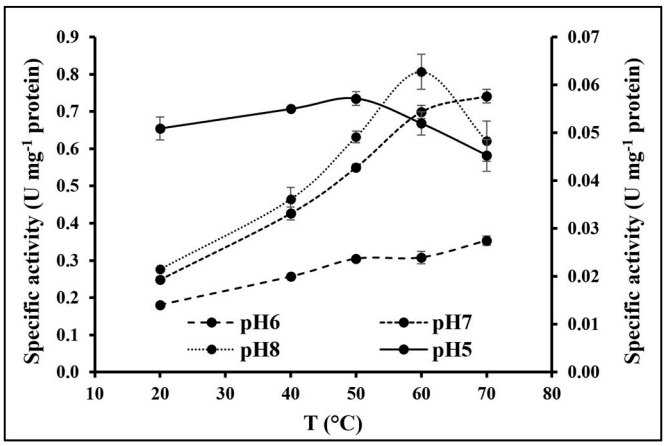

Fig. 4. Temperature and pH-dependent PME activity of PapainPME-Prep2 towards lemon pectin. Lemon pectin (LR DM70_DA1) was incubated with PapainPME-Prep2 at temperatures ranging from 20 °C to 70 °C using four pH values (5.0–7.0) for 1 h. The PME activity (U mg^{-1} protein) was determined using pH stat. The Y-Axis on the left refers to the measured activity at pH 6.0, 7.0 and 8.0, whereas the Y-Axis on the right refers to the measured activity at pH 5.0. The experiments were conducted in duplicate. See "M&M" for details.

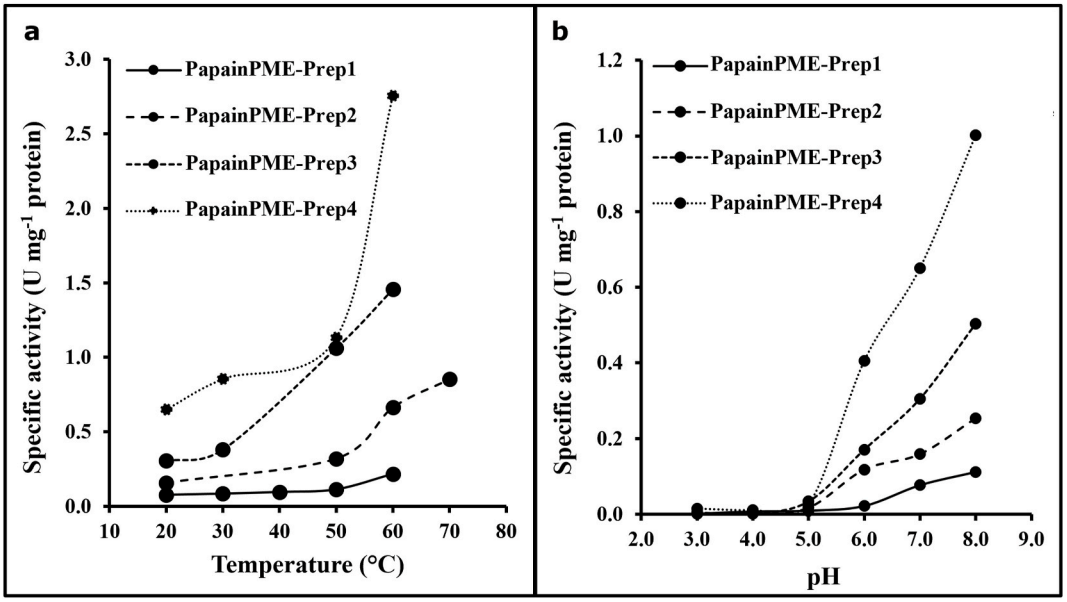

Fig. 3. a Temperature- and **b** pH-dependent PME activity of four papain preparations towards lemon pectin. Lemon pectin (LR DM70_DA1) was incubated with PapainPME-Prep1-4 at temperatures ranging from 20 to 60 °C (at pH 5.0) and pH values ranging from 3.0 to 8.0 at 20 °C for 1 h. The PME activity (U mg^{-1} protein) was determined using pH stat. PME activity determination has been performed using data points within the linear range of the reaction. Analyses were performed singularly. See "M&M" for details.

Therefore, a more detailed assessment of the pH and temperature dependent PME activity was performed by incubating lemon pectin (LR DM70_DA1) with PapainPME-Prep2 (Fig. 4). An elevation of the incubation temperature from 20 to 70 °C altered the PME activity at pH 5.0 and 6.0 to a lower extent compared to the up to 3-fold enhanced activity at pH 7.0 and 8.0. Based on these results, the effect of temperature on the PME activity depends strongly on the pH at which the incubations were performed. According to the evaluation of the thermal stability, the PME activity determined in the four Papain-PME preparations was stable upon pre-incubation at 20–60 °C for 1 h (Fig. 5). Notably, this stability range closely align with the one (30–70 °C) of papaya CpL-PME, which was conducted at pH 7.0 (Vasu et al., 2012). The PME activity of PapainPME-Prep2 drastically declined at pre-incubation temperatures above 60 °C, which is also reflected in the determined rapid decline in PME activity of PapainPME-Prep2 at 70 °C and pH 8 (Fig. 4). The pre-incubation of PapainPME-Prep2 at 80 °C for 1 h led to an activity decrease of 95% towards lemon pectin compared to the activity of the non-pre-incubated preparation.

In summary, the pH and temperature optimum of the PapainPME-Prep1-4 ranged from 7.0 to 8.0 and 50–70 °C, respectively. A temperature treatment above 80 °C is required to enable a full deactivation of the PME activity.

3.4. Batch-to-batch variation

The three batches of PapainPME-Prep2A, 2B and 2C did not differ in their protein content and overall appearance of protein bands using SDS-PAGE (Suppl. Fig. S1). Likely, the determined protein bands primarily corresponded to non-PME proteins, considering previous data demonstrating a 92-fold increase in PME activity of CpL-PME after purification from papaya latex (Vasu et al., 2012). However, the determined PME activity towards lemon pectin (LR DM70_DA1) using pH stat was about 40 and 20% lower for batch 2A and 2B, respectively, compared to batch 2C (Fig. 6a). This difference in PME activity was further investigated in detail by the analysis of released MeOH after the incubation of lemon pectin with the three different batches. In comparison to the incubation of lemon pectin with Batch 2C, 50 and 20% less MeOH was released for the incubation with batch 2A and 2B, respectively (Fig. 6b). Obviously, the protein content of PapainPME-Prep2A, 2B and 2C was consistent, but the PME activity varied to a larger extent which limits the use of the protein content to predict the intrinsic PME activity.

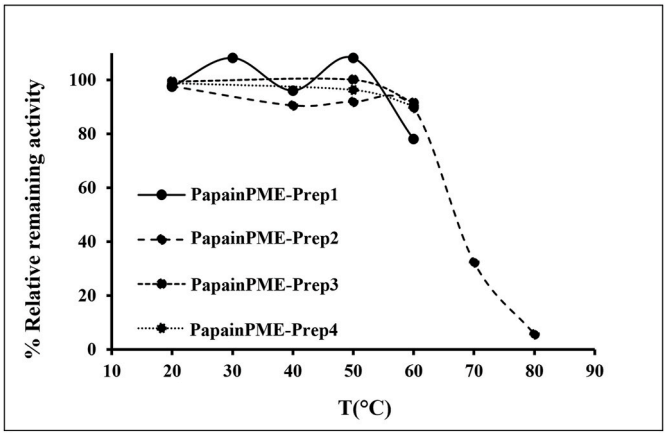

Fig. 5. Thermal stability of the PME activity present in four papain preparations. PapainPME-Prep1-4 were pre-incubated at temperatures ranging from 20 °C to 80 °C at the initial pH (~5.0) for 1 h. Subsequently, the residual enzyme activity was analysed at pH 7.0 and 20 °C using lemon pectin (LR DM70_DA1). The PME activity (U mg^{-1} protein) was determined using pH stat and is represented as remaining relative activity (%) based on the initial activity of the non-treated papain preparations using the same experimental setup and concentrations. PME activity determination has been performed using data points within the linear range of the reaction. Analyses were performed singularly. See "M&M" for details.

Based on batch-to-batch variations of the PME activity, we recommend interpreting the determined PME activity among various papain preparations from different suppliers with caution, like presented in Fig. 1. To define which one of PapainPME-Prep1-4 comprises the highest PME activity, it would be necessary to analyse batch-to-batch variations of each preparation. Next to the protein content, papain preparations are typically standardized based on their proteolytic activity, like tyrosine U (TU), but not based on their PME activity.

To facilitate the application of papain preparations to obtain reproducible results in industrial processes, we suggest quantifying the PME activity of each batch, as the latter cannot be estimated from the determined protein content and proteolytic activity.

3.5. Substrate specificity towards various types of pectin

PapainPME-Prep2 released MeOH from all tested pectins, which included high methylesterified (randomly and blockwise distributed) lemon pectin (LR DM70_DA1 and LB DM70_DA1), low methylesterified (randomly distributed) lemon pectin (LR DM30_DA1), apple pectin (DM55_DA7), moderate methylesterified and moderate acetylated SBP (DM55_DA25), and low methylesterified and low acetylated SBP (DM35_DA13). The achieved relative MeOH release (%) based on the total amount of bound MeOH in these pectins ranged from 26 to 91% after an incubation time of 81 min (Fig. 7a and b).

In comparison to the relative amount of released MeOH from randomly methylesterified lemon pectin (LR DM70_DA1), which was 40 (pH 5.0) and 55% (pH 7.0) after 81 min, PapainPME-Prep2 released about 20% less MeOH from blockwise methylesterified pectins (LB DM70) (Fig. 7a and b; Suppl. Fig. S6). A similar PME specificity was also determined for a *Cs*PME from orange peel (*Citrus sinensis*) and for *At*PME3 and *At*PME31 from *Arabidopsis thaliana* (L'Enfant et al., 2015; Sénéchal et al., 2015). The slightly lower PME activity towards blockwise methylesterified lemon pectin may result from a higher local charge density due to the presence of consecutive non-methylesterified GalA units of the HG backbone, which strongly interacted with positively charged PMEs, assuming that the PMEs present in this preparation have alkaline pIs, as reported for some papain PMEs (Hocq et al., 2023; Lim & Chung, 1993). However, PapainPME-Prep2 released a higher percentage MeOH of the bound MeOH present from relatively low methylesterified pectins, like lemon pectin (LR DM30_DA1) (Fig. 7; Suppl. Fig. S6). As we did not determine the degree of blockiness, we can only speculate if larger blocks of non-methylesterified GalA units were present in the HG backbone of this pectin. In addition, 0.1 M NaCl addition would have decreased such charge interactions. Therefore, this specificity could result from the binding preference of the active site of Papain PMEs, which might be hindered by pectin surfaces comprising larger areas of consecutive methylesterified GalA units (Hocq et al., 2023). It is important to note that in this experiment, the reactions were stopped after 81 min (Fig. 7), resulting in only partial demethylesterification. The latter was observed to be within the linear range of the lemon pectins comprising a DM of 70, while an almost complete demethylesterification of the DM30 pectin was already achieved (Suppl. Fig. S6). The absolute quantity of methylesters present in lemon pectin (LR DM30_DA1) and apple pectin (DM55_DA7) was lower compared to the amount of methylesters in lemon pectin (LR DM70_DA1). Consequently, when using equal amount of protein of PapainPME-Prep2, the time-dependent release of MeOH decreased after 36 min, as about 60–70% of the percentage of methylesters present in lemon pectin (LR DM30_DA1) and apple pectin (DM55_DA7) had already been released. The MeOH release was further studied by incubating various lemon pectins with PapainPME-Prep2 for 300 min. Based on the relative MeOH release, low methylesterified lemon pectin (LR DM30_DA1) was almost completely demethylesterified by PapainPME-Prep2 at both pH 5.0 and 7.0, after an incubation time of 81 min (Suppl. Fig. S6c & d). In contrast,

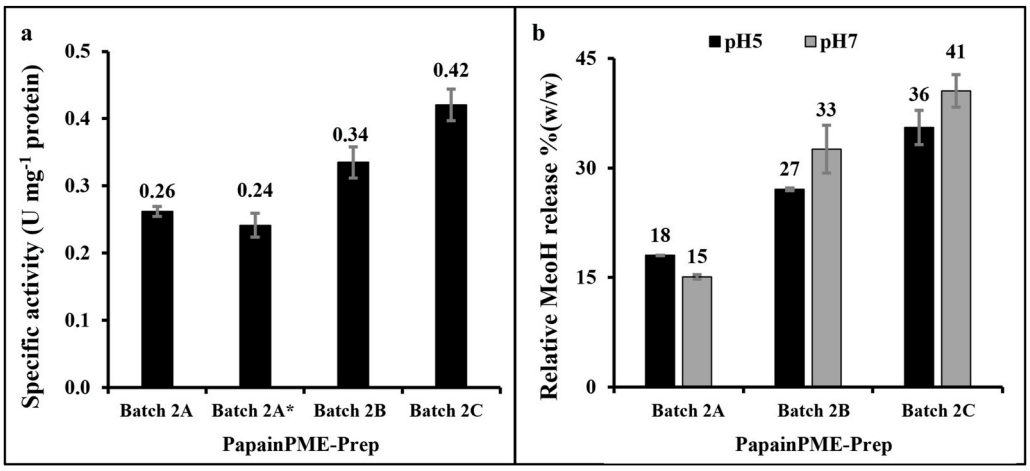

Fig. 6. Batch-to-batch variation of PME activity in PapainPME-Prep2A, 2B and 2C. **a** The PME activity (U mg^{-1} protein) as determined by using pH stat. Lemon pectin (LR DM70_DA1) was incubated with PapainPME-Prep2A, 2B and 2C (1 mg mL^{-1}) using a 10% (w/w) protein per substrate weight at 7.0. **a*** Same Batch 2A, albeit stored at distinct locations for approximately 6 months **b** Relative MeOH release (%) based on the total amount of MeOH present in 100 g of lemon pectin using HS-GC-FID. Lemon pectin (5 mg mL^{-1}) was incubated PapainPME-Prep2A, 2B and 2C using a 5% (w/w) protein per substrate weight at pH 5.0 and 7.0 for 24 min. The experiments were conducted in duplicate. See "M&M" for incubation details.

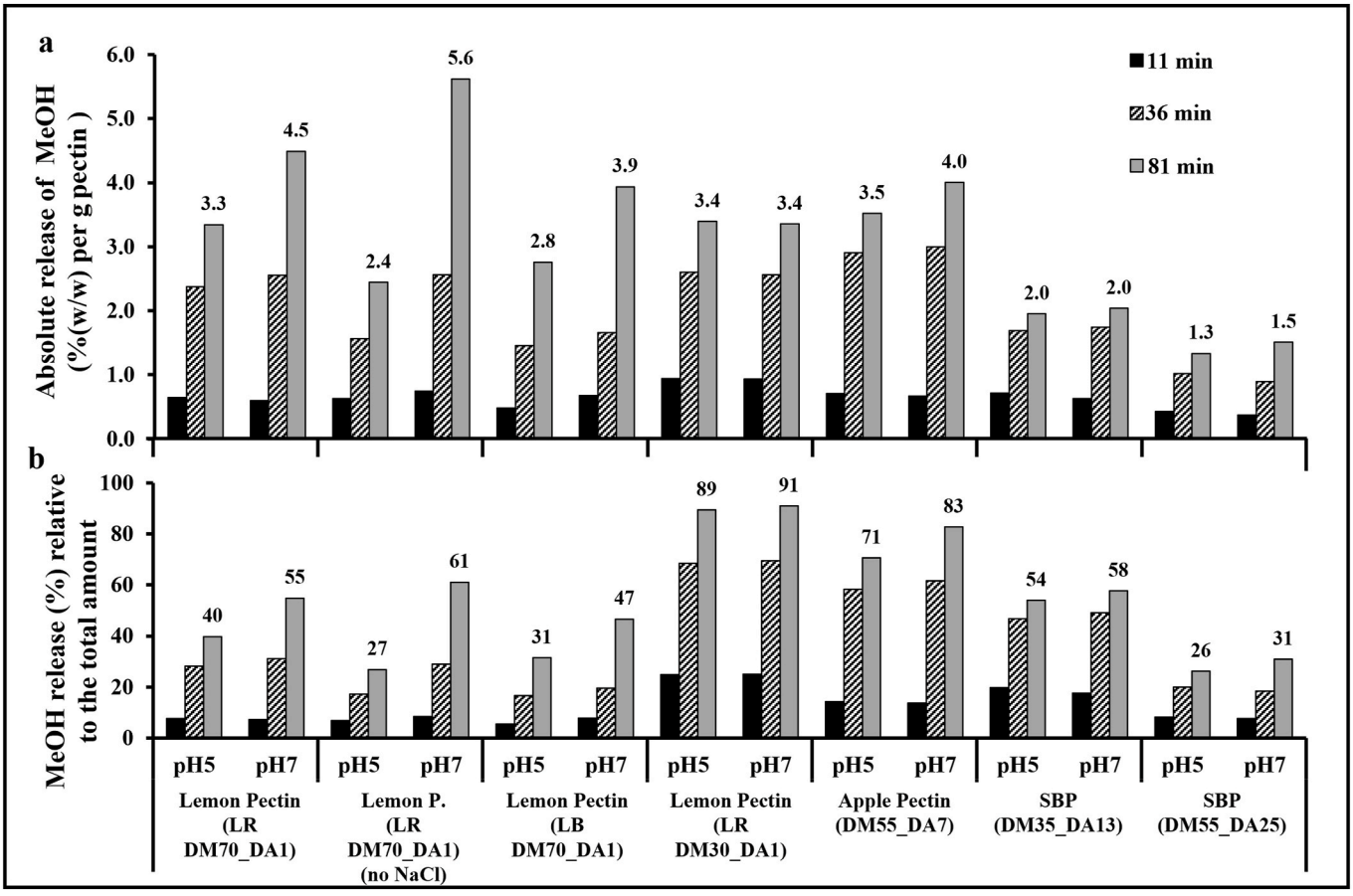

Fig. 7. **a** Absolute and **b** relative release of MeOH from different types of pectin after incubation with PapainPME-Prep2. Pectins (5 mg mL^{-1}) originating from lemon, apple and sugar beet were incubated with 5% (protein per substrate weight (w/w)) PapainPME-Prep2 at 40 °C, pH 5.0 and 7.0, for 11, 36 and 81 min. The MeOH release was determined using HS-GC-FID. **a** Absolute MeOH release expressed as %(w/w) per g of pectin and **b** relative MeOH release (%) based on total amount of MeOH present in 100 g of pectin. The experiments were conducted singularly. See "M&M" for incubation details.

the complete demethylesterification of lemon pectin (LR DM70_DA1) by PapainPME-Prep2 at pH 7.0 was only achieved after 300 min. At the same incubation temperature, only 75% of the total MeOH was released from this pectin at pH 5.0 (Suppl. Fig. S6c & d). The lower PME activity of this preparation towards pectin at pH 5.0 compared to pH 7.0 was already presented in Chapter 3.3.

Obviously, the above described determined PME activity of PapainPME-Prep2 towards lemon pectin using pH stat analyses differed

from the rates of MeOH release from lemon pectin which was analysed by HS-GC-FID. As mentioned in Chapter 3.3, these differences may have resulted from variations of the experimental setup, as 1 mg mL^{-1} pectin was dissolved in MQ containing 0.1 M NaCl for pH stat, whereas 5 mg mL^{-1} pectin was dissolved in 0.15/0.18 M McIlvaine buffer containing 0.1 M NaCl for the HS-GC-FID method.

PapainPME-Prep2 released MeOH from both SBPs, but exhibited a lower activity towards highly acetylated SBP (DM55_DA25) compared to low acetylated SBP (DM35_DA13) (Oosterveld, Beldman, Searle-van Leeuwen, & Voragen, 2000; Remoroza, Wagenknecht, et al., 2014). In brief, PapainPME-Prep2 removed 54 and 58% of the total MeOH from SBP DM35_DA13 at pH 5.0 and 7.0, respectively, after an incubation time of 81 min. In contrast, only 26 and 31% of the total MeOH was removed from SBP (DM55_DA25) at pH 5.0 and 7.0, respectively. The disparity observed in the MeOH release indicated that the PME activity of PapainPME-Prep2 may be more influenced by the quantity of acetylesters bound to the galacturonic acid backbone of SBP, rather than being dependent on the pH conditions.

In conclusion, PapainPME-Prep2 exhibited a high PME activity towards high and low methylesterified pectins. Furthermore, PapainPME-Prep2 exhibited a broad specificity towards both randomly (LR DM70_DA1) and blockwise (LB DM70) methylesterified lemon pectins. It remains a subject of debate whether the potential presence of several papain PMEs in PapainPME-Prep2 could have contributed to a diverse substrate specificity, resulting in the release of MeOH from lemon, apple, and sugar beet pectins. Based on an industrial applicational point of view, this broad specificity is seen as an advantage, as it enables a modification of a diversity of pectins comprising various methylesterification patterns.

3.6. Acetyl esterase activity

An initial experimental setup demonstrated the liberation of MeOH from SPB by both plant and fungal preparations (Suppl. Fig. S5). Therefore, we further determined the presence of acetyl esterases in PapainPME-Prep1 and 2, as well as FungalPME-Prep1. The incubation of SBP (SBP DM35_DA13) with PapainPME-Prep1, PapainPME-Prep2 and FungalPME-Prep1 at pH 5.0 and 7.0 led to an acetic acid release of 9–10, 12–14 and 21–42%, respectively, based on the total acetic acid amount present as acetyl groups bound to this SBP (Suppl. Table S2). The latter results raised the question if the PME activity towards SBP comprising a high DA (SBP DM55_DA25) was either enabled or enhanced by the presence of the intrinsic acetyl esterase activity in PapainPME-Prep2. As the determined deesterification activity was rather low, a purification of these preparations would be recommended to determine the relevance of the acetyl esterase activity for the PME activity. Furthermore, the latter approach enables the isolation and characterization of the enzyme (s) that can release acetic acid from HG and RG-I, like pectin acetylesterases and rhamnogalacturonan acetylesterases (Carpita & Gibeaut, 1993; Oosterveld, Beldman, Schols, & Voragen, 2000; Remoroza, 2014; Remoroza, Broxterman, et al., 2014; Schols, Posthumus, & Voragen, 1990).

In comparison, BromelainPME-Prep1, BromelainPME-Prep2, KiwiPME-Prep1, and PapainPME-Prep3 did not comprise any acetyl esterase activity (Suppl. Table S2).

3.7. Determination of the mode of action using enzymatic fingerprinting

3.7.1. Partial demethylesterification

As stated in Chapter 3.1, the mode of action of PapainPME-Prep2 was of mainly investigated and compared to the one of PapainPME-Prep1 and FungalPME-Prep1. Therefore lemon pectin (LR DM70_DA1) was incubated with predefined amounts of these PME preparations, which were calculated based on measured PME activities using pH stat at pH 5.0 and 7.0. We aimed at obtaining lemon pectin fractions comprising DM ranges from 65 to 45. It is noteworthy that the initial DM of lemon pectin was already 63, which was lower to the previous determination by (Daas, et al., 1999, 2000) (Suppl. Table S3). The subsequent partial demethylesterification using the PME preparations resulted in fractions with a limited DM, specifically ranging only from 32 to 48. Nevertheless, our objective was to investigate the mode of action of the PME preparations at pH 5.0 and 7.0, and to compare it to the mode of action of FungalPME-Prep1. To achieve this, we conducted incubation experiments using partially demethylesterified lemon pectin fractions with an *endo*-PG derived from *K. fragilis*, following a previously established protocol (Daas et al., 2000; Jermendi et al., 2022).

3.7.2. HPSEC-RI analyses of endo-PG digested PME-demethylesterified pectins

The deesterification of lemon pectin alone using the PME-containing preparations did not lower the M_w distribution, based on the HPSEC elution profiles (Fig. 8; Suppl. Figs. S7 and S8). The elution profiles of all incubations shared the presence of a large peak eluting around 14–15 min that corresponded to the presence of small M_w compounds and likely originated from the NaCl addition (0.1 M). As a consequence of the limited DM range (32–48), no observable impact of individual DM variations of the fungal or plant PME demethylesterified lemon pectin fractions was determined when comparing HPSEC elution profiles of *endo*-PG digests (Suppl. Table S3).

The *endo*-PG digest of the FungalPME-Prep1 treated lemon pectin at pH 5.0 comprised a larger pectin fraction having a medium M_w which eluted between 11 and 13 min (Fig. 8a). The determined M_w distribution pattern is typical for an *endo*-PG digest of randomly methylesterified pectins, as the lower presence of adjacent non-methylesterified GalA units on the pectin backbone limits the release of small M_w compounds, like mono-, di-, tri-GalA units by the *endo*-PG, which requires at least four consecutive non-methylesterified GalA residues for its activity (Daas et al., 1999; Pasculli, Voragen, & Pilnik, 1991). The elution profile of the *endo*-PG digests of PapainPME-Prep1 and 2 treated lemon pectin showed the presence of a large M_w fraction (9–11 min), which resembled the one of *endo*-PG digested lemon pectin without prior PME incubation (Fig. 8c and e; Suppl. Figs. S7 and S8). In comparison to the FungalPME-Prep1 treated pectin fractions, the *endo*-PG-digests of PapainPME-Prep1 and 2 treated pectin fractions comprised lower quantities of small M_w compounds eluting from 12 to 13.5 min, which indicated that *endo*-PG cleaved larger blocks of consecutive non-methylesterified GalA units releasing mono- or smaller oligomers. The elution profile of this fraction between 12 and 13.5 min was almost identical for *endo*-PG digests of PapainPME-Prep1 and 2 treated lemon pectin at pH 7.0 (Suppl. Fig. S8b &8d), whereas there was discernible difference in the intensity of this fraction treated at pH 5.0 (Suppl. Fig. S8a &8c). This observation provides compelling evidence that the mode of action of the PMEs present in these papain preparations is pH dependent (Hocq et al., 2023; Kim et al., 2013).

3.7.3. HPAEC-PAD analyses of PG digested PME-demethylesterified pectins

The based on HPSEC determined medium M_w compounds in the *endo*-PG digest of lemon pectin treated with FungalPME-Prep1, which eluted between 11 and 13 min, corresponded to high amounts of released oligo-GalA units (DP > 3) that were identified by HPAEC-PAD (Fig. 8b; Suppl. Fig. S9). More precisely, 60% of the released GalA units were oligomers comprising a DP > 3, whereas only 40% were released as mono-, di-, or trimers (Fig. 9a & b). The latter ratio was similar compared to the determined release of mono- and oligomers in the *endo*-PG digest of the lemon pectin alone, which was not partially demethylesterified. Therefore, we concluded that FungalPME-Prep1 comprised a typical PME activity which exhibited a multiple-chain mechanism leading to a random methylester distribution that did not enhance the accessibility of these partially demethylesterified pectin fractions for the *endo*-PG (Duvetter et al., 2006; Ishii et al., 1979).

In comparison, GalA units released as mono-, di- and trimers in the *endo*-PG digest of lemon pectin treated with PapainPME-Prep1 and 2

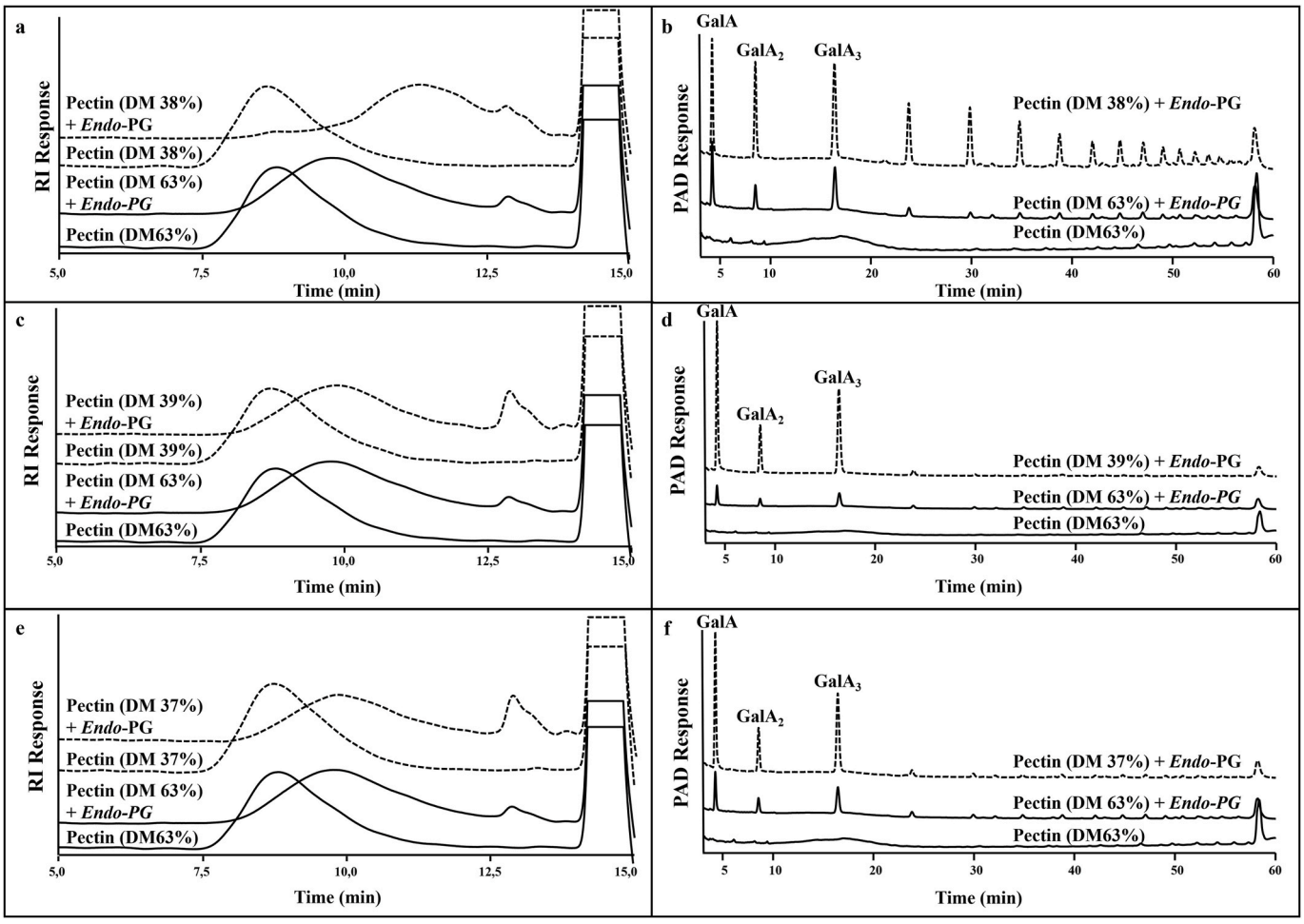

Fig. 8. **a, c** & **e** HPSEC-RI and **b, d** & **f** HPAEC-PAD elution pattern of lemon pectin before and after *endo*-PG digestion of lemon pectins having different DM values. The pectins have been obtained by incubating lemon pectin (LR DM70_DA1) with either **a** & **b** FungalPME-Prep1 at pH 5.0, **c** & **d** PapainPME-Prep1 or **e** & **f** PapainPME-Prep2 at pH 7.0 under defined conditions (Suppl. Table S3). See "M&M" for details.

ranged from 60 to 95%, compared to relatively low amounts of released longer GalA units (DP > 3) (Fig. 8d and f, 9b; Suppl. Fig. S10). The latter profile of released GalA units in the *endo*-PG digest indicated the presence of long(er) demethylesterified GalA blocks in the pectin backbone after incubation with PapainPME-Prep1 and 2, which is a typical result of PMEs exhibiting a processive mode of action as described for many plant PMEs (Duvetter et al., 2006; Hocq et al., 2023).

The *endo*-PG digest of lemon pectin which was treated with PapainPME-Prep2 at pH 5.0 revealed a lower activity of the *endo*-PG itself, as indicated by the lower amount of released GalA units, compared to the one treated at pH 7.0 (Fig. 9a). In addition, higher ratios of released longer GalA oligomers (DP > 3) to mono-, di-, trimers were determined in the *endo*-PG digests of PapainPME-Prep2-modified pectin fractions at pH 5.0 compared to the digests of modified fractions at pH 7.0, which indicated a pH-dependency of this preparation, as reported for other plant PMEs (Denès, Baron, Renard, Péan, & Drilleau, 2000; Hocq et al., 2023). Whether the observed pH dependency is attributed to the presence of different PME isoforms in PapainPME-Prep2 remains to be demonstrated, as only CpL-PME has been isolated from a commercial papaya latex preparation thus far (Vasu et al., 2012). Still, other PMEs have already been isolated and characterized in papaya fruit flesh and pulp after peeling (Fayyaz et al., 1994; Lim & Chung, 1993; Lourenco & Catutani, 1984).

Compared to the *endo*-PG digests of PapainPME-Prep2, we did not determine such an impact of the pH on released GalA ratios of longer GalA oligomers (DP > 3) to mono-, di-, trimers in the *endo*-PG digests of PapainPME-Prep1 modified pectin fractions at pH 5.0 and 7.0. At pH 7.0, the mode of action of both PapainPME-Prep1 and 2 was the same, as the ratios of released longer GalA units (DP > 3) to mono-, di-, trimers determined in the *endo*-PG digests did not differ. Both preparations were sourced from distinct suppliers and variations in the mode of action of the PME could be attributed to different factors, such as variations in the extraction method or the origin of the raw material. As above mentioned, the presence of different PME isoforms in these papaya latex preparation has yet to be demonstrated (Vasu et al., 2012).

Based on the obtained results we would recommend to use purified PMEs from papain preparations and partially demethylesterified pectin comprising DM values ranging from 30 up to 80 (Ngouémazong et al., 2011) to reveal whether papain PMEs exhibit either only a single chain mechanism or also a multiple attack mechanism (Cameron et al., 2011; Grasdalen et al., 1996; Hotchkiss et al., 2002; Savary et al., 2003). Furthermore, a recently published HPSEC-MS-based method has been described, which offers enhanced sensitivity for profiling of (non) methylesterified GalA units in *endo*-PG digests (Hocq et al., 2020; Voxeur et al., 2019). This method holds promise for further investigating the mechanism of action of purified papain PMEs of the preparation used in this work, which likely comprise CpL-PME that has been previously reported (Luzio et al., 2002). The detection of both non and methylesterified GalA units until DP16 and will provide valuable insights into the mode of action of the *endo*-PG itself, distinguishing between possessive and non-processive PG activity. An alternative approach for improved detection of non-methylesterified and methylesterified GalA

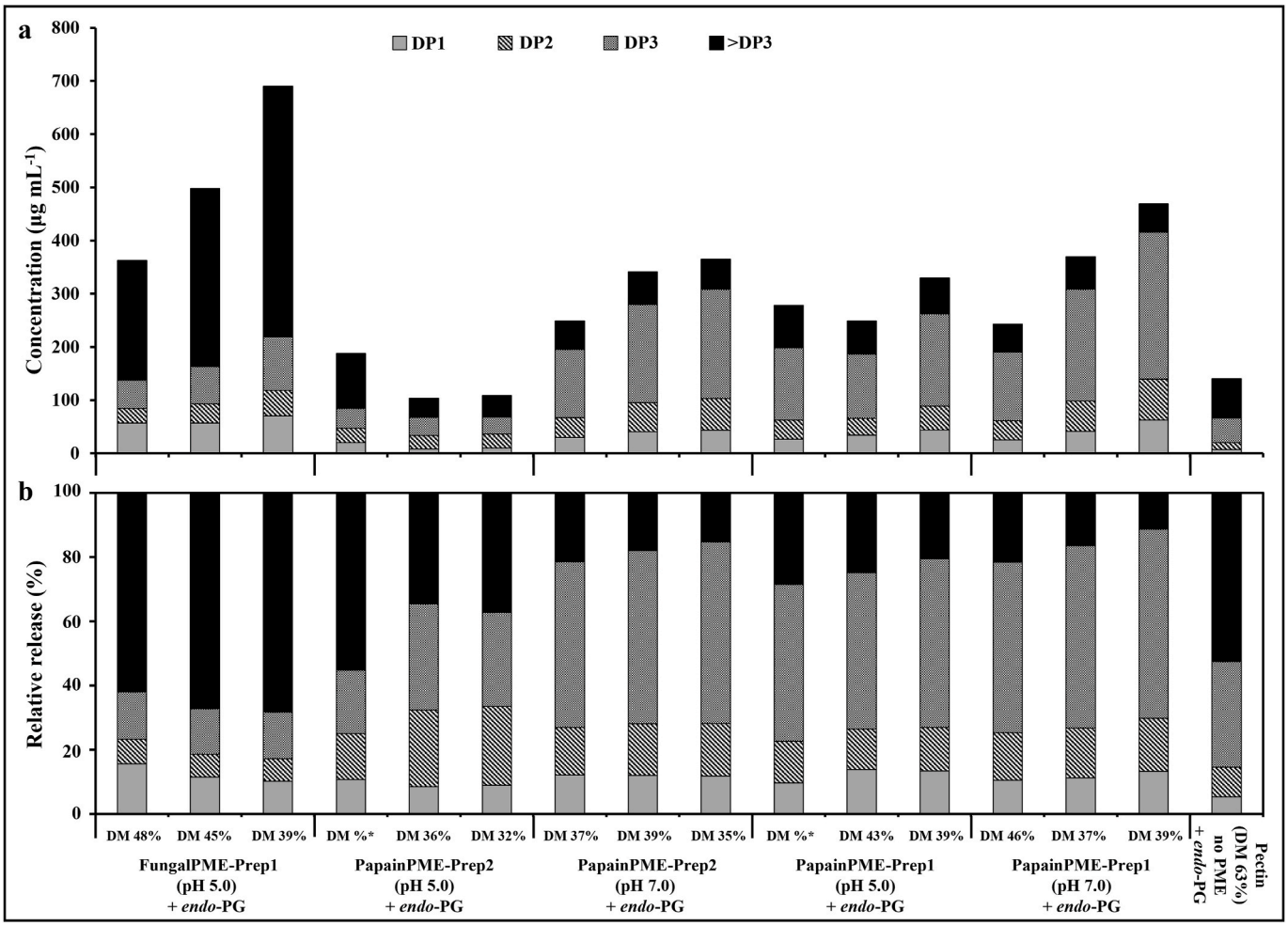

Fig. 9. **a** Absolute and **b** relative amounts of GalA units released as mono- or oligomers after *endo*-PG digestion of lemon pectins having different DM values. These pectins have been obtained by incubating lemon pectin (LR DM70_DA1) with either FungalPME-Prep1, PapainPME-Prep1 or PapainPME-Prep2 at pH 5.0 and 7.0 or without a PME at pH 7.0 under defined conditions (Suppl. Table S3). The total amount of GalA released as mono- or oligomers thereof was set to 100% based on the quantified amounts using HPAEC-PAD/UV (Suppl. Fig. S9 and S10). *Due to the small sample size of partially demethylesterified lemon pectin fraction, the DM was not further determined. See "M&M" for details.

units after *endo*-PG treatment involved the combination of SEC fractionation followed by MALDI-TOF-MS analyses (Ralet et al., 2012) or using HPAEC-ELSD for DP40 to 50 (Cameron, Kim, Galant, Luzio, & Tzen, 2015). An assessment at varying pH values would provide additional insights into pH-dependency of these PMEs. In accordance with the previously published data, employing the *endo*-PG digest method, we were able to discriminate between different mode of actions of two commercial papain preparations and a fungal PME via analyzing the methylester distribution pattern using HPSEC and HPAEC.

4. Conclusions

In this study, we investigated if commercial crude plant extracts comprising PMEs can be applied to modify pectins via de-esterification and alterations of the methylester distribution pattern.

Four papain preparations (PapainPME-Prep1-4) from three different suppliers, including the three batches of PapainPME-Prep2A, 2B and 2C, and preparations that originated from pineapple (bromelain) and kiwi (actinidin) were included in this assessment. The protein content based on dry matter of these preparations varied vastly and ranged from 2 to 93% (w/w) and was highest for PapainPME-Prep2 and 3 (90–93% (w/w)). The highest PME and acetylesterase activity towards pectin was determined in papain preparations, which were chosen for a more detailed assessment. The PME activity of these preparations was stable and not affected by auto-proteolysis. Moreover, the papain preparation did not contain any pectin-degrading side activity.

The protein content was constant among three PapainPME-Prep2 batches, whereas the PME activity varied up to 40%. The determined PME activity of the papain preparations showed a strong pH dependency and was highest between pH 7.0 to 8.0. Preincubation from 20 °C to 80 °C at the initial pH (~5.0) for 1 h showed that the PME activity remained stable until 60 °C. All papain preparations contained salt-dependent PMEs.

PapainPME-Prep2 had a broad substrate specificity, as up to 91, 83 and 58% of the bound MeOH was released from lemon, apple and sugar beet pectin comprising various DMs and DAs, respectively. The release of acetic acid after the incubation of SBP with PapainPME-Prep1 and 2 indicated the presence of acetyl esterases. Based on enzymatic fingerprinting, it was shown that PMEs present in PapainPME-Prep1 and 2 exhibited a processive mode of action towards lemon pectin. In contrast, the Fungal-PME-Prep1 comprised a typical PME activity which exhibits a multiple-chain mechanism leading to a random methylester distribution pattern.

The determined mode of action and the absence of pectin-degrading side activities enables the application of crude plant extracts for the modification of pectins that are used as ingredients or are a part of pectin-rich food matrixes, such as fruit and vegetable puree-containing products. In the presence of divalent metal ions, like calcium, and

introducing blockwise methylesterification pattern using papain PMEs may improve industrially relevant techno-functional properties of pectin leading to an improved gelation and texturization.

CRediT authorship contribution statement

Matthias Frommhagen: Writing – original draft, Visualization, Validation, Project administration, Methodology, Conceptualization. **Natalia Hutnik:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation. **Henk A. Schols:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110714.

Data availability

The data that has been used is confidential.

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