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A *Bacillus licheniformis* pectin acetylesterase is specific for homogalacturonans acetylated at *O*-3

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ABSTRACT

A recombinant acetylesterase from *Bacillus licheniformis* DSM13, belonging to carbohydrate esterase family 12, was purified and biochemically characterized. The purified enzyme, termed *Bli*PAE, was capable of deacetylating acetylated pectins, e.g. sugar beet pectin (SBP). Contrary to its provisional annotation as rhamnogalacturonan acetylesterase, the enzyme specifically removed acetyl groups from the homogalacturonan region classifying it as a PAE. The recombinant enzyme has a molecular mass of 26.7 kDa and shows optimal activity at pH 8.0 and 50 °C. It is stable in the range pH 5.0–7.0 and below 50 °C. Methylesterification of the galacturonic acid (GalA) moieties reduces the deacetylation efficacy of *Bli*PAE. The enzyme efficiently removes acetyl groups from SBPs with low degree of methylesterification (DM) 9-30, releasing about 75% of the acetyl groups present in the homogalacturonan. Furthermore, ^{1}H NMR of polymer and LC-HILIC-MS^{n} after endo-PGII and PL degradation were used to structurally characterize the *Bli*PAE-modified pectins. The results show that *Bli*PAE removes acetyl groups specifically when substituted at the *O*-3 position of GalA moieties.

1. Introduction

Traditionally, commercial pectin is extracted from citrus peels and apple pomace. The primary structural elements of pectin are homogalacturonan (HG) and rhamnogalacturonan I (RG-I). The HG is composed of (1→4) linked α-D-GalA residues, which can be methylesterified at the C-6 position, while the RG-I backbone is composed of the disaccharide repeating unit [(1→2)-α-L-Rha-(1→4)-α-D-GalA]. Between 20 and 80% of the rhamnosyl residues can be substituted with side chains consisting of neutral sugars. The GalA residues can be acetylated at positions *O*-2 and/or *O*-3in both RG-I and HG (Quéméner, Cabrera Pino, Ralet, Bonnin, & Thibault, 2003). The level of acetylation in commercial pectins from citrus peels and apple pomace is negligible (Thibault & Ralet, 2003).

Nowadays, sugar beet pulp and sunflower residue are raw materials seen as potential source of commercial pectins. However, these pectins are rich in acetyl groups and commercial sugar beet pectin (SBP) may have a degree of acetylation (DA) of 30 (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004). It was reported that about 75% of all acetyl groups are located in the HG and 25% in the RG-I of acid extracted (Ralet, Cabrera, Bonnin, Quémenér, Hellín, & Thibault, 2005). The high degree of acetylation in pectins adversely affects their functional properties (Kim, Sosulski, & Lee, 1978). Hence, commercialization of SBP is limited due to its poor gelling capability (Buchholt et al., 2004). Enzymatic deacetylation may overcome the poor gelling properties of SBP and could provide a promising alternative way for the commercialization of SBP and other acetylated pectins. So far, only a low number of acetylesterases active on acetylated pectin have been identified and characterized. In addition, the effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium shows that the gelation of the modified SBP is dependent on the presence or modification by pectin methylesterase (PME) (Oosterveld, Beldman, Searle-Van Leeuwen, & Voragen, 2000). This limits the possibilities for specifically modifying the level of acetylation of SBP and other acetylated pectins.

Pectin acetylesterase (PAE) removes acetyl groups from the HG region of pectin (Benen, Alebeek, Voragen, & Visser, 2003) and belongs to carbohydrate esterase (CE) family 12 (CAZy database, www.cazy.org). Two PAEs have been detected in orange peel, which exhibit a maximum activity at acidic pH and deacetylate a synthetic substrate more efficiently than the low methylesterified HG of SBPs (Christensen, Nielsen, Mikkelsen, Visser, & Voragen, 1996; Williamson, 1991). Another type of acetylesterase in the same CE

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family is the class of rhamnogalacturonan acetylesterase (RGAE). *Aspergillus aculeatus* RGAE is known to specifically remove acetyl groups bound to GalA residues in the RG-I of pectins and showed an optimal activity at acidic pH (Searle-van Leeuwen, Broek, Schols, Beldman, & Voragen, 1992). Another acetylesterase from *A. aculeatus* was able to deacetylate both the HG and RG-I of SBP (Bonnin, Clavurier, Daniel, Kauppinen, Mikkelsen, & Thibault, 2008). Aside from plants and fungi, CE family 12 acetylesterases have been identified in bacteria. PaeY and PaeX, two PAEs from *Erwinia chrysantemi* (Molgaard, Kauppinen, & Larsen, 2000; Shevchik & Hugouvieux-Cotte-Pattat, 2003) and PAE YxiM from *Bacillus subtilis* (Bolvig, Pauly, Orfila, Scheller, & Schnorr, 2003) were highly active towards several synthetic substrates and preferred these substrates over GalA oligomers and acetylated low methylesterified SBP. None of the above mentioned acetylesterases has been distinguished for a possible preference for *O*-2 or *O*-3 acetylation.

Bacillus licheniformis DSM13 is known to be an important source for a multitude of biotechnologically important enzymes, among them several pectin degrading enzymes, e.g. pectate lyases (Veith, Herzberg, Steckel, Feesche, Maurer, Ehrenreich et al., 2004). Pectin acetylesterases, however, have not yet been reported for this species. In this study, we cloned a *B. licheniformis* DSM13 gene encoding a PAE classified in CE family 12 and expressed it heterologously in *Escherichia coli*. The recombinant enzyme, termed *Bli*PAE, was purified and characterized for its biochemical properties. ^{1}H NMR analysis and enzymatic fingerprinting of modified SBP using pectolytic enzymes were carried out, followed by hydrophilic interaction liquid chromatography (HILIC) with online mass spectrometry (MS) analysis in order to determine *Bli*PAE's mode of action and specificity.

2. Experimental

2.1. Characterization of the acetylated pectins from different sources

2.1.1. Substrates

Sugar beet pectin, (SBP6230) with degree of methylesterification (DM) of 62 and DA of 30 was extracted from sugar beet pulp and this pectin was further de-esterified by alkali or by plant or fungal pectin esterases to yield different series of pectins (Table 1). SBP6230 modified by plant pectin methylesterase (p-PME) yielded the P-series of SBP (P5328, P3429) and SBP6230 modified by fungal pectin methylesterase (f-PME) yielded the F-series (F5129, F3331). For the B-series, SBP6230 was alkali de-esterified yielding B5326, B4626, B3124 and B0915. Partial deacetylation of SBP6230 in a sodium methylate/methanol solution resulted in B′6126, B′6109, B′6023, B′5803. The chemical preparation and composition of these SBPs have been described elsewhere (Buchholt et al., 2004).

Pectic material from sunflower and potato material (DuPont), Okra (Sengkhamparn, Bakx, Verhoef, Schols, Sajjaanantakul, & Voragen, 2009) and apple modified hairy region (MHR) (Schols, Voragen, & Colquhoun, 1994) (Table 1), acetylated xanthan (Kool, Schols, Delahaije, Sworn, Wierenga, & Gruppen, 2013) and acetylated eucalyptus xylan oligomers (Van Gool, Vancsó, Schols, Toth, Szakacs, & Gruppen, 2011) were also used as substrates for testing enzyme specificity. Mild alkali-treated SBP5519 (B5519) and SBP1713 (B1713) was prepared following the procedure for preparation of the B-series of sugar beet pectin (Buchholt et al., 2004).

2.1.2. Constituent monosaccharide composition analysis

Determination of the neutral monosaccharide and uronic acid contents of sunflower and potato material was carried out as previously described elsewhere (Remoroza, Cord-Landwehr, Leijdekkers, Moerschbacher, Schols, & Gruppen, 2012).

2.1.3. Determination of the DM and DA

Pectin samples (2 mg/ml) were saponified in 100 mM NaOH and after neutralization the DM was determined using a colorimetric method (Guillotin, Van Loey, Boulenguer, Schols, & Voragen, 2007) while the DA was analyzed using the Megazyme acetic acid kit (Megazyme, Wicklow, Ireland).

2.2. Production and purification of BliPAE

2.2.1. Bacterial strains, media, and growth conditions

B. licheniformis DSM13 and *E. coli* strains NEB 5-alpha (New England Biolabs, Frankfurt a. Main, Germany) and Rosetta 2(DE3) (pLysSRARE2) (Merck, Darmstadt, Germany) were grown in Luria-Bertani (LB) medium at 37 °C. For plasmid maintenance, *E. coli*

Table 1
Chemical characteristics of pectin from various sources used in this study.

Pectin	GalA % (w/w)	Rha	Ara	Xyl	Gal	Glc	DM (%)[a]	DA (%)[a]
SBP6230[b]	55	5	12	0.3	10	0.5	62	30
B5519[c]	62	4	7	0.1	8	0.5	55	19
B1713[c]	62	4	7	0.1	8	0.5	17	13
B′6126	56	5	11	0.3	9	0.5	61	26
B′6109	59	5	11	0.3	8	0.5	61	09
B′6023	57	5	11	0.3	9	0.6	60	23
B′5803	60	5	10	0.3	8	0.5	58	03
B5326	58	6	12	0.5	10	0.4	53	26
B4626	60	6	11	0.3	10	0.4	46	26
B3124	55	6	12	0.3	10	0.4	31	24
B0915	57	5	10	0.2	9	0.3	9	15
F5129	57	5	12	0.3	9	0.2	51	29
F3331	57	5	12	0.3	9	0.3	33	31
P5328	58	6	11	0.2	10	0.4	53	28
P3429	66	5	11	0.2	10	0.4	34	28
Sunflower pectin[c]	75	1	1	n.d.	1	n.d	60	11
Potato material[c]	34	1	4	n.d	40	2	40	18
Apple MHR[d]	30	14	29	10	16	1	16	35
Okra RG region[e]	40	25	n.d	n.d	32	10	24	58

n.d. not detected; MHR: modified hairy region.

[a] Moles acetyl or methanol per 100 moles of galacturonic acid.
[b] Monosaccharide composition of SBP6230, B-, B′-, P- and F series (Buchholt et al., 2004).
[c] Monosaccharide composition was determined in this study.
[d] Monosaccharide composition (Schols et al., 1994b).
[e] Monosaccharide composition (Sengkhamparn et al., 2009).

strains were grown in the presence of ampicillin and/or chloramphenicol at a final concentration (f.c.) of 100 μg/ml and 34 μg/ml, respectively. After transformation of the latter strain with the *Bli*PAE expression plasmid, 40 mM glucose was added to the medium when the strain was grown on LB agar plates.

2.2.2. Cloning of the BliPAE coding sequence

The cell pellet of 0.5 ml of a *B. licheniformis* DSM13 overnight culture was resuspended in 50 μl 10 mM Tris/HCl buffer (pH 8.0) and incubated at 99 °C for 5 min. Next, the suspension was centrifuged (13,000 × *g*, 1 min, room temperature (RT)) and the supernatant was used as template for the PCR amplification of the *Bli*PAE open reading frame (ORF; GenBank accession number (acc. no.) AAU42913). For this, Phusion High-Fidelity DNA Polymerase (Fermentas, St. Leon-Rot, Germany) and primers 5′-CCGCAATTCCATATGATGCGAGACATTCAGTTGTTTTTG-3′ (forward) and 5′-CCCGAGCTCTAAAGGAATTCCCGCTTCTTTTG-3′ (reverse) were used. Additionally, an *NdeI* recognition site, overlapping with the start codon, and a SacI recognition site, concomitantly removing the ORF's stop codon, were introduced. The amplicon obtained was cut with *NdeI/SacI* and ligated with the likewise cut and dephosphorylated expression vector pET22b-StrepIIc, being a pET-22b(+) (Merck, Darmstadt, Germany) derivative additionally containing a sequence coding for the StrepII affinity tag. Competent cells of *E. coli* NEB 5-alpha were transformed with the ligation reaction and plasmids were kit-isolated from the transformants obtained. Correctness of insertion and coding sequence was verified by determination of the nucleotide sequence of the insert and the flanking regions (Eurofins MWG, Ebersberg, Germany). The *Bli*PAE expression plasmid generated was termed pET22b-*Bli*PAE-StrepIIc. PCR, agarose gel electrophoresis, DNA restriction endonuclease digestion, ligation and transformation of *E. coli* were done as described in Sambrook and Russell (2001).

2.2.3. Synthesis and purification of BliPAE

E. coli Rosetta 2(DE3) (pLysSRARE2) was transformed with pET22b-*Bli*PAE-StrepIIc and grown in 500 ml auto-induction medium (Studier, 2005) complemented with ampicillin and chloramphenicol. Incubation was done in a shaking incubator for 4 h at 180 rpm and 37 °C and further for 20 h at 120 rpm and 26 °C. Alternatively, for additional induction, isopropyl-β-D-thiogalactopyranoside (IPTG; f.c. 0.5 mM) was added after 15 h of incubation. Cells were harvested by centrifugation (4200 × *g*, 10 min, 4 °C), the pellet was resuspended in 30 ml 40 mM triethanolamine buffer (pH 8.0) containing 0.4 M NaCl and frozen at −20 °C. The thawed suspension was incubated for 30 min at RT, 75 U benzonase (Merck) were added and the suspension was sonicated. Centrifugation (40,000 × *g*, 40 min, 4 °C) yielded the crude extract, and *Bli*PAE was purified via affinity chromatography using a 1-ml Strep-Tactin Superflow Plus Column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For enzyme concentration and buffer exchange, Vivaspin columns (MWCO 10 kDa; Sartorius, Göttingen, Germany) and 20 mM triethanolamine buffer (pH 7.0) were used. Finally, the enzyme solution was complemented with glycerol (f.c. 10%) and stored at 4 °C.

2.3. Verification of enzyme purity

2.3.1. Protein analysis

Protein contents were determined using the Pierce BCA Protein Assay Kit (Fisher Scientific, Schwerte, Germany) and BSA as the calibration protein according to the manufacturer's instructions. SDS-PAGE was carried out using 12% polyacrylamide gels. peqGOLD Protein Marker II (PeqlabBiotechnologie, Erlangen, Germany) served as molecular mass standard. Gels were stained with Coomassie brilliant blue G-250. For Western blot, proteins were transferred to a nitrocellulose membrane. Immuno detection of StrepII-tagged *Bli*PAE was performed using a Strep-Tactin HRP conjugate (1:1000 dilution; IBA, Göttingen, Germany) according to the manufacturer's instructions. The signal of the horseradish peroxidase coupled to the antibody was imaged in a Fusion-SL VilberLourmat (PeqlabBiotechnologie, Erlangen, Germany) by chemiluminescence detection using luminol-based ECL substrate.

2.3.2. Colorimetric determination of acetic acid released

*Bli*PAE activity was measured colorimetrically using SBP, sunflower pectin, potato material, apple MHR and okra pectin. The pectin sample (~5 mg) was dissolved in 1 ml 50 mM McIlvaine's buffer (pH 6.5) incubated at 40 °C for 24 h with an enzyme concentration of 0.02% (w/w, on protein-substrate basis). The enzyme was inactivated by heating for 6 min at 100 °C. The amount of acetic acid released was analyzed spectrophotometrically using the Megazyme acetic acid kit (Megazyme). The assay was done in duplicate.

2.3.3. Determination of acetylesterase activity on pNPA

Esterase activity was measured using 4-nitrophenol acetate (pNPA) (Sigma, MO, USA). The reaction mixture of 240 μl contained 12 μl of 10 mM pNPA in DMSO, 1 μl of *Bli*PAE (1.1 mg/ml) and 227 μl of 100 mM potassium phosphate buffer (pH 7.3). The hydrolysis of pNPA and the formation of p-nitrophenol at 40 °C was continuously monitored using a spectrophotometer for 10 min at 405 nm in a 96 well microtiter plate. Esterase activity measured was expressed in nanokatal (1 nkat is defined as the amount of enzyme required to release 1 nmol product per second under the defined assay conditions).

2.3.4. Determination of acetylesterase activity by pH-stat

Esterase activity was determined by pH-stat titration using a thermostated auto-titration system (719 S Titrino, Metrohm, Herisau, Switzerland) as described elsewhere (Pouvreau, Jonathan, Kabel, Hinz, Gruppen, & Schols, 2011). Pectin solution (5 mg/ml) containing 100 mM NaCl was maintained at pH 6.5 at 40 °C by the addition of 25 mM NaOH.

2.3.5. Determination of optimum temperature and pH

Temperature and pH optima were determined using SBP as the substrate. SBP (B3124, DM 31, DA 24) solution (5 mg/ml) containing 50 mM McIlvaine's buffers at pH 3.0–8.0. Enzymes were added at a dose of 0.02% (w/w on protein-substrate basis). The optimum pH was determined by analyzing the enzyme activity at pH 3.0–8.0 at 40 °C. The temperature profile was determined within the temperature range 25–80 °C at pH 6.5. The incubation was performed for 10 min. The background level for the spontaneous deacetylation of the B3124 (control) was subtracted for all data points. All measurements were done in duplicate. At pH 8.0, the substrate was found to be slightly unstable and released about 2% of the total acetyl groups present by auto-hydrolysis.

2.3.6. Determination of temperature and pH stability

To determine its stability, the enzyme was first incubated at pH 3.0–8.0 using 50 mM McIlvaine's buffers or at temperatures 25–80 °C for 1 h. The residual activity of *Bli*PAE was measured by substrate (B3124) addition (5 mg/ml) and incubation at pH 6.5 and 40 °C for 10 min. The residual activity was expressed as the remaining activity of the enzyme after incubation for 1 h at given conditions without the substrate, relative to the activity of the fresh enzyme under these conditions.

2.4. Enzymatic fingerprinting of BliPAE-modified pectins

2.4.1. Pectin preparations

SBP sample (5 mg/ml) was treated with *Bli*PAE at a dose of 0.02% (w/w on protein-substrate basis) in 50 mM McIlvaine's buffers (pH 6.5). The incubation was set at 40 °C for 24 h. Blanks without enzyme addition were used as reference. The reaction was stopped by boiling for 6 min. Further addition of enzyme did not increase the final acetic acid release indicating that the endpoint had been achieved. Subsequently, the *Bli*PAE-modified SBP was dialyzed in milli-Q water overnight, followed by freeze-drying. The freeze-dried SBP obtained was re-dissolved (5 mg/ml) in 50 mM sodium citrate buffer (pH 5.0) and digested using the enzyme cocktail of purified RG-I and HG degrading enzymes at 40 °C for 24 h followed by LC–MS analysis described elsewhere (Remoroza, Buchholt, Gruppen & Schols, 2014).

For ^{1}H NMR analysis, SBPs (B5519 and B1713, 5 g) was dissolved in 500 ml demineralized water containing 0.1 g sorbic acid and 2.9 g sodium chloride. The sample was adjusted to pH 6.5 with 1 M NaOH in a 1 l stirred thermostated reactor and heated to 40 °C. A *Bli*PAE enzyme dose at 1% (w/w protein-substrate basis) was added and the pH was kept at 6.5 by adding of 0.1 M NaOH. Incubation of the substrates (B5519, B1713) with *Bli*PAE was 7 h and 4 h, respectively. The endpoint reaction was monitored directly by the pH-stat. The reaction was stopped by adjusting pH to 3.5 with 1 M hydrochloric acid and heating to 75 °C for 5 min. After cooling to room temperature pectin was precipitated by mixing into 1 l isopropyl alcohol. The precipitate was separated on a screen, dried in an oven at 40 °C overnight and the dry pectin was milled using a 0.5 mm sieve. The freeze-dried pectins (B5519, B1713) were characterized by ^{1}H NMR.

2.4.2. Hydrophilic interaction liquid chromatography (HILIC)-MS^n

SBP digests, diluted to 1 mg/ml in 50% (v/v) acetonitrile, were analysed using an Accela UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to an evaporative light scattering detector (Agilent 1200 series, Gen Tech Scientific, NY, USA) and an ESI-IT-MS^n-detector (LTQ Velos Pro, Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed on an Acquity UPLC BEH Amide column as described previously (Remoroza et al., 2014). Various pectin samples within the SBP series were modified by *Bli*PAE and analyzed by HILIC-MS^n but only one sample was chosen as the representative of SBPs in Table 1.

2.4.3. NMR spectroscopy

Liquid state ^{1}H NMR spectroscopy was conducted at 14.095 Tesla (600 MHz ^{1}H frequency) with a 5 mm smart probe and a Bruker Advance III spectrometer. The samples (B5519, B1713) before and after *Bli*PAE treatment were dissolved (5 mg/ml) in D_2O (99.9 atom% D, Sigma–Aldrich, St. Louis, MO, USA). NMR spectra were recorded at a probe temperature of 353 K collecting 64 scans. The spectra were acquired using standard pulse sequences delivered by Bruker. Chemical shifts are expressed in parts per million (ppm) relative to internal trimethylsilyl propanoic acid (TMSP).

3. Results and discussion

3.1. Sequence analysis, production and purification of BliPAE

*Bli*PAE, originating from *B. licheniformis* DSM13, comprises 220 amino acids (aa) corresponding to a calculated molecular mass of 25.0 kDa and based on homology, *Bli*PAE (acc. no. AAU42913) has been assigned to CE family 12 and annotated as a putative RGAE. *Bli*PAE revealed closest similarity to a 245-aa protein of *Bacillus* sp. BT1B_CT2 (acc. no. WP_009329846; 99.6% identity), annotated as a RGAE. Significant similarity was also seen for a putative PAE of *B. licheniformis* SVD1 (acc. no. BAL46009; 224 aa, 89.1% identity). Subjecting the deduced aa sequence of *Bli*PAE to a SignalP analysis (Petersen, Brunak, von Heijne, & Nielsen, 2011), a signal peptide was not to be predicted.

The coding sequence of *Bli*PAE was cloned and heterologously overexpressed in an inducible T7-based *E. coli* expression strain. In order to facilitate purification, *Bli*PAE was synthesized with a C-terminally attached StrepII tag. Employing conventional IPTG-induced gene expression as well as self-inducing auto-induction medium, the latter yielded the highest quantities of *Bli*PAE. Using this method, about 2 mg of purified enzyme per 500-ml production culture were obtained.

*Bli*PAE was purified by affinity chromatography. Verification of enzyme purity and integrity was done using SDS-PAGE and Western blot analysis (Fig. 1A,B). To unravel faint impurities or degradation products and to exclude similar-sized contaminating proteins, different amounts of *Bli*PAE were loaded. The stained SDS gel revealed a single band only, the size of which matching the calculated molecular mass of tagged *Bli*PAE (26.7 kDa), illustrating homogeneity of the enzyme purified. In the Western blot a corresponding strong *Bli*PAE signal was observed. An additional, but very faint signal represented the biotin carboxyl carrier protein (Choi-Rhee & Cronan, 2003), a biotinylated 16.7-kDa *E. coli* host protein that is co-purified with Strep-tagged proteins, however, in negligible quantities only.

3.2. Biochemical characterization of BliPAE

3.2.1. pH & temperature optima

Optimum pH and temperature of *Bli*PAE were determined on SBP (B3124) (Fig. 2A,B). The enzyme was tested in a pH range 3.0–8.0. The purified enzyme was active between pH 5.0–8.0 and the highest activity was measured at pH 8.0 (Fig. 2A). The activity profile of *Bli*PAE was also determined in the temperature range 25–80 °C at pH 6.5. The results showed that the enzyme has an optimum temperature at 50 °C. In addition, the enzyme has an activity of >50% of its maximum activity at 25 °C until 60 °C (Fig. 2B).

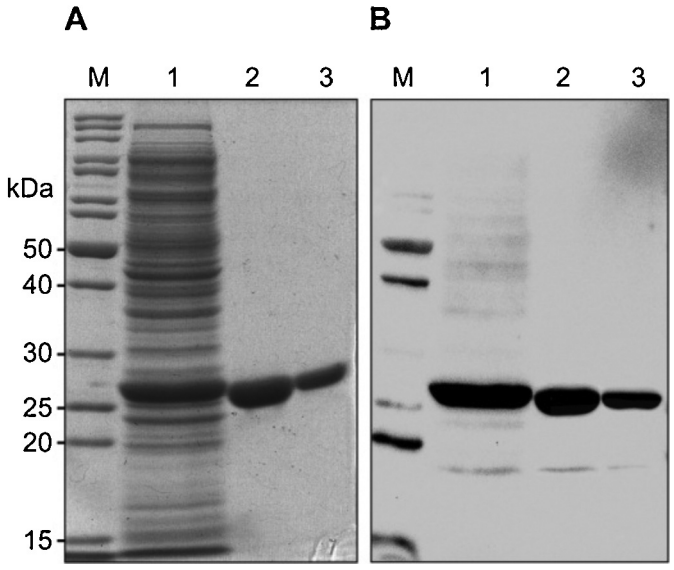

Fig. 1. SDS-PAGE and corresponding Western blot of purified *Bli*PAE. A) 30 μg crude extract from *E. coli* Rosetta 2(DE3) (pET22b-*Bli*PAE-StrepIIc, pLysSRARE2) (lane 1) and purified *Bli*PAE (6 μg, lane 2; 2 μg, lane 3) were separated on a 12% SDS polyacrylamide gel followed by Coomassie blue staining.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

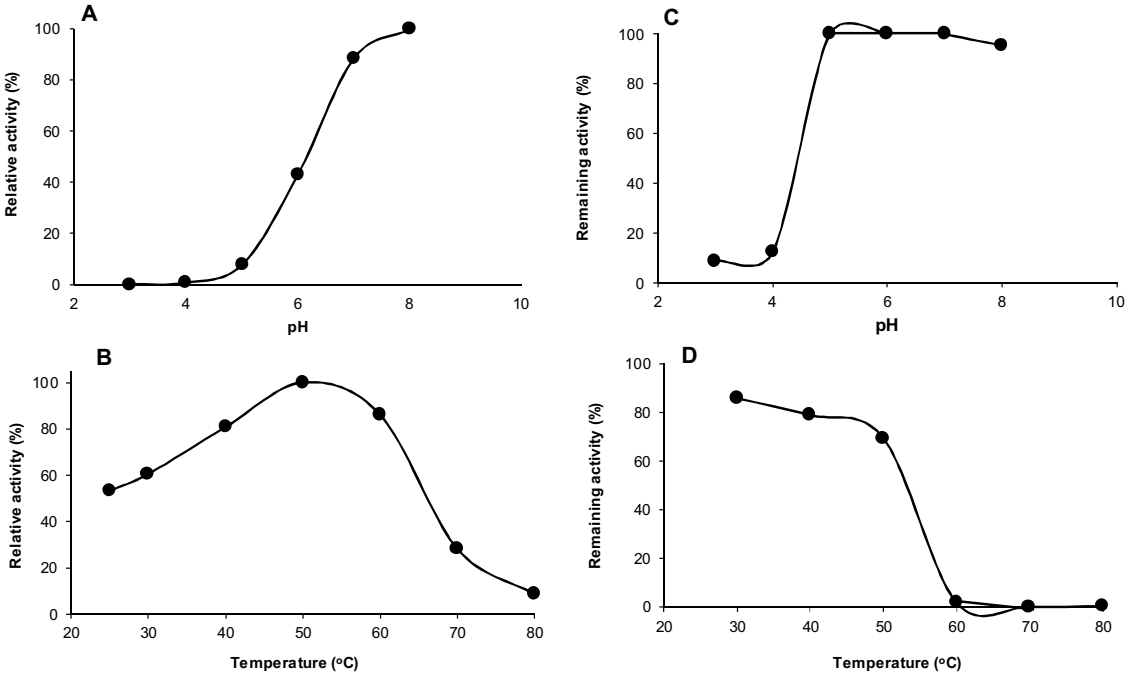

Fig. 2. Influence of different pH and temperatures on the activity and stability of *Bli*PAE. (A) pH 3.0–8.0 at 40 °C and (B) temperatures (25–80 °C) incubated at pH 6.5 for 10 min. *Bli*PAE stability was measured after the pre-incubation of the enzyme for 1 h at different pH 3.0–8.0 at 40 °C (C) and (D) temperature (30–80 °C) at pH 6.5. Relative activities are determined on SBP (DM 31, DA 24). The activity is expressed as a percentage of the maximum activity.

3.2.2. pH and temperature stability

To eliminate the role of auto hydrolysis occurring at pH 8.0 (material and methods) and to allow an extended digestion of the substrate, incubation at pH 6.5 and 40 °C for 24 h was performed. At these conditions, the enzyme maintained its activity of 70–90%. Fig. 2C shows that the enzyme retained more than 90% of its initial activity towards SBP (B3124) between pH 5.0 and 7.0. The enzyme was relatively stable up to 50 °C for 1 h with a residual activity of 70–90% (Fig. 2D).

The findings for *Bli*PAE are consistent with those reported for PAEs of *B. subtilis and E. chrysanthemi* (Bolvig et al., 2003). The optimum temperature found for *Bli*PAE is also similar to *Aspergillus* RGAE and PAE (Searle-van Leeuwen, Vincken, Schipper, Voragen, Beldman, Visser et al., 1996) and 10 °C higher compared to the optimal temperature found for *E. chrysanthemi* PAEs (Shevchik & Hugouvieux-Cotte-Pattat, 2003).

3.2.3. Specificity of BliPAE towards different substrates

Table 2 shows the specific activity of *Bli*PAE and total acetic acid released from different acetylated pectins. Incubation of the enzyme with pNPA showed an activity of 847 nkat/mg. The specific activity towards SBP6230, sunflower pectin, potato material and apple modified hairy region (MHR) was 302, 247, 243,107 nkat/mg, respectively. No activity was observed towards acetylated xanthan and acetylated eucalyptus xylan oligomers indicating that *Bli*PAE is specific towards acetylated pectins. The relatively lower specific activity of the enzyme for natural substrates (e.g. SBP, potato material and sunflower pectin) compared to pNPA is in agreement with the previous results for *Aspergillus* acetylesterases (Searle-van Leeuwen et al., 1996).

3.2.4. Efficiency of BliPAE towards various acetylated pectins

Endpoint incubation was carried out to explore the specificity of *Bli*PAE towards the RG-I and HG regions of pectin in detail. About 75% of the acetyl groups in SBP (DM 62, DA 30) are located on the HG region (Ralet et al., 2005). Digestion of SBP6230 by *Bli*PAE released 19% of the acetyl groups. A two-fold increase in activity (37%) was found when a low DM PME-modified pectin (F3331) was used. Sunflower pectin has high proportions of HG and low proportions of RG-I as indicated by the low levels of neutral sugars (Table 1) (Iglesias & Lozano, 2004; Miyamoto & Chang, 1992). *Bli*PAE removed 71% of the acetyl groups of the sunflower pectin (DM 60, DA 11) (Table 2). The results also suggest that the enzyme is able to cleave the acetyl groups, which are hypothesized to be in the HG region of sunflower pectin. Despite the high DM value, *Bli*PAE was able to remove a significant proportion of acetyl groups from sunflower pectin without PME modification. Table 2 shows that *Bli*PAE released 40% of the total acetyl groups present in potato material. The presence of the acetyl groups in potato homogalacturonan segments was described by Ishii (1997). Interestingly, *Bli*PAE only released 6% of acetyl groups from the RG-I rich apple MHR (Schols & Voragen, 1994), suggesting that the enzyme could not release the acetyl groups

Table 2
Specific activity and final acetate release of *Bli*PAE for different acetylated substrates.

Substrate	Specific activity nkat/mg protein[a]	Final acetate released[b] (%)
pNPA[c]	847	n.d
Sugar beet pectin (SBP 6230)	302	19
Sugar beet pectin (F3331)	302	37
Sunflower pectin	247	71
Potato material	243	40
Apple MHR	107	6
Okra RG region	0	n.d
Xanthan	0	n.d
Eucalyptus xylan oligomer	0	n.d

n.d: not determined.

[a] Specific activity: nanomol of acetate per second per mg protein. Incubation for 10 min, pH 6.5 at 40 °C.

[b] Acetic acid release: incubation for 24 h in 50 mM McIlvaine's buffer pH 6.5 at 40 °C.

[c] pNPA: incubation for 10 min in 0.05 M sodium phosphate buffer pH 7.3 at 37 °C.

of the RG region. The low amount of acetic acid release could even originate from the small proportion of acetylated and non-methylesterified GalA residues within the HG segments in apple MHR (Schols, Posthumus, & Voragen, 1990). This result is in accordance with the observation of Searle-van Leeuwen et al. (1992) for the deacetylation (10%) of the same substrate by *A. aculeatus* PAE. In addition, *Bli*PAE showed no detectable activity towards RG-I rich okra material fraction (Sengkhamparn et al., 2009), indicating that *Bli*PAE is unable to remove acetyl groups bound to rhamnosyl residues within the RG backbone of okra material.

In the *B. licheniformis* DSM13 genome annotation, *Bli*PAE was previously classified as an RGAE. However, our experimental findings show that *Bli*PAE acts specifically on the HG backbone of the pectin polymer and is therefore a PAE. Furthermore, the enzyme's activity towards various acetylated pectins is proven to be more efficient than the previously reported *Bacillus* PAEs, which removed only 5–25% of the maximum acetyl groups present in SBP1124 (Bolvig et al., 2003; Shevchik & Hugouvieux-Cotte-Pattat, 1997).

3.3. Mode of action of BliPAE

Since the proportion of methylester substitution as well as their distribution patterns are factors that can influence the efficiency of *Bli*PAE, the mode of action of *Bli*PAE was examined using differently methylesterified and acetylated SBP substrates.

3.3.1. Effect of methylesterification on the activity of BliPAE

SBP samples with different DM values (31–62) were used as substrates (Fig.3A). The maximum release of acetate from SBP observed with *Bli*PAE was 37% for low methylesterified SBP (F3331), suggesting that high levels of methylester substitution hindered the action of the enzyme. Furthermore, Fig. 3A shows that the level of total acetic acid release was slightly different for SBP samples having rather similar DA and DM. This variation in acetate release suggested that the action of *Bli*PAE is influenced by the different methylesterification patterns present in these pectins (Remoroza et al., 2014).

3.3.2. Effect of acetylation on the activity of BliPAE

The effect of acetylation towards *Bli*PAE activity was examined using different SBP series having a rather constant DM values (51–62) and different DA values (3–30) (Fig. 3B). The enzyme released 22% of the total acetyl groups from B5326 while only 13% of all acetyl groups were released for B′5803. This suggests that the activity of *Bli*PAE varies for pectins having different DA. It is postulated that it is not due to the level of acetyl groups but due to different positions of the acetyl groups (*O*-2 or/and *O*-3) within the HG part of the substrates. Hence, the site specificity of the enzyme was determined by enzymatic fingerprinting of the *Bli*PAE-modified SBP.

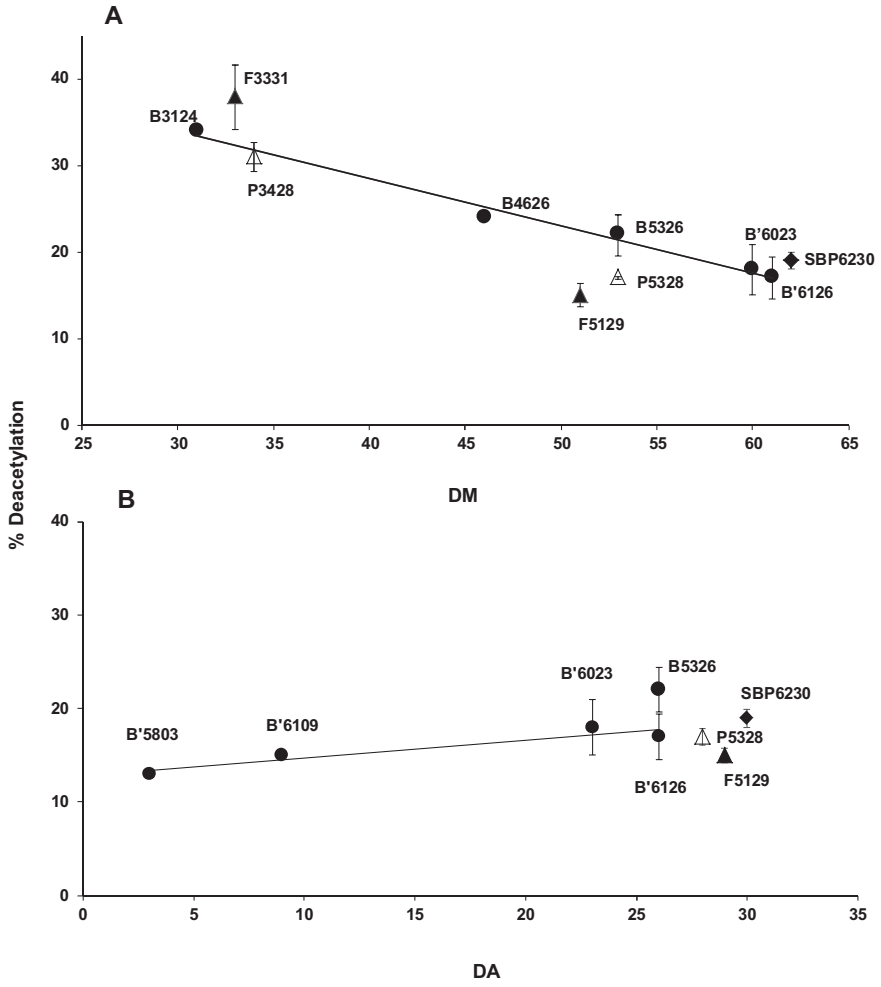

Fig. 3. Level of deacetylationof SBPs by *Bli*PAE as a function of DM (A) and DA (B). Solid line is the correlation for the B-series sugar beet pectins. SBP6230 (♦); B-series (□); F-series (▲); P-series (Δ). B, P and F series of SBP have different distributions of methylesters while maintaining the same level of acetyl groups (Remoroza et al., 2014).

3.3.3. Specificity of BliPAE towards the acetyl group position

It was hypothesized that the *Bli*PAE-modified SBP will lead to a more complete degradation of the HG backbone by endo-PGII and PL compared to the unmodified SBP. *Bli*PAE released a maximum of 34% of the total acetyl groups from P3429 resulting in a pectin with a DA 19. In order to elucidate the precise position of acetyl groups not removed by the enzyme, the *Bli*PAE-modified P3429 was digested using endo-PGII and PL followed by HILIC-MS^n analysis as described elsewhere (Remoroza et al., 2014) (Fig. 4). The RG-I region of the unmodified pectin was degraded by RG-I degrading enzymes added into the enzyme cocktail and generated $(Rha\text{-}GalA)_n$ and $(Rha\text{-}GalA)_2Ac$ (Fig. 4A). Fig. 4B shows that the $(Rha\text{-}GalA)_2Ac$ oligomers were still present after modification of P3429 by *Bli*PAE, which supports the hypothesis that *Bli*PAE removes only the acetyl groups from the HG segments of pectin. Non- and partially methylesterified and acetylated GalA oligomers ranging from DP 2 to 7 were released from the unmodified pectin by endo-PG and PL. The HILIC elution profile of unmodified P3429 digest shows the presence of different levels of acetylated GalA oligomers, e.g. pentamer DP 5 with a single acetyl group (5^{01}), hexamer (6^{01}) (Fig. 4A) and these GalA oligomers were acetylated either at the *O*-2 or *O*-3 position as annotated using MS^n (Remoroza et al., 2012). An overview of structures identified is presented in Table 3. In the *Bli*PAE-modified P3429 digest, GalA oligomers having *O*-2 acetylation were dominantly present, e.g. 4^{11}, 5^{11}, 6^{11}, 6^{21}. On the contrary, no GalA oligomers with *O*-3 acetylation were found. In addition, *Bli*PAE was not able to remove the acetyl group from a single GalA residue having both a methylester and acetyl group as exemplified for GalA oligomer 5^{11} $(GalA)_2GalA_{MeAc}(GalA)_2$ (Fig. 4). The reason for its resistance to *Bli*PAE degradation is not to have the methylester and acetyl group on a single GalA but to have the acetyl group on *O*-2 position. Most of the *O*-3 acetylated GalA units in the oligomers initially present in unmodified P3429 were not observed in the *Bli*PAE-modified P3429 digest. This result shows that the enzyme specifically removes acetyl groups from GalA residues within HG having the acetylation at the *O*-3 position. Moreover, it is interesting to note that whenever the methylester and acetyl group are present on a single residue, the acetyl group is located on *O*-2.

^1H NMR analysis allows the localization of acetyl groups on GalA residues (Ishii, 1997) and can confirm the site specificity established by MS^n analysis for *Bli*PAE. Fig. 5 shows the ^1H NMR data for the acetyl proton region for two different SBPs before and after *Bli*PAE modification. As seen from NMR patterns of the unmodified B5519 and B1713, alkali de-esterification resulting in different positions of the remaining acetyl groups and can be visualized by the ratio of *O*-3 and *O*-2 acetylation. Three well distinct chemical shifts for acetyl groups at 2.20, 2.10 and 2.02 ppm were observed for B5519 (Fig. 5A). The ^1H NMR analysis of B5519 shows mainly one (*O*-3) acetyl signal (2.10 ppm; ~30% of the total) next to the *O*-2 acetyl group (2.02 ppm; ~5% of the total) within the nonmethylesterified GalA residues. For B1713, the *O*-2 acetyl signal at 2.02 ppm disappeared (Fig. 5B). This might be explained by the fact that this acetyl group is more distant from the (negatively charged) carboxylate group and is easier to be removed by extended treatment with mild alkali. This indicates that chemical de-esterification might be more specific than expected.

Upon treatment with *Bli*PAE, B5519 and B1713 pectins resulted in a removal of 30% and 75% of the total acetyl groups present, respectively. After treatment by *Bli*PAE, an almost complete disappearance of the signal at 2.10 ppm was observed. The signal at 2.20 ppm remained unchanged whereas the signal around 2.02 ppm was slightly increased. From previous ^1H NMR studies on acetylated pectins, the chemical shift at 2.09–2.11 ppm are known to correspond to single acetylation at the *O*-3 position of nonmethylesterified GalA residue in homogalacturonan (Ishii, 1997; Perrone, Hewage, Thomson, Bailey, Sadler, & Fry, 2002). The chemical shift around 2.00–2.04 ppm has been reported to represent the nonmethylesterified GalA residues with acetylation at the *O*-2 position whereas various signals at 2.18–2.21 ppm represent the *O*-2/*O*-3 acetyl positions from methylesterified GalA residues and

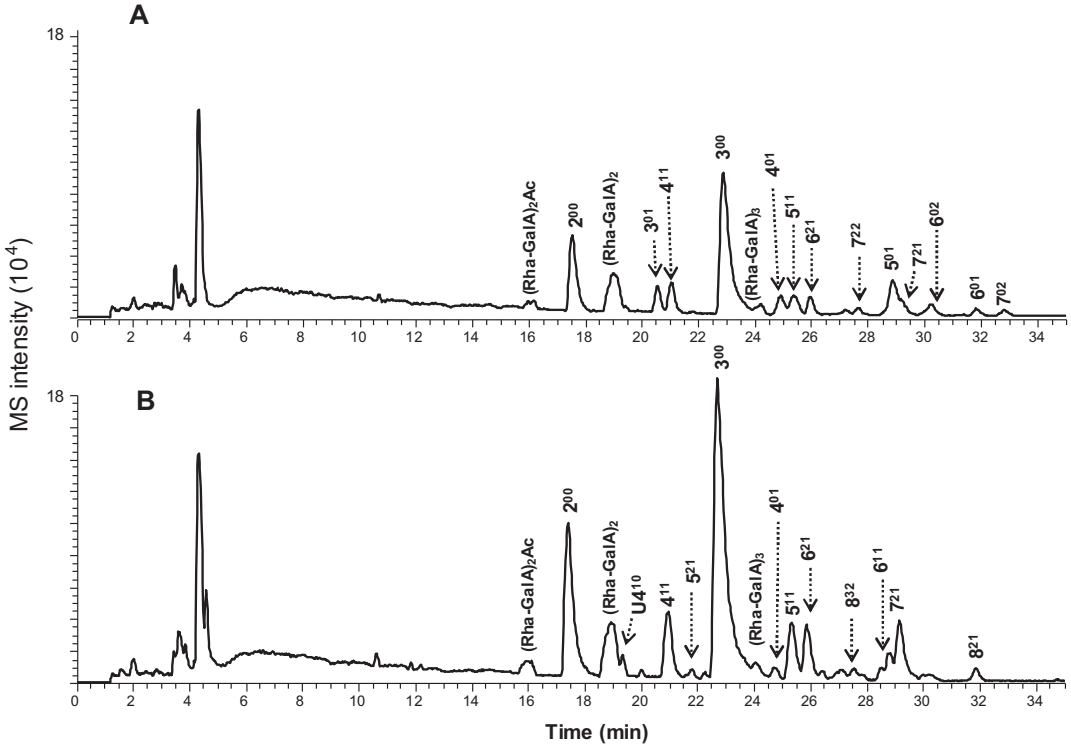

Fig. 4. HILIC elution patterns of (A) P3429 (DM 34 DA 29) and (B) *Bli*PAE-modified P3429 after digestion by a cocktail of RG-I and HG degrading enzymes using ESI-IT-MS^n detection. Peak annotation: 5^{11}, DP 5, 1 *O*-methylester, 1 *O*-acetyl group. Rha: rhamnose, GalA, galacturonic acid; Ac: acetyl group.

Table 3
Structures of different methylesterified and/or acetylated GalA oligomers released from *Bli*PAE modified and unmodified P3429 after digestion by a cocktail of enzymes as analyzed by LC-HILIC-MS^n (Fig. 4).

Component	Structure	Unmodified P3429	*Bli*PAE modified P3429
3^{01}		+	–
3^{01}		+	–
4^{11}		+	+
4^{01}		+	–
4^{01}		+	+
5^{01}		+	–
5^{11}		+	+
5^{11}		+	+
5^{21}		–	+
6^{11}		+	+
6^{21}		+	+
6^{01}		+	–
6^{02}		+	–
7^{21}		–	+
7^{02}		+	–

Nonesterified GalA ; methylester ; acetyl group ▲ *O*-2, Δ *O*-3.
Peak annotation: 5^{11}, DP 5, 1 *O*-methylester, 1 *O*-acetyl group; + detected; – not detected.

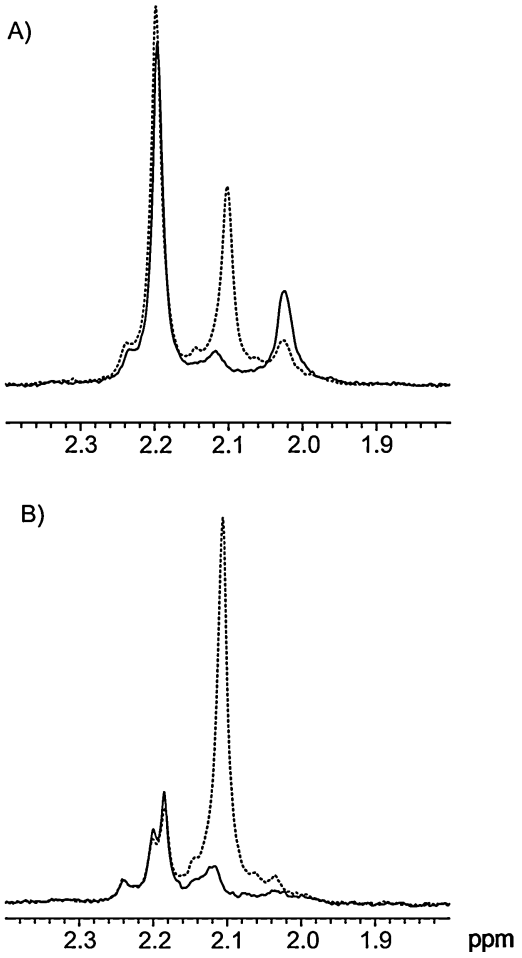

Fig. 5. ^{1}H NMR data of two SBPs samples before and after modification by *Bli*PAE (A) B5519 (DM 55, DA 19) and (B) B1713 (DM 17, DA 13). Unmodified SBP (-----); *Bli*PAE-modified SBP (—).

the double acetylated GalA residues (Perrone et al., 2002; Renard & Jarvis, 1999). In conclusion, NMR spectroscopy clearly proves that *Bli*PAE specifically deacetylates the acetyl group from the *O*-3 position of acetylated pectins.

SBP substrate (B1713) was also examined by NMR spectroscopy. In this sample, the signals at 2.20 and 2.10 ppm were present (Fig. 5B). This pectin had the majority of acetyl group substituted to GalA *O*-3 (2.10 ppm), which was removed upon incubation by *Bli*PAE. From the NMR signals before and after *Bli*PAE treatment, it is calculated that *Bli*PAE removed about 75% of all acetyl groups present and almost all acetyl groups substituted to GalA *O*-3. Obviously, the 75% removal of acetyl groups was much higher for B1713 compared with the deacetylation levels of the mother pectin SBP6230 and the alkali de-esterified SBPs (B-series) by *Bli*PAE (Section 3.3) and by AspacAE (Bonnin et al., 2008).

The difference in level of deacetylation of B1713 can be explained by the different position of acetyl groups present in the various sugar beet pectins (Fig. 5A versus B). It is now clear that *Bli*PAE specifically deacetylates the *O*-3 acetyl group in GalA residues in nonmethylesterified HG region. The removal of acetyl groups from B5519 and B1713, 5.7 DA units was removed from B5519 while 9.7 DA units was removed from B1713. The significant released of acetate is relevant for the two substrates. This can be explained by the positions of the acetyl groups at the *O*-3. The removal of acetyl groups by *Bli*PAE from sunflower pectin (Table 2) and from potato material are located at the *O*-3 position of GalA residues in HG region (unpublished results; (Ishii, 1997). Hence, the remaining acetyl groups from the potato material (~10 DA units) are not mainly located at *O*-3 position of the HG region.

4. Conclusions

A recombinant acetylesterase, *Bli*PAE, originating from *B. licheniformis* DSM13, has been identified and characterized to be a new PAE member of the carbohydrate esterase family 12. It was demonstrated for the first time that a *Bacillus* PAE specifically removes *O*-3 linked acetyl groups from the nonmethylesterified HG region of SBP. *Bli*PAE has proven to be more efficient in catalyzing the removal of acetyl groups in SBPs and other *O*-3 acetylated pectins compared to other bacterial PAEs. Also, one may infer from this study that this enzyme has the capability of being used in future studies to provide addition information on pectin fine structure.

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