Carbohydrate Polymers 115 (2015) 540–550

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Mode of action of *Bacillus licheniformis* pectin methylesterase on highly methylesterified and acetylated pectins

Connie Remoroza[a,1], Martin Wagenknecht[b,1], Hans Christian Buchholt[c], Bruno M. Moerschbacher[b], Harry Gruppen[a], Henk A. Schols[a,*]

[a] *Wageningen University, Laboratory of Food Chemistry, Postbus 17, 6700 AA Wageningen, The Netherlands*
[b] *Westphalian Wilhelms—University of Münster, Department of Plant Biology and Biotechnology, Schlossplatz 8, 48143 Münster, Germany*
[c] *DuPont Nutrition Biosciences ApS, Edwin Rahrsvej 38, DK-8220 Brabrand, Denmark*

ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 25 June 2014
Accepted 1 September 2014
Available online 19 September 2014

Keywords:
HILIC–MS/ELSD
Fingerprinting
Blockwise
De-methylesterification
Enzymology

ABSTRACT

A gene encoding a putative pectinesterase from *Bacillus licheniformis* DSM13 was cloned and expressed in *Escherichia coli*. The resulting recombinant enzyme (*Bli*PME) was purified and characterized as a pectin methylesterase. The enzyme showed maximum activity at pH 8.0 and 50 °C. *Bli*PME is able to release up to 100% of the methylesters from lime pectin (DM 34–76 → DM 0) and up to 73% of all methylesters from SBPs (DM 30–73 → DM 14). *Bli*PME efficiently de-methylesterifies lemon pectins and SBPs in a blockwise manner and is quite tolerant towards the acetyl groups present within the SBPs. Detailed analysis of the *Bli*PME-modified pectins using HILIC–MSn and the classical calcium reactivity measurement showed that the enzyme generates pectins with low methylesterification (lime and SBP) and high acetyl content (SBP) while creating blocks of nonmethylesterified galacturonic acid residues. The high activity of *Bli*PME towards highly methylesterified and acetylated pectins makes this novel esterase more efficient in removing methylesters from highly esterified beet pectin compared to other PMEs, e.g. *Aspergillus niger* PME.

1. Introduction

Due to the commercial interest of pectin as gelling and thickening agent for the food industry, pectin is extracted on an industrial scale from by-products. Pectic substances are predominantly present in citrus peel, apple pomace, sugar beet pulp and sunflower by-products, which are seen as sources of a low cost raw material for the pectin industry (May, 1990). The main structural elements in pectin are homogalacturonan (HG) and rhamnogalacturonan I (RG-I). The HG consists of galacturonic acid (GalA) residues, which can be methylesterified at the C-6 of the GalA unit. The RG-I region has repeating units of α-1,4-linked D-galacturonosyl-α-1,2-L-rhamnose and the rhamnose units may be substituted with neutral side chains (Voragen, Coenen, Verhoef, & Schols, 2009). The GalA unit in both RG-I and HG can be acetylated at positions *O*-2 and/or *O*-3 (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). The degree of methylesterification (DM) and degree of acetylation (DA) as well as the distribution of these esters along the pectin backbone are major factors influencing the functional properties of pectin (Willats, Knox, & Mikkelsen, 2006).

Commercially extracted pectin (DM ~80) can be lowered in methylesterification by alkali de-methylesterification (May, 1990). However, alkali treatment could affect the molecular weight of pectin due to depolymerization by β-elimination of the pectin backbone (Renard & Thibault, 1996). Chemical de-methylesterification is considered to remove methylesters and acetyl groups randomly (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004). Enzymatic de-methylesterification of SBP by pectin methylesterases (PMEs) after deacetylation by pectin acetylesterases (PAEs) could create blocks of nonmethylesterified GalA residues and thereby enhance the gelling properties in the presence of Ca^{2+} ions. However, *Aspergillus niger* PME was only efficient in removing methylesters in acetylated pectin when combined with *A. niger* PAE (Oosterveld, Beldman, Searle-Van Leeuwen, & Voragen, 2000). The use of pure enzymes to tailor pectin's esterification has the advantage that depolymerization is avoided.

PMEs (EC: 3.1.1.11) are a well-studied group of enzymes, which belong to carbohydrate esterase (CE) family 8 (CAZy database, www.cazy.org). They catalyze the removal of methylesters at the C-6 position of the GalA residues (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010) and create random or blockwise

* Corresponding author. Tel.: +31 317 482239.
E-mail address: henk.schols@wur.nl (H.A. Schols).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.carbpol.2014.09.016
0144-8617/

Table 1
Chemical characteristics of pectin samples used in this study.

Mother pectin	De-esterification method	Pectin	GalA	Rha (%)[a]	Ara	Gal	DM (%)[b]	DA (%)[b]
Lime pectin								
Grinsted™		E 8100	88	1	0.3	4	81	0
Pectin URS								
	p-PME	P5300	90	1	0.3	4	53	0
	f-PME	F7600	90	2	0.3	3	76	0
		F5800	88	1	0.3	3	58	0
	Alkali	B3400	92	1	0.3	2	34	0
Lime pectin[c]		A6100	75	1	1	5	61	0
Sugar beet pectin								
SBP6230		SBP6230	59	5	12	10	62	30
	p-PME	P5328	58	5	11	10	53	28
	f-PME	F5129	59	5	12	9	51	29
	Alkali	B3124	55	6	12	10	31	24
		E7329	58	5	8	9	73	29

[a] Monosaccharide composition in % (w/w) (Buchholt et al., 2004; Ralet et al., 2001).
[b] Moles methanol (DM) or acetyl groups (DA) per 100 mol of galacturonic acid.
[c] Monosaccharide composition determined in this study.

patterns of methylesterification in the HG region of pectin. At alkaline conditions (pH 7.0–9.0), PMEs derived from plants generally de-methylesterify the HG region of pectin in a processive way, also termed blockwise or single-chain manner (Micheli, 2001). Fungal PMEs, e.g. from *Aspergillus*, de-methylesterify pectin in a random manner (Kim et al., 2013; Limberg et al., 2000a) whereas a bacterial PME from *Erwinia chrysanthemi* (PemA) de-methylesterifies pectin-derived oligosaccharides in a blockwise manner (Fries, Ihrig, Brocklehurst, Shevchik, & Pickersgill, 2007). Previous studies have shown that plant PME (*Citrus sinensis*) and fungal PME (*Aspergillus*) are hindered by the presence of acetyl groups in acetylated SBP (Buchholt et al., 2004; Duvetter et al., 2006), whereas the bacterial PME from *E. chrysanthemi* never has been characterized towards various acetylated and non-acetylated pectins. Consequently, there is a considerable interest in the application of PMEs with broad substrate specificities, able to process pectin-rich materials and quite tolerant for the presence of the acetyl groups.

Bacillus licheniformis DSM13 is a significant source for a multitude of biotechnologically important enzymes (Veith et al., 2004). In the databases, several (putative) pectinesterases and PMEs are listed for strain DSM13 and other strains of *B. licheniformis*. However, for the majority of these enzymes experimental data is lacking and a PME, however, has not yet been characterized at all for this species. In the present work, production, purification and characterization of *B. licheniformis* DSM13 pectin methylesterase (*Bli*PME) were carried out. The purified enzyme was tested on acetylated and non-acetylated pectins with different methylesterification. *Bli*PME-modified pectins were analyzed by enzymatic fingerprinting in combination with HPLC–HILIC–ESI–MSn. The tolerance of *Bli*PME towards the presence of acetyl groups was compared with the well characterized *A. niger* PME.

2. Materials and method

2.1. Substrates

Pectins originated from lime pectin, (Mother pectin Grinsted™ Pectin URS 1200: E8100, DM 81, DA 0) and SBP (DM 62, DA 30) were de-esterified by alkali, plant or fungal PMEs by Dupont (Brabrand, Denmark). Esterification of SBP under methanol in acidic solution converted SBP6230 into E7329 pectin (Table 1). The chemical properties of the different have been published elsewhere (Buchholt et al., 2004; Ralet, Dronnet, Buchholt, & Thibault, 2001) and are also given in Table 1. Methylesterified (saturated and unsaturated) GalA oligomers with degree of polymerization (DP) 2–6 were purified from endo-polygalacturonase II (endo-PGII) and pectin lyase (PL) digests of lime pectin (DM 70 DA 0) as described previously (Van Alebeek, Zabotina, Beldman, Schols, & Voragen, 2000).

2.2. Enzymes

Purified and well characterized RG-I and HG degrading enzymes were used to hydrolyse sugar beet pectins. The enzymes used in this study were *Aspergillus aculeatus* endo-galactanase (EC 3.2.1.89) (Schols, Posthumus, & Voragen, 1990), endo-arabinanase (EC 3.2.1.99) (Beldman, Searle-van Leeuwen, De Ruiter, Siliha, & Voragen, 1993), RG-hydrolase (EC 3.2.1.B9) (Mutter, Renard, Beldman, Schols, & Voragen, 1998), *Chrysosporium lucknowense* (C1) exo-arabinase (EC 3.2.1.1) (Kühnel et al., 2010), *A. niger* fungal pectin methyl esterase (fungal PME) (EC 3.1.1.11) (Van Alebeek, Van Scherpenzeel, Beldman, Schols, & Voragen, 2003), pectin lyase (EC 4.2.2.10) (Harmsen, Kusters-van Someren, & Visser, 1990) and endo-polygalacturonase II (EC 3.2.1.15) (Limberg et al., 2000b). Purified fungal PME from *A. niger* (*Ani*PME) (Van Alebeek et al., 2003) was used as a comparison to *Bli*PME.

2.3. Amino acid sequence analysis

Homology searches were done using BLAST and databases provided by the NCBI (www.ncbi.nlm.nih.gov). Conserved protein domains were identified using CD-Search, likewise provided by the NCBI. Identification of conserved domains was done also using Pfam (Punta et al., 2012). For the compilation of multiple alignments and the calculation of identity values ClustalW2 (Larkin et al., 2007) was used. GeneDoc (Nicholas, Nicholas, & Deerfield, 1997) was employed for alignment editing and illustration. Secondary structure prediction was done using PredictProtein (Rost, Yachdav, & Liu, 2004), and SignalP (Petersen, Brunak, von Heijne, & Nielsen, 2011) and TatP (Bendtsen, Kiemer, Fausbøll, & Brunak, 2005) were employed for signal peptide predictions.

2.4. Production and purification of BliPME

2.4.1. Bacterial strains, media, and growth conditions

B. licheniformis DSM13 and *Escherichia coli* strains NEB 5-alpha (New England Biolabs, Frankfurt a. Main, Germany) and Rosetta 2(DE3)(pLysSRARE2) (Merck, Darmstadt, Germany) were cultivated under conditions specified (Remoroza et al., 2014b).

2.4.2. Cloning of the BliPME coding sequence

Template preparation, PCR amplification of the *Bli*PME open reading frame (ORF; GenBank accession number (acc no.)

AAU42325) using primers 5′-GCAATTCCATATGGTTAATCGGGAGGCAGCTG-3′ (forward) and 5′-CCCGAGCTCTGATTCAACTTTAGGATTCCAGCC-3′ (reverse) and cloning of the ORF were done as described (Remoroza et al., 2014b). The *Bli*PME expression plasmid generated was termed pET22b-*Bli*PME-StrepIIc.

2.4.3. Synthesis and purification of BliPME

E. coli Rosetta 2 (DE3) (pLysSRARE2) was transformed with pET22b-*Bli*PME-StrepIIc and cultivated in 500 ml auto-induction medium (Studier, 2005) supplemented with ampicillin and chloramphenicol. Synthesis conditions and details of the purification process can be found elsewhere (Remoroza et al., 2014b). Other than described there, additional induction using isopropyl-β-D-thiogalactopyranoside was not applied.

2.5. Characterization of BliPME

2.5.1. Protein analysis

Determination of protein contents, SDS-PAGE, staining of gels, Western blot and signal imaging were done as described elsewhere (Remoroza et al., 2014b).

2.5.2. Determination of DM

Pectin solution (≈2 mg/ml) was saponified in 100 mM NaOH to determine the degree of methylesterification (DM) using a colorimetric method, in which the methanol in the solution is oxidized to formaldehyde with 2,4-pentanedione to the coloured product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (Wood & Siddiqui, 1971). This coloured product was analyzed spectrophotometrically at 410 nm as previously described (Guillotin, Van Loey, Boulenguer, Schols, & Voragen, 2007).

2.5.3. Measurement of BliPME activity by titration

The PME activity was determined using a thermostated autotitration system (pH-stat, 719 S Titrino, Metrohm, Herisau, Switzerland) as previously described (Remoroza et al., 2014b). Pectin solution (5 mg/ml) was maintained at 40 °C, at pH 6.5 (*Bli*PME) and pH 4.7 (*Ani*PME) by the continuous addition of 25 mM NaOH for 10 min. All reaction mixtures were supplemented by NaCl of 100 mM (final concentration) for PME stability and activity enhancement. Enzymes were added at a dose of 0.06% (w/w on protein-substrate basis). PME activity was measured in nanokatal (nkat). One nanokatal is defined as the amount of enzyme required to release methanol equivalent to 1 nmol NaOH per second under the assay conditions defined.

2.5.4. Determination of optimum temperature and pH

Temperature and pH optima were determined using SBP as substrate. SBP E7329 (Table 1) was dissolved (5 mg/ml) in 50 mM McIlvaine's buffers of different pH. Enzyme was added at a dose of 0.06% (w/w on protein-substrate basis). The pH optimum was determined in a pH range 3.0–9.0 at 40 °C and for 10 min incubation time. Using the same substrate, the temperature optimum was determined within the temperature range 30–80 °C at pH 6.5 for 10 min. The reaction was stopped by heating at 100 °C for 6 min. The methanol released was analyzed spectrophotometrically using the colorimetric method described previously (Guillotin et al., 2007). PME activities were calculated relative to the activity at optimal conditions. The background level for the spontaneous de-esterification of E7329 (control) was subtracted for all the data points. After 10 min at pH 8.0–9.0 incubation, about 2% of the total methylesters present was released.

2.5.5. Enzymatic de-methylesterification of pectins

Pectin solution (5 mg/ml) containing 100 mM NaCl was maintained at 40 °C and pH 6.5 by the continuous addition of 25 mM NaOH using pH stat titration. Lime and sugar beet pectins having different DM values were treated with *Bli*PME at a dose of 0.06% (w/w on protein-substrate basis). The incubation was performed for 10 min, 30 min and 24 h. Blanks without enzyme addition were included as references. The reaction was stopped by heating at 100 °C for 6 min. Subsequently, the *Bli*PME-modified and reference pectins were dialyzed against demineralized water overnight, followed by freeze-drying. Methylesterified lime pectin oligomers (5 mg/ml) were digested by *Bli*PME at a dose of 0.06% (w/w on protein-substrate basis) in 50 mM McIlvaine's buffers (pH 6.5) containing 100 mM NaCl at 40 °C. The samples were incubated for different time periods (10 min, 1 h and 24 h) and the reaction was stopped by heating at 100 °C for 6 min.

The determination of calcium reactivity test was carried out using another commercial lime pectin (DM 61, DA 0), which was re-precipitated before use. Pectin sample (10 mg/ml) was maintained at pH 5.0 and at 40 °C by the continuous addition of 100 mM NaOH for 25 h. *Bli*PME was added at a dose of ∼0.02% (w/w on protein-substrate basis). Using lime pectin (DM 61, DA 0) sample (10 mg/ml) containing 100 mM NaCl was maintained at pH 5.0 (by addition of 100 mM NaOH) and incubated for 2.5 h. All reactions were stopped by heating at 100 °C for 6 min.

2.5.6. Fingerprinting of BliPME-modified pectins

Freeze-dried *Bli*PME modified pectins were dissolved (5 mg/ml) in 50 mM sodium citrate buffer (pH 5.0) at 40 °C for 24 h by RG-I (endo-galactanase + endo/exo arabinase + RG hydrolase) and HG (endo-PGII + PL) degrading enzymes. The reaction was stopped by heating at 100 °C for 6 min. The total digest was analyzed by LC–HILIC–MS (Remoroza et al., 2014b).

2.5.7. Hydrophilic interaction liquid chromatography (HILIC)

Pectin digests, diluted to 1 mg/ml in 50% (v/v) acetonitrile, were analysed using an UHPLC system coupled to an evaporative light scattering detector (ELSD) and an ESI–IT–MSn-detector. Chromatographic separation was performed on an Acquity UPLC BEH Amide column in combination with a Van Guard pre-column (Waters Corporation, Milford, MA, USA) as described previously (Remoroza et al., 2014b).

2.5.8. Calcium reactivity measurement of the modified lime pectins

Non-acetylated *Bli*PME-modified pectin samples were dissolved (10 mg/ml) in 0.5 M sodium acetate buffer (pH 5.0) in the absence or presence of 20–40 mM of Ca^{2+} ions according to the method described elsewhere (Buchholt et al., 2004).

3. Results

3.1. Amino acid sequence analysis of BliPME

*Bli*PME, originating from *B. licheniformis* DSM13, comprises 317 amino acids (aa) and has a calculated molecular mass of 35.1 kDa. In the corresponding GenBank entry (acc no. AAU42325) it is denominated as a putative pectinesterase and in the CAZy database (www.cazy.org) it is assigned to CE family 8.

Apart from several 317-aa (putative) pectinesterases and PMEs originating from different *B. licheniformis* strains (e.g., acc no. AGN37876, strain 9945A; acc no. BAL46005, strain SVD1; acc no. EQM25835, strain CG-B52) and, hence, not unexpectedly sharing 97.2 to 100.0% aa identity with *Bli*PME, BLASTP analysis revealed closest relation of *Bli*PME to a likewise 317-aa-comprising pectinesterase of *B. sonorensis* L12 (acc no. EME73130; 79.8% aa identity). Similarity was also found for a putative pectinesterase from *B. infantis* NRRL B-14911 (acc no AGX04064; 313 aa, 60.1% aa identity) and for two larger PME proteins from *B. endophyticus*

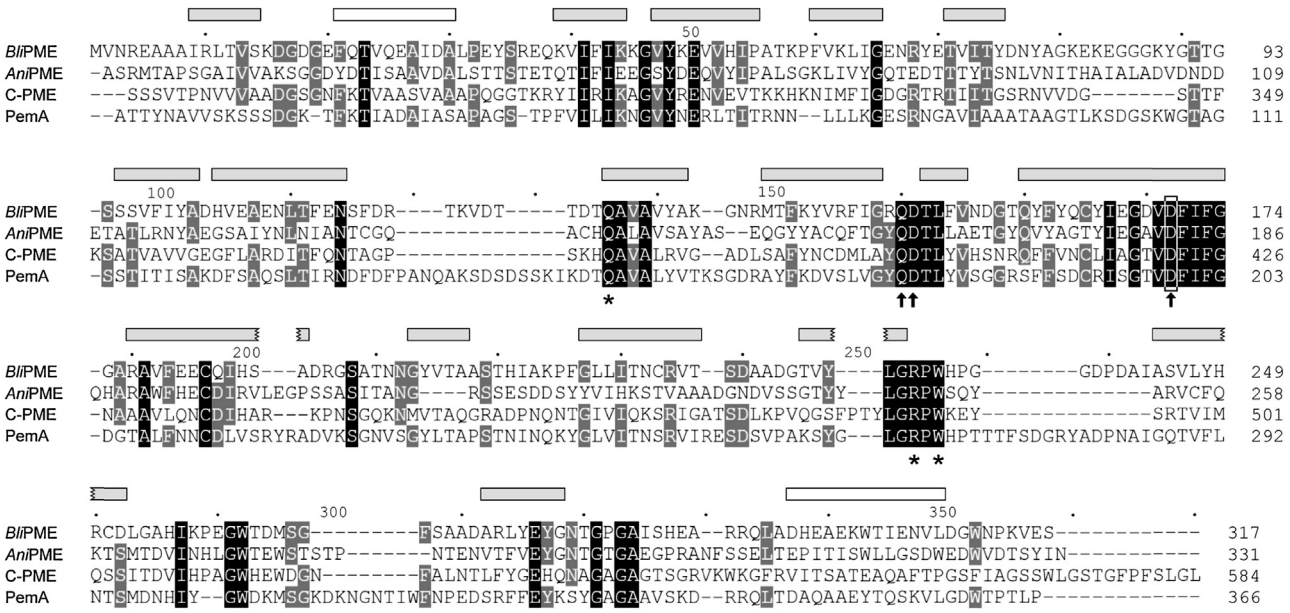

Fig. 1. Multiple sequence alignment of *Bli*PME, *Ani*PME (acc no. CAA38084), C-PME (acc no. P83948) and PemA (acc no. CAA68628). Only mature proteins were used, signal peptide sequences of the latter three enzymes (17, 50 and 24 aa, respectively) and an additional N-terminal propeptide of C-PME (216 aa) were omitted in this alignment. Residues conserved in four and three enzymes are highlighted in black and gray, respectively. Conserved catalytic residues and residues involved in the substrate binding (Fries et al., 2007) are marked by vertical arrows and asterisks, respectively. The catalytic nucleophile is additionally highlighted by a box. Secondary structure elements predicted for *Bli*PME are represented by gray (β-strands) and white (α-helices) rectangles, respectively.

(acc no. WP_019394632; 495 aa, 59.3% aa identity) and from *C. thermocellum* DSM1313 (acc no. YP_005687040; 567 aa, 49.8% aa identity), respectively. For the esterase from *B. endophyticus*, similarity was seen for the C-terminus (aa position 185–495) only, while for the esterase from *C. thermocellum* DSM1313 solely the N-terminus (aa position 27–327) showed significant sequence similarity.

The homology search also revealed numerous related proteins annotated as PMEs, suggesting *Bli*PME being a PME. Hence, the aa sequence of the enzyme was compared with the well-characterized PMEs from *A. niger* (*Ani*PME) (Van Alebeek et al., 2003), *C. sinensis* (C-PME) (Christensen, Nielsen, Kreiberg, Rasmussen, & Mikkelsen, 1998; Versteeg, Rombouts, & Pilnik, 1978) and *E. chrysanthemi* (PemA) (Fries et al., 2007; Jenkins, Mayans, Smith, Worboys, & Pickersgill, 2001). Despite their different phylogenetic affiliations, the mature enzymes *Ani*PME (314 aa, 34.0 kDa), C-PME (318 aa, 34.0 kDa) and PemA (342 aa, 36.9 kDa) match in size with the *Bacillus* enzyme. It shows highest aa identity for PemA (38.5%) and less for *Ani*PME (28.7%) and C-PME (22.4%).

Other than for the above enzymes, each having a signal peptide, sequence analysis with SignalP and TatP did not predict a signal peptide for *Bli*PME. However, as supposed for *B. subtilis* proteins, the training of the SignalP algorithm on data sets originating from gram-positive bacteria might lead to omission of signal peptides of deviating character (Tjalsma, Bolhuis, Jongbloed, Bron, & van Dijl, 2000). Here, the PSORT programs (http://www.psort.org) may provide an alternative localization prediction tool. For instance, for the *Bacillus* enzyme YesX, a RG lyase, PSORT suggested extracellular localization (Ochiai, Itoh, Kawamata, Hashimoto, & Murata, 2007), while both SignalP and TatP did not (our own observation). Applying PSORT to *Bli*PME, extracellular localization is likewise predicted, being consistent with the accessibility of the enzyme's substrate.

Subjecting *Bli*PME to a search for conserved domains revealed significant similarity (alignment region aa 11–309) with member PLN02432 of the putative-pectinesterases-comprising superfamily cl01911. Almost the same segment (alignment region aa 11–305) of *Bli*PME aligned with the related consensus sequence of Pfam protein family PF01095 (pectinesterases). Similarly, analysis of *Ani*PME, C-PME and PemA revealed their affiliation to the above protein families. A multiple sequence alignment of *Bli*PME with these enzymes disclosed several conserved and semi-conserved regions (Fig. 1). A secondary structure prediction for *Bli*PME revealed a large number of β-strands, distributed along the polypeptide chain, and only two α-helical regions at the N- and C-terminus, respectively. The overall secondary structure of *Bli*PME is predicted to be similar to that of *Ani*PME, C-PME and PemA.

3.2. Biochemical characterization of BliPME

*Bli*PME was heterologously produced using a T7-based inducible system and *E. coli* as the expression host. The enzyme was synthesized with a C-terminally fused StrepII tag allowing its purification by means of affinity chromatography. Verification of *Bli*PME purity as well as integrity was done by SDS-PAGE (Fig. 2A) and Western blot (Fig. 2B). Different quantities of purified enzyme were analyzed allowing both the detection of degradation products or faint impurities and of similar-sized protein contaminations. The stained SDS gel revealed that *Bli*PME was purified to near electrophoretic homogeneity. An intense band, the size of which matching the calculated molecular mass of the tagged enzyme (36.8 kDa), and a second, faint band were seen. In the Western blot, signals for both were observed, suggesting the former being the full-length enzyme and the latter being a fragment of *Bli*PME. An additional, but very faint signal became only visible in the Western blot analysis. It represents a biotinylated 16.7-kDa *E. coli* host protein, the biotin carboxyl carrier protein (Choi-Rhee & Cronan, 2003), which is co-purified with Strep-tagged proteins, however, in negligible amounts only.

The expression system used allowed the synthesis of large quantities of soluble enzyme, as it is apparent from the highly pronounced *Bli*PME band in the crude extract. By means of self-inducing auto-induction medium about 7 mg of purified *Bli*PME per 500-ml production culture were obtained.

To elucidate the intrinsic activity of *Bli*PME, the enzyme was incubated with acetylated pectin and the production of acetate or methanol was measured. Release of methanol only, confirmed the

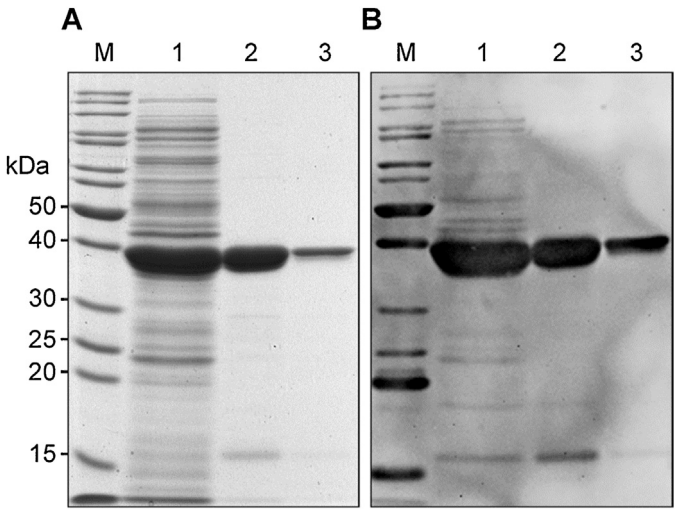

Fig. 2. SDS-PAGE and corresponding Western blot of purified *Bli*PME. (A) 30 μg crude extract from *E. coli* Rosetta 2(DE3) (pET22b-*Bli*PME-StrepIIc, pLysSRARE2) (lane 1) and purified *Bli*PME (6 μg, lane 2; 1 μg, lane 3) were separated on a 12% SDS polyacrylamide gel followed by Coomassie blue staining. M, molecular mass standard. (B) Western-blot analysis of crude extract and purified *Bli*PME using a Strep-Tactin HRP conjugate. For allocation of lanes see (A).

Table 2
Activity of PMEs from *Bacillus licheniformis* DSM13 and *Aspergillus niger* on lime pectin and SBP substrates.

Substrate	V_{max} (nkat/mg)[a]	
	*Bli*PME	*Ani*PME
Lime pectin (DM 76 DA 00)	2727	2339
Sugar beet pectin (DM 51 DA 29)	1035	468

[a] V_{max}: nanomoles of methanol release per second per mg protein.

Table 3
Percentage de-esterification of acetylated and non-acetylated pectins by PMEs from *Bacillus licheniformis* DSM13 and *Aspergillus niger*.

Substrates	Methanol release (% mole)	
	*Bli*PME	*Ani*PME
Non-acetylated pectin		
F58	96	72
P53	95	86
B34	99	51
Acetylated pectin		
SBP6230	63	39
F5129	69	42
P5328	69	44
B3124	73	39

homology-based prediction, namely *Bli*PME being a PME. Subsequently, the enzyme was subjected to a detailed characterization.

3.2.1. *pH & temperature optima*

Fig. 3A shows that the purified enzyme was active between pH 3.0–9.0 and displayed the highest activity at pH 8.0. At pH 9.0, *Bli*PME retained 70% of its maximum activity. The optimum temperature was at 50 °C and the enzyme retained 80% of its maximum activity at temperatures between 30 °C and 50 °C (Fig. 3B). To omit the role of auto hydrolysis, it was decided to performed the subsequent incubations at pH 5.0–6.5 and 40–50 °C in the presence of salt (NaCl), where the enzyme has >80% of its residual activity even after an extended incubation for 24 h (result not shown).

3.2.2. *Activity of BliPME towards different substrates*

Table 2 shows the specific activity of *Bli*PME towards non-acetylated (F7600) and acetylated (F5129) pectins being 2727 and 1035 nkat/mg, respectively. The activity of *Bli*PME on methylesterified pectin oligomers was determined to be 1869 nkat/mg showing that the enzyme was 1.5 times more active towards methylesterified polymer compared to methylesterified oligomers. The activity of *A. niger* PME (*Ani*PME) was determined on both polymeric substrates in order to compare both PMEs. Table 2 shows that the specific activity of *Ani*PME (2339 nkat/mg) towards the non-acetylated pectin (F7600) was quite comparable to *Bli*PME and 2 times lower (468 nkat/mg) towards acetylated pectin (F5129).

3.2.3. *De-methylesterification efficiency of BliPME towards lime pectins and SBPs*

An extended incubation of acetylated and non-acetylated pectins with *Bli*PME and *Ani*PME was performed in order to determine the limits of de-methylesterification. Table 3 presents the efficacy of *Bli*PME towards lime pectin and SBPs at the endpoint of the reaction. Except for SBP6230, these pectin substrates are pretreated differently, e.g. alkali and plant and fungal PMEs (Buchholt et al., 2004). Table 3 shows that *Bli*PME almost completely de-esterified the methylesters present in non-acetylated pectins (>95%), whereas *Ani*PME was only able to remove 51–86% of the total methylesters. When acetylated (DA 24–30) *and* methylesterified (DM 31–62) substrates were used, *Bli*PME removed 63–73% of the total methylesters, while *Ani*PME removed only 39–44% (Table 3). To fully understand the mode of action of *Bli*PME on various substrates, characterization of the *Bli*PME-modified pectins was performed via enzymatic fingerprinting.

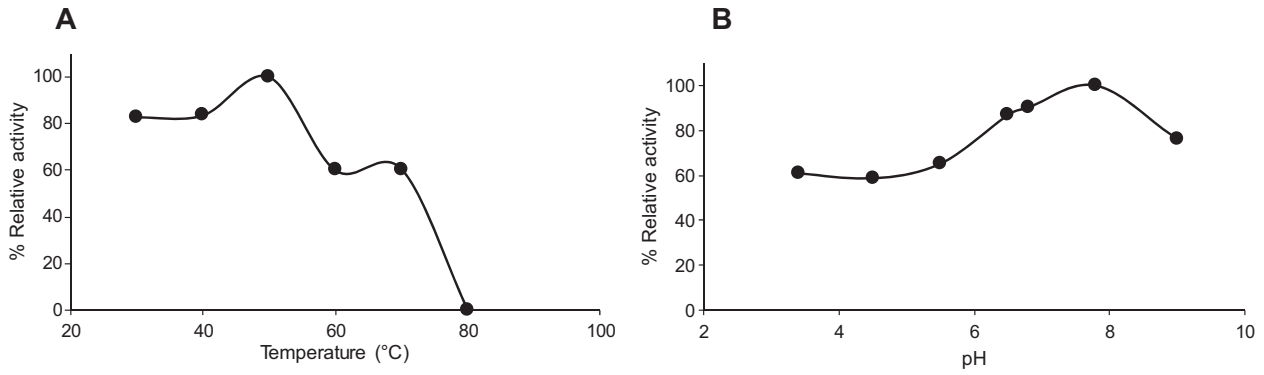

Fig. 3. Influence of pH and temperature on the activity (μmoles/mg min) of *Bli*PME on SBP (DM 73, DA 29). (A) pH 3.0–9.0 at 40 °C and (B) temperature25–80 °C at pH 6.5. Samples were incubated for 10 min. The relative activity is expressed as a percentage of the maximum activity.

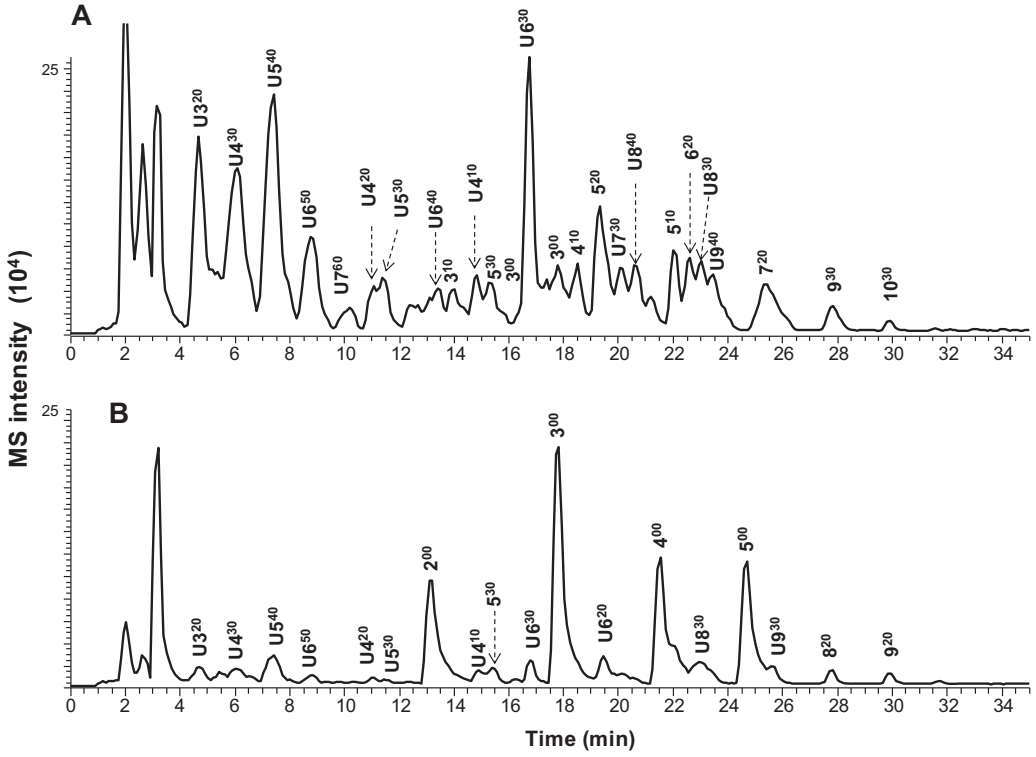

Fig. 4. HILIC–MSn profiles of non-acetylated pectin F7600 (DM 76, DA 0) before (A) and (B) after *Bli*PME treatment digested by endo-PGII and PL. Peak annotation: 5^{30}, DP 5, 3 methylester, 0 acetyl group.

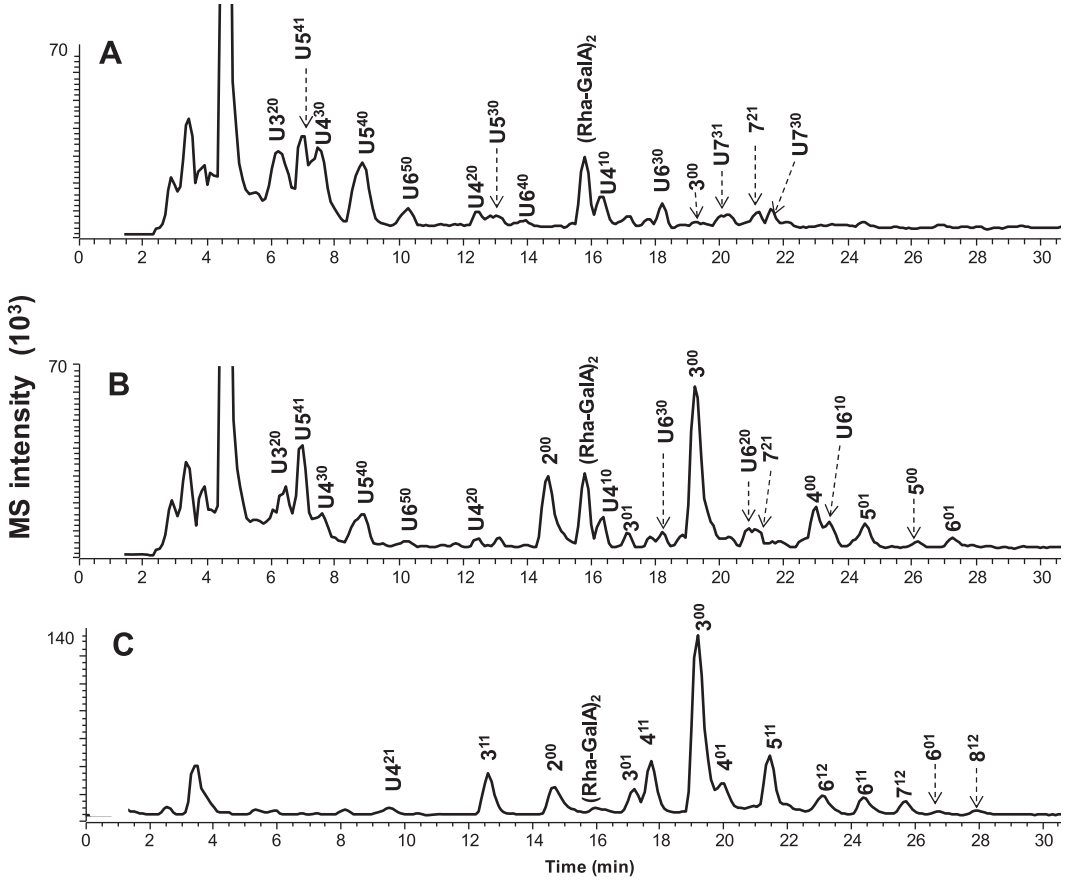

Fig. 5. HILIC–MSn profiles of acetylated pectin SBP E7329 (DM 73, DA 29) (A) before and (B) after 30 min and; (C) 24 h *Bli*PME treatment digested by RG-I degrading enzymes, endo-PGII and PL. Peak annotation: 7^{21}, DP 7, 2 *O*-methylester, 1 *O*-acetyl.

Table 4
Structures of different GalA oligomers released by endo-PGII and pectin lyase after digestion of non-treated and *Bli*PME-treated F7600 as analyzed by HILIC–ESI–MSn (Fig. 4).

Components	Structure	unmodified F7600 pectin	*Bli*PME-modified F7600 pectin (30 min)
2^{00}		–	++
3^{00}		+	+++
3^{10}		–	+
$U3^{20}$		+	+
4^{00}		–	++
4^{10}		–	+
$U4^{10}$		+	+
$U4^{20}$		+	+
$U4^{30}$		+	+
5^{00}		–	++
5^{10}		+	–
5^{20}		+	+
5^{30}		–	+
$U5^{30}$		+	+
$U5^{30}$		+	+
$U5^{40}$		++	+
$U6^{20}$		+	+
$U6^{30}$		+++	+
$U6^{40}$		+	–
$U6^{50}$		+	+
7^{20}		+	–
$U7^{30}$		+	–
$U7^{60}$		+	–
8^{20}		+	+
$U8^{30}$		+	+
$U8^{40}$		+	–
9^{20}		–	+
9^{30}		+	–
$U9^{30}$		–	+
$U9^{40}$		+	–
10^{30}		+	–

Nonesterified GalA ○; methylester ⬮; unsaturated GalA ●.

3.3. *Oligosaccharides formed by endo-PGII and PL from pectin with or without BliPME treatment*

3.3.1. *Non-acetylated pectin*

The non-acetylated lemon pectin (DM 76, DA 0) was incubated with *Bli*PME until 30% of the total methylesters present were removed resulting in a DM of 53. Fig. 4 illustrates the HILIC–MSn elution patterns of the endo-PGII and PL digest of unmodified and *Bli*PME-modified pectins. The products released from the unmodified pectin consist mostly of unsaturated GalA oligomers having a DP of 3–9. Only a few *saturated* oligomers (partly methylesterified) and a nonesterified trimer (3^{00}) were present in the unmodified pectin digest (Fig. 4A, Table 4). In contrast, the presence of nonesterified GalA oligomers (DP 2–5), e.g. 2^{00}, 3^{00}, 4^{00}, 5^{00} was much more pronounced compared to unsaturated oligomers (e.g. $U3^{20}$, $U5^{40}$, $U6^{20}$, $U6^{30}$) in the digest of *Bli*PME-modified pectin (Fig. 4B).

Table 5
Structures of different GalA oligomers released by endo-PGII and pectin lyase after digestion of unmodified and *Bli*PME-modified E7326 as analyzed by HILIC–ESI–MSn (Fig. 5).

Components	Structure	Unmodified	*Bli*PME-modified E7329	
			30 min	24 h
2^{00}		–	+	+
3^{00}		+	+++	+++
3^{11}		–	–	+
3^{01}		–	+	+
$U3^{20}$		+	+	–
4^{00}		–	+	–
4^{01}		–	+	+
4^{11}		–	–	+
$U4^{10}$		+	+	–
$U4^{20}$		+	+	–
$U4^{21}$		–	–	+
$U4^{30}$		+	+	–
5^{00}		–	+	–
5^{01}		–	+	–
5^{11}		–	–	+
$U5^{30}$		+	–	–
$U5^{30}$		+	–	–
$U5^{40}$		+	+	–
$U5^{41}$		+	+	–
6^{01}		–	+	+
6^{11}		–	–	+
6^{12}		–	–	+
$U6^{10}$		–	+	–
$U6^{30}$		+	+	–
$U6^{40}$		+	–	–
$U6^{50}$		+	+	–
7^{21}		+	–	–
7^{12}		–	–	+
$U7^{30}$		+	–	–

Nonesterified GalA ○; methylester ⬮; acetyl group *O*-2 ▲, *O*-3 △; unsaturated GalA ●.

3.3.2. Acetylated pectin

The modification and enzymatic fingerprinting of highly methylesterified and acetylated SBP (E7329) were carried out to reveal the mode of action of *Bli*PME in the presence of acetyl groups. A short incubation of E7329 with the enzyme released 10% of the total methylesters present resulting in a reduction of DM to 66. Fig. 5 illustrates the HILIC–MSn elution patterns of the endo-PGII and PL digests of the *Bli*PME-modified and unmodified acetylated pectin. Analysis of the *Bli*PME-modified SBP (E7329) digest showed the production of nonesterified GalA oligomers (2^{00}, 3^{00}) as major products (Fig. 5B). A number of highly methylesterified unsaturated (e.g. $U3^{20}$, $U4^{30}$,$U5^{41}$, $U5^{40}$, $U6^{50}$) and acetylated (e.g. 3^{01}, 5^{01}, 6^{01}) GalA oligomers were identified in the digest. The latter oligomers are acetylated at the *O*-3 position (Table 5). In addition, the unsaturated oligomers released after the endo-PGII and PL digestion of slightly *Bli*PME-modified SBP (Fig. 5B) and untreated SBP (Fig. 5A) were similar in structure but in different amounts as indicated by HILIC–MS profiles. Furthermore, minor amounts of oligomers originated from the RG-I segment, e.g. $(Rha\text{-}GaA)_2$ were also present in the SBP digests (Fig. 5).

The incubation of E7329 with *Bli*PME was extended until the reaction endpoint. De-methylesterification continued until a low DM 22 pectin (DA 29) was obtained indicating the enzymatic release of about 70% of all the methylesters present and the Fig. 5C shows the HILIC–MSn profiles of the products present in the digest. Besides the significant amount of trimer (3^{00}) as the major product, GalA oligomers with a single methylester and acetyl group; e.g. 3^{11}, 4^{11}, 5^{11} were also observed (Table 5). The latter oligomers have *O*-2 acetylation either at the same GalA residue or next to a methylesterified GalA residue, e.g. $GalA\text{-}GalA_{MeAc}\text{-}GalA$ or $UGalA_{Me}\text{-}GalA_{Ac}\text{-}GalA_{Me}\text{-}GalA$.

Oligosaccharides with *O*-3 acetylation (3^{01}, 4^{01}, 6^{12}, 7^{12}) have been identified. Fig. 5B and C shows that the endo-PGII and PL digestion of the more extensively *Bli*PME treated pectin clearly creates oligomers with longer sequences of nonesterified GalA residues compared to the degradation products of pectin after short treatment by the *Bli*PME.

3.4. Mode of action of BliPME towards pectin oligomers

Fig. 6 shows the HILIC analysis of unsaturated and saturated GalA oligomers (DP 2–6) with 1-3 methylesters treated with *Bli*PME for 0 (Fig. $6A_1$), 10, 60 min. It can be seen that the de-methylesterification rate of the enzyme towards unsaturated DP 5 oligomer ($U5^{30}$) was faster compared to unsaturated DP 4 ($U4^{20}$) and saturated DP 4 (4^{20}) (Fig. $6A_2$). Due to the conditions, intermediate de-methylesterification products (4^{10} from 4^{20}) as well as non-accessible methylesterified GalA oligomers ($U4^{10}$ and $U5^{10}$) were observed even after 60 min (Fig. $6A_3$). Fig. 6B shows that *Bli*PME is able to completely remove the methylesters within the saturated GalA oligomers, creating nonmethylesterified GalA oligomers (2^{00}, 3^{00}, 4^{00}). On the contrary, the enzyme could not remove the methylester from the unsaturated GalA residues at the nonreducing end including DP 3 ($UGalA_{Me}\text{-}GalA_{Me}\text{-}GalA$) even after extended incubation times (endpoint) (Fig. $6B_2$).

3.5. Calcium reactivity test of the modified lime pectins

Lime pectin (DM 61, DA 0) and *Bli*PME-modified lime pectins (DM 58, DA 0) and (DM 40, DA 0) were subjected to a calcium reactivity test. The *Bli*PME-modified pectin (DM 58, DA 0) slightly precipitated when Ca^{2+} ions were added at concentrations of 20 and 40 mM, whereas the unmodified pectin (DM 61, DA 0) did not precipitate upon Ca^{2+} ions addition. Moreover, the strongly *Bli*PME-treated pectin, which resulted to low methylesterified pectin (DM 40, DA 0), showed precipitation in the presence of 20 and 40 mM Ca^{2+} ions.

4. Discussion

In this study, we produced and characterized a bacterial PME from *B. licheniformis* DSM13. Comparison of its protein sequence

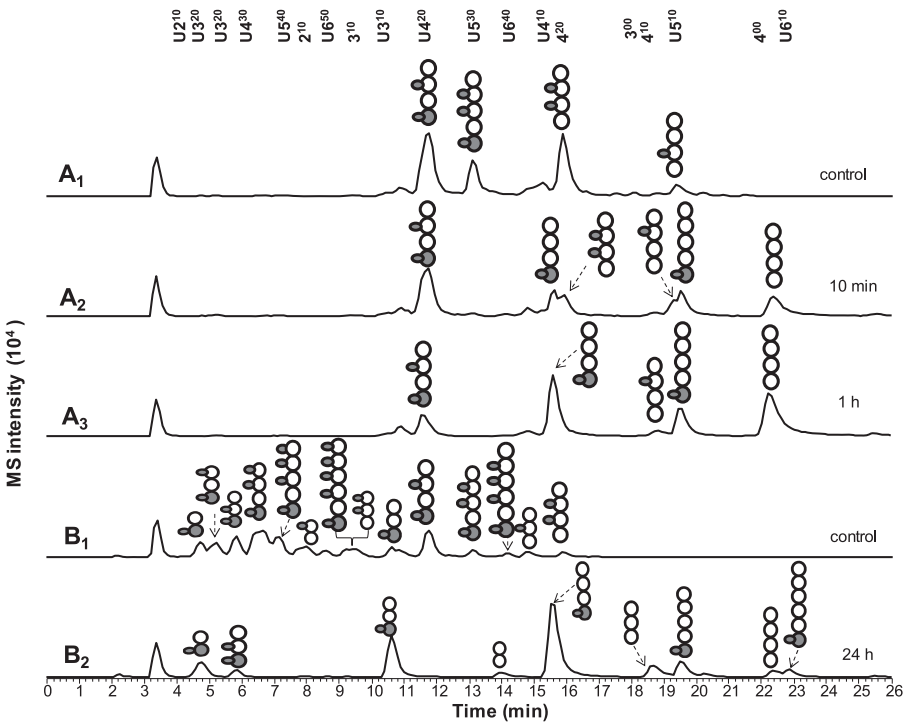

Fig. 6. HILIC–MSn profiles of saturated and unsaturated pectin oligomers (A) degree of polymerization (DP) 4–5; (B) DP 2–6 digested with *Bli*PME at different times. Peak annotation: Galacturonic acid (GalA) ○; unsaturated GalA ●; methylesters ⬮.

with the well-studied PMEs PemA, *Ani*PME and C-PME revealed similar sizes and several conserved and semiconserved regions (Fig. 1). Among such enzymes, highest overall aa sequence identity (38.5%) was seen for *Bli*PME and the likewise bacterial PemA of *E. chrysanthemi*. This result was not unexpected, as phylogenetic analyses revealed that bacterial PMEs, though quite heterogenous in sequence and size, form a separate clade (Markovič & Janeček, 2004). Such clade is clustered with the one composed of the fungal enzymes, but rather distinct from the eight clades formed by plant PMEs. This classification is in agreement with the calculated aa identity values when *Bli*PME was compared with *Ani*PME (28.7%) and C-PME (22.4%), respectively.

Based on crystallographic studies of PemA, a right-handed parallel β-helix structure was found (Jenkins et al., 2001), being distinct from the common α/β hydrolase fold and of the catalytic Ser-His-Asp triad of most esterases (Huo et al., 2012). The high level of resemblance in the predicted secondary structures of the *Bacillus* enzyme and PemA suggests *Bli*PME to be a parallel β-helix protein as well.

In PemA, Asp178 acts as the general acid-base catalyst while Asp199 is the nucleophile in the reaction mechanism (Fries et al., 2007). Gln177 is suggested to form the oxyanion hole, stabilizing the negative charge of the transition state. Gln153, Arg267 and Trp269 are involved in the substrate binding. Equivalents of all above six residues are likewise present in *Bli*PME (Asp149, Asp170, Gln148; Gln126, Arg232, Trp234) (Fig. 1), suggesting these two bacterial PMEs to share the same reaction mechanism. Besides the high similarity of conserved regions as well as the predicted secondary structure between *Bli*PME and PemA, both bacterial enzymes have their optimal activity at neutral and alkaline conditions (Fries et al., 2007; Laurent, Kotoujansky, & Bertheau, 2000). On the contrary, *Aspergillus* PMEs are generally active at more acidic conditions (Plaza et al., 2008; Semenova, Grishutin, Gusakov, Okunev, & Sinitsyn, 2003) compared to bacterial or plant- derived PMEs. Moreover, *Bli*PME performed optimally at low and moderately high temperatures. Although quite similar, both bacterial PMEs (*Bli*PME and PemA), regrettably, cannot be compared in great detail, since PemA (or other PMEs) was not analyzed on various methylesterified acetylated and non-acetylated pectins.

As far as we know, this is the first time that detailed experimental evidence is provided on an enzyme from *B. licheniformis* DSM13 having PME activity. Obviously, *Bli*PME has distinct features compared with the bacterial PemA, fungal *Ani*PME and plant C-PME enzymes. Apart from the substantial higher de-methylesterification activity towards acetylated pectins tested, *Bli*PME de-methylesterifies the pectins in a blockwise manner irrespective of the methylesters and/or acetyl groups distribution.

In this study, *Bli*PME shows to be 2.5 times more efficient in catalyzing the removal of methylesters in non-acetylated pectin compared to acetylated pectin and methylesterified pectin oligomers. Although the degree of methylesterification (DM 34–58) and distribution of methylesters are different in the non-acetylated lime pectins (Limberg et al., 2000a), *Bli*PME rapidly de-methylesterified the lime pectins almost completely. This can be attributed to high binding affinity of *Bli*PME towards the substrate. In addition, the bacterial enzyme was not limited by the methylester distribution in non-acetylated lime pectins and could also release the last methylesters from modified low DM pectins. On the contrary, the de-methylesterification of the same pectins using *Ani*PME stopped when DM reached ~16. These values are in agreement with previous studies (Limberg et al., 2000a; Ralet et al., 2001).

The rate of de-methylesterification was different when acetylated substrates were used. *Bli*PME is clearly active towards methylesterified and acetylated pectin and its activity was two times higher compared to *Ani*PME. The findings for *Bli*PME demonstrate that the enzyme has a high tolerance on highly acetylated SBPs, hence it is able to release ~70% of the total methylesters present in SBPs. Apparently, *Bli*PME is quite tolerant for the presence of acetyl groups. So far, this information has not been reported for other bacterial PME.

Enzymatic fingerprinting of non-acetylated and acetylated pectin was done in order to understand the mode of action of *Bli*PME. From the oligosaccharides profile as shown in Figs. 4 and 5 it can be concluded that the enzyme creates blocks of nonmethylesterified GalA sequences. Although the blockwise de-methylesterification pattern was observed theoretically leading to blockwise methylesterified segments remaining, unsaturated GalA oligomers were present in rather lower amounts compared to the nonesterified GalA oligomers in the digests of non-acetylated and acetylated pectins. This indicates the formation of relative short blocks of nonmethylesterified sequences in the HG region of *Bli*PME-treated pectin. The blockwise acting *Bli*PME strongly binds to non-acetylated lime pectin (F7600) and acetylated SBP (F5129) having a random distribution of methylesters.

The influence of acetyl groups on *Bli*PME activity was also demonstrated. After *Bli*PME treatment and enzymatic fingerprinting (Fig. 5), generation of both nonesterified and non-methylesterified *O*-3-acetylated GalA oligomers such as 4^{01}, 5^{01}, 6^{01} was observed. Such degradation products have also been observed after the enzymatic fingerprinting of plant PME-modified SBPs having a low DM as described elsewhere (Remoroza, Buchholt, Gruppen, & Schols, 2014a). On the contrary, *Bli*PME could not completely remove the methylesters from the GalA residues within a pectin, especially when the adjacent or the same GalA residue is *O*-2 acetylated, e.g. 3^{11} GalAGalA$_{MeAc}$GalA. This indicates that *Bli*PME can release more methylesters from the methylesterified GalA residues in case that the acetylation is at the *O*-3 position and not at the *O*-2 position (Table 5). Based on the above results, it can be hypothesized that a steric shielding effect of the acetyl groups at the *O*-2 position hinder the enzyme from binding with the substrate.

When testing saturated and unsaturated methylesterified GalA oligomers, *Bli*PME de-methylesterify completely the methylesters from the saturated oligomers, which generates nonmethylesterified oligomers. *Bli*PME could not remove the methylester from the unsaturated GalA residue within a GalA oligomer, which is in contrast with previous findings (Van Alebeek et al., 2003). *Ani*PME de-methylesterifies the methylester from the unsaturated GalA on its non-reducing end. Unsaturated DP 3 oligomer with 2 methylesters, UGalA$_{Me}$-GalA$_{Me}$-GalA could be one of the limitations of the *Bli*PME enzyme. It can be predicted that the presence of a nonesterified GalA residue and a methylesterified GalA residue is the enzyme requirement as -1 and +1 subsites for binding. From the available methylesterified GalA oligomers (Fig. 6), it cannot be concluded whether *Bli*PME is able to remove a methylester from both the non-reducing end or reducing end of the saturated GalA oligomers. Based on the above observation, *Bli*PME seemed to introduce a single-chain or blockwise removal of methylesters on non-acetylated pectins. This is confirmed by the results of the Ca^{2+} precipitation.

5. Conclusion

A pectin methylesterase (*Bli*PME) from *B. licheniformis* DSM13 prefers to de-esterify methylesterified pectin polymers rather than oligomers. In contrast to plant PMEs, *Bli*PME does not form large de-methylesterified blocks of GalA residues, but creates many small blocks of nonesterified GalA residues. Moreover, *Bli*PME is quite tolerant to the presence of acetyl groups at the *O*-3 position of a GalA residue in the HG region of SBP and the enzyme has been proven to be more efficient in creating a blockwise structure on acetylated pectins compared to other reported PMEs.

Acknowledgments

This research was supported by the European Community within a consortium PolyModE KBBE-2007-3-3-07. We are grateful to Dr. Stephan Kolkenbrock for gene selection and helpful advice as to enzyme production and purification. We also like to thank Rianne Willems of the Laboratory of Food Chemistry, Wageningen University, The Netherlands for providing the pectin oligomers used in the study.

References

Beldman, G., Searle-van Leeuwen, M. J. F., De Ruiter, G. A., Siliha, H. A., & Voragen, A. G. J. (1993). Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. *Carbohydrate Polymers*, *20*(3), 159–168.

Bendtsen, J. D., Kiemer, L., Fausbøll, A., & Brunak, S. (2005). Non-classical protein secretion in bacteria. *BMC Microbiology*, *5*(1), 58.

Buchholt, H. C., Christensen, T. M. I. E., Fallesen, B., Ralet, M. C., & Thibault, J. F. (2004). Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers*, *58*(2), 149–161.

Choi-Rhee, E., & Cronan, J. E. (2003). The biotin carboxylase-biotin carboxyl carrier protein complex of *Escherichia coli* acetyl-CoA carboxylase. *Journal of Biological Chemistry*, *278*(33), 30806–30812.

Christensen, T. M. I. E., Nielsen, J. E., Kreiberg, J. D., Rasmussen, P., & Mikkelsen, J. D. (1998). Pectin methyl esterase from orange fruit: Characterization and localization by in-situ hybridization and immunohistochemistry. *Planta*, *206*(4), 493–503.

Duvetter, T., Fraeye, I., Sila, D. N., Verlent, I., Smout, C., Hendrickx, M., et al. (2006). Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. *Journal of Agricultural and Food Chemistry*, *54*(20), 7825–7831.

Fraeye, I., Duvetter, T., Doungla, E., Van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, *21*(5), 219–228.

Fries, M., Ihrig, J., Brocklehurst, K., Shevchik, V. E., & Pickersgill, R. W. (2007). Molecular basis of the activity of the phytopathogen pectin methylesterase. *European Molecular Biology Organization*, *26*(17), 3879–3887.

Guillotin, S. E., Van Loey, A., Boulenguer, P., Schols, H. A., & Voragen, A. G. J. (2007). Rapid HPLC method to screen pectins for heterogeneity in methyl-esterification and amidation. *Food Hydrocolloids*, *21*(1), 85–91.

Harmsen, J. A. M., Kusters-van Someren, M. A., & Visser, J. (1990). Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pelA*): Indications of a pectin lyase gene family in *A. niger*. *Current Genetics*, *18*(2), 161–166.

Huo, Y., Miao, J., Liu, B., Yang, Y., Zhang, Y., Tahara, Y., et al. (2012). The expression of pectin methylesterase in onion flower buds is associated with the dominant male-fertility restoration allele. *Plant Breeding*, *131*(1), 211–216.

Jenkins, J., Mayans, O., Smith, D., Worboys, K., & Pickersgill, R. W. (2001). Three-dimensional structure of *Erwinia chrysanthemi* pectin methylesterase reveals a novel esterase active site. *Journal of Molecular Biology*, *305*(4), 951–960.

Kim, Y., Williams, M. A. K., Galant, A. L., Luzio, G., Savary, B. J., Vasu, P., et al. (2013). Nanostructural modification of a model homogalacturonan with a novel pectin methylesterase: Effects of pH on nanostructure, enzyme mode of action and substrate functionality. *Food Hydrocolloids*, *33*(1), 132–141.

Kühnel, S., Hinz, S. W. A., Pouvreau, L., Wery, J., Schols, H. A., & Gruppen, H. (2010). *Chrysosporium lucknowense* arabinohydrolases effectively degrade sugar beet arabinan. *Bioresource Technology*, *101*(21), 8300–8307.

Larkin, M., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947–2948.

Laurent, F., Kotoujansky, A., & Bertheau, Y. (2000). Overproduction in *Escherichia coli* of the pectin methylesterase A from *Erwinia chrysanthemi 3937*: One-step purification, biochemical characterization, and production of polyclonal antibodies. *Canadian Journal of Microbiology*, *46*(5), 474–780.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000a). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*. *Carbohydrate Research*, *327*(3), 293–307.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000b). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research*, *327*(3), 321–332.

Markovič, O., & Janeček, Š. (2004). Pectin methylesterases: Sequence-structural features and phylogenetic relationships. *Carbohydrate Research*, *339*(13), 2281–2295.

May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, *12*(1), 79–99.

Micheli, F. (2001). Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. *Trends in Plant Science*, *6*(9), 414–419.

Mutter, M., Renard, C. M. G. C., Beldman, G., Schols, H. A., & Voragen, A. G. J. (1998). Mode of action of RG-hydrolase and RG-lyase toward rhamnogalacturonan oligomers. Characterization of degradation products using RG-rhamnohydrolase and RG-galacturonohydrolase. *Carbohydrate Research*, *311*(3), 155–164.

Nicholas, K., Nicholas, H., Jr., & Deerfield, D., II. (1997). GeneDoc analysis and visualization of genetic variation. *EMBNEW News*, *4*, 14.

Ochiai, A., Itoh, T., Kawamata, A., Hashimoto, W., & Murata, K. (2007). Plant cell wall degradation by saprophytic *Bacillus subtilis* strains: Gene clusters responsible

for rhamnogalacturonan depolymerization. *Applied and Environmental Microbiology*, *73*(12), 3803–3813.
Oosterveld, A., Beldman, G., Searle-Van Leeuwen, M. J. F., & Voragen, A. G. J. (2000). Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium. *Carbohydrate Polymers*, *43*(3), 249–256.
Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nature Methods*, *8*(10), 785–786.
Plaza, L., Duvetter, T., Plancken, I., Meersman, F., Loey, A., & Hendrickx, M. (2008). Influence of environmental conditions on thermal stability of recombinant *Aspergillus aculeatus* pectinmethylesterase. *Food Chemistry*, *111*(4), 912–920.
Punta, M., Coggil, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., et al. (2012). The Pfam protein families database. *Nucleic Acids Research*, *40*(D1), D290–D301.
Ralet, M.-C., Dronnet, V., Buchholt, H. C., & Thibault, J.-F. (2001). Enzymatically and chemically de-esterified lime pectins: Characterisation, polyelectrolyte behaviour and calcium binding properties. *Carbohydrate Research*, *336*(2), 117–125.
Remoroza, C., Buchholt, H. C., Gruppen, H., & Schols, H. A. (2014). Descriptive parameters revealing substitution patterns in differently prepared sugar beet pectins. *Carbohydrate Polymers*, *101*, 1205–1215.
Remoroza, C., Wagenknecht, M., Gu, F., Buchholt, H. C., Moerschbacher, B. M., Schols, H. A., et al. (2014). A *Bacillus* pectin acetylesterase is specific for homogalacturonan's acetylated at *O*-3. *Carbohydrate Polymers*, *107*(0), 85–93.
Renard, C., & Thibault, J.-F. (1996). Pectins in mild alkaline conditions: β-Elimination and kinetics of demethylation. In A. G. J. Voragen, & J. Visser (Eds.), *Progress in biotechnology: 14 Pectins and pectinases* (pp. 603–608). Amsterdam, The Netherlands: Elsevier.
Rost, B., Yachdav, G., & Liu, J. (2004). The predict protein server. *Nucleic Acids Research*, *32*(suppl 2), W321–W326.
Schols, H. A., Posthumus, M. A., & Voragen, A. G. J. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research*, *206*(1), 117–129.
Semenova, M., Grishutin, S., Gusakov, A., Okunev, O., & Sinitsyn, A. (2003). Isolation and properties of pectinases from the fungus *Aspergillus japonicus*. *Biochemistry (Moscow)*, *68*(5), 559–569.
Studier, F. W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expression and Purification*, *41*(1), 207–234.
Tjalsma, H., Bolhuis, A., Jongbloed, J. D., Bron, S., & van Dijl, J. M. (2000). Signal peptide-dependent protein transport inBacillus subtilis: A genome-based survey of the secretome. *Microbiology and Molecular Biology Reviews*, *64*(3), 515–547.
Van Alebeek, G. J. W. M., Zabotina, O., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2000). Structural analysis of (methyl-esterified) oligogalacturonides using post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Mass Spectrometry*, *35*(7), 831–840.
Van Alebeek, G. J. W. M., Van Scherpenzeel, K., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2003). Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*. *Biochemical Journal*, *372*(1), 211–218.
Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K. H., Ehrenreich, P., et al. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *Journal of Molecular Microbiology and Biotechnology*, *7*, 204–211.
Versteeg, C., Rombouts, F. M., & Pilnik, W. (1978). Purification and some characteristics of two pectinesterase isoenzymes from orange. *Lebensmittel-Wissenschaft & Technologie*, *11*, 267–274.
Voragen, A. G. J., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263–275.
Voragen, A. G. J., Pilnik, W., Thibault, J.-F., Axelos, M. A. V., & Renard, C. M. G. C. (1995). Pectins. In A. M. Stephen (Ed.), *Food polysaccharides and their applications* (pp. 287–339). New-York, Basel, Hong Kong: M. Dekker.
Willats, W. G. T., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: New insights into an old polymer are starting to gel. *Trends in Food Science & Technology*, *17*(3), 97–104.
Wood, P. J., & Siddiqui, I. R. (1971). Determination of methanol and its application to measure of pectin ester content and pectin methyl esterase activity. *Analytical Biochemistry*, *39*(2), 418–428.