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

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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

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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.)