Food Hydrocolloids 39 (2014) 319–329

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Characterization of charged functional domains introduced into a modified pectic homogalacturonan by an acidic plant pectin methylesterase (*Ficus awkeotsang* Makino) and modeling of enzyme mode of action☆

Yang Kim [a], Martin A.K. Williams [b,c,d], Jason T.C. Tzen [e], Gary A. Luzio [a], Ashley L. Galant [a], Randall G. Cameron [a,*]

[a] Citrus and Other Subtropical Products Research Unit, US Horticultural Research Laboratory, US Department of Agriculture, Agricultural Research Service, 2001 South Rock Road, Fort Pierce, FL 34945, USA
[b] Institute of Fundamental Sciences, Massey University, Science Tower C4.09, Turitea Site, Palmerston North Campus, Private Bag 11222, Palmerston North 4442, New Zealand
[c] The Riddet Institute, Palmerston North, New Zealand
[d] The MacDiarmid Institute for Advanced Materials and Nanotechnology, New Zealand
[e] Graduate Institute of Biotechnology, National Chung-Hsing University, 250 Kuo Kuang Rd., Taichung 402, Taiwan, ROC

ARTICLE INFO

Article history:
Received 17 September 2013
Accepted 14 January 2014

Keywords:
Pectin
Nanostructure
Pectin methylesterase
Enzyme mode of action
Rheology

ABSTRACT

An acidic plant pectin methylesterase from *Ficus awkeotsang* achenes (*Fa*PME) was used to demethylesterify a model homogalacturonan (HG) at pH 4.5 and 7.5. Introduced demethylesterified blocks (DMBs) were released by a limited endo-polygalacturonase (EPG) digestion, separated and quantified by HPAEC. The average DMB size ($\overline{BS}$) and number of such blocks per molecule ($\overline{BN}$) differed depending on the degree of methylesterification (DM) and reaction pH ($P < 0.05$). Significant increases in $\overline{BS}$ and $\overline{BN}$ were observed in HGs of 30% DM compared to higher DMs. HGs demethylesterified to 30% and 50% DM at pH 4.5 showed significantly larger $\overline{BS}$ compared to pH 7.5. Absolute degree of blockiness (DB_{abs}), obtained using exhaustive EPG digestions, displayed a linear relationship with the DM regardless of reaction pH ($P < 0.001$). The distribution of DMBs released by the limited EPG digest was predicted by mathematical modeling and compared with the experimental results. The *in silico* modeled enzyme mode of action suggested that a random, multiple chain, non-processive mode of action best explains the distributions of small blocks ($\overline{BS} \geq 11$) and a processive multiple attack mechanism best explains the distributions of longer blocks. Decreasing the DM of the HGs by the *Fa*PME increased the G' and G'' of calcium-mediated gels. Pearson's correlation displayed significant correlation coefficients between $\overline{BS}$, $\overline{BN}$, DB_{abs}, DM, and G'. The results suggest the possibility to control $\overline{BS}$ and to produce a uniform population of demethylesterified pectin molecules, particularly in acidic environments where most basic plant PMEs are less active.

Published by Elsevier Ltd.

1. Introduction

Pectin is a natural substance found in plant cell walls and one of the most abundant polysaccharides on earth (Atmodjo, Hao, & Mohnen, 2013). Galacturonic acid (GalA) is a main constituent of the three major pectic domains, homogalacturonan (HG), rhamnogalacturonan I and II (RG I and RG II). HG, the dominant pectic domain, is a linear polymer of 1,4-linked α-D-galacturonic acid (~100 GalA residues which would be ~41 nm in length) (Ralet et al., 2008; Round, Rigby, MacDougall, & Morris, 2010; Thibault, Renard, Axelos, Roger, & Crepeau, 1993; Yapo, Lerouge, Thibault, & Ralet, 2007). The functionality of pectin directly depends largely

* Corresponding author. Tel.: +1 772 462 5856; fax: +1 772 462 5986.
E-mail addresses: ya_kim@hotmail.com (Y. Kim), m.williams@massey.ac.nz (M.A.K. Williams), TCTZEN@dragon.nchu.edu.tw (J.T.C. Tzen), Gary.Luzio@ars.usda.gov (G.A. Luzio), Ashley.Galant@ars.usda.gov (A.L. Galant), Randall.Cameron@ars.usda.gov (R.G. Cameron).

0268-005X/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.foodhyd.2014.01.022

on the amount and distribution of contiguous unmethylesterified GalAs in the linear polymeric backbone. Currently, commercial pectins are classified solely by their DM into high (>50%) or low methoxyl pectin (<50%) however, the pattern of methylesterification has important commercial implications due to its impact on the functional properties of pectins (Duvetter et al., 2009; Thibault et al., 1993; Van Buggenhout et al., 2006).

Pectin methylesterase (PME; EC 3.1.1.11), a ubiquitous plant enzyme that hydrolyzes the C6 methyl ester on GalA residues of pectic HG, has been successfully applied to modify the physical properties (charge distribution) and resulting functionality of pectins (Giovane et al., 1996; Kim, Yoo, Kim, Park, & Yoo, 2008; Willats, Knox, & Mikkelsen, 2006). Owing to its pectin modifying properties PME is regarded as an essential enzyme in many food, cosmetic, and pharmaceutical applications (Denes, Baron, Renard, Pean, & Drilleau, 2000; Hotchkiss et al., 2002) and has been regularly used in wine and juice industries (Kashyap, Vohra, Chopra, & Tewari, 2001; Savary, Hotchkiss, Fishman, Cameron, & Shatters, 2003). It is known that processive, blockwise demethylesterification commonly observed with plant PMEs with alkaline isoelectric points produces polymers that are more sensitive to calcium cross-linking compared to those of the same DM generated by random demethylesterification by chemical saponification or fungal PMEs having acidic isoelectric points (Ngouémazong et al., 2012).

Both enzymatic and physical methods have been used to characterize the charge distribution within a population of pectin molecules, including exploiting digestion with endo-polygalacturonase (EPG) (Cameron, Luzio, Goodner, & Williams, 2008; Cameron, Luzio, Vasu, Savary, & Williams, 2011; Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Guillotin et al., 2005; Ngouémazong et al., 2011; Tanhatan-Nasseri, Crepeau, Thibault, & Ralet, 2011), pectin lyase (Ralet et al., 2012) and using NMR to estimate relative frequencies of nonmethylesterified diads and triads (Grasdalen, Andersen, & Larsen, 1996; Kim, Teng, & Wicker, 2005; Winning, Viereck, Salomonsen, Larsen, & Engelsen, 2009).

EPG has been used in two distinct ways: one method utilizes a *limited* EPG digest to excise random sections of the *in-pectin* demethylesterified blocks (DMBs) and by characterizing their distributions in pectin by estimating average block size ($\overline{BS}$) and block number per molecule ($\overline{BN}$). The original block distribution is then inferred by matching the distribution of excised fragments to that obtained by modeling the PME enzyme mode of action under different reaction conditions (Cameron et al., 2008, 2011; Kim et al., 2013). It was suggested by these studies that $\overline{BS}$ and $\overline{BN}$ could be manipulated not only by the final DM, but also by reaction conditions such as pH. The second EPG-based method involves an *exhaustive* digest to estimate the so-called degree of blockiness (DB) and the absolute degree of blockiness (DB_{abs}) parameters by assessing how much of the unmethylesterified galacturonic acid can be liberated as monomer, dimer and trimer (Daas et al., 1999; Daas, Voragen, & Schols, 2000; Guillotin et al., 2005; Kim et al., 2013; Ngouémazong et al., 2011; Tanhatan-Nasseri et al., 2011). It has been reported that the higher the DB of pectins of a similar DM, the more blockwise the distribution of the nonmethylesterified GalAs in the pectin (Daas et al., 1999, 2000). However, pectins having similar DM and DB values may still differ in the size of the blocks.

Studies on the manipulation of charge distribution within a population of pectin molecules have centered on characterizing the differences obtainable when using enzymatic and chemical methods of demethylesterification and descriptions of the charge distribution within a pectin population have therefore focused on the ordered vs. random nature. Plant PMEs with a basic isoelectric point (p*I*) have been shown to produce ordered contiguous distributions of demethylesterified GalAs within HG regions while fungal PMEs (*Aspergillus niger* PME, *An*PME), with an acidic p*I*, as well as base-catalyzed demethylesterification produce random distributions (Cameron et al., 2008, 2011; Duvetter et al., 2006; Kim et al., 2013; Willats et al., 2001). Three generalized PME modes of action have been described based largely on the description of starch depolymerizing enzymes: 1) a single chain mechanism in which the enzyme binds the substrate and hydrolyzes methyl esters on contiguous GalA residues until an nonmethylesterified residue or branch point is reached before the enzyme–substrate complex dissociates, 2) a multiple chain mechanism in which only a single methyl ester is hydrolyzed for each enzyme–substrate interaction, and 3) a multiple attack mode of action which is intermediate between the other two and in which some number of reactions are catalyzed by the enzyme between formation of the enzyme–substrate complex and dissociation from the polymer. These three models describe a continuum of enzyme processivity, from dissociation following a single catalytic event to dissociation following tens, hundreds or thousands of catalytic events (Campa et al., 2004; Greenwood & Milne, 1968; Robyt & French, 1970). A molecular basis for the processive action of an *Erwinia* PME has been recently elucidated (Fries, Ihrig, Brocklehurst, Shevchik, & Pickersgill, 2007; Mercadante, Melton, Jameson, Williams, & Simone, 2013). The report by Mercadante et al. (2013) suggests that the degree of processivity (p) may change as the size of the demethylesterified block increases.

Over the last twenty years, an acidic plant PME isolated from jelly fig (*Ficus awkeotsang*) achenes has been purified and well-characterized (Ding, Hsu, Wang, & Tzen, 2002; Ding, Lee, Wang, Tai, & Tzen, 2000; Lin, Liu, Chen, & Wang, 1989; Winning, Viereck, Norgaard, Larsen, & Engelsen, 2007). Interestingly, the jelly fig PME (*Fa*PME) possesses a p*I* of pH 3.5 as established by isoelectric focusing of the purified enzyme (Lin et al., 1989) which is in contrast with the much higher p*I* of most known plant PMEs (pH 9–11) (Bordenave, 1996). Micheli, Sundberg, Goldberg, and Richard (2000) described alkaline, neutral and acidic PME isoforms in various tissues present in cross sections through the cambial regions of hybrid Aspens. Analysis of 99 entries for PMEs or putative PMEs (fragments excluded) in the ExPASy proteomics database (Artimo et al., 2012) using the ProtParam tool to estimate p*I*, including the 66 identified from *Arabidopsis*, also identified the three charge classes of PMEs. Of the total number of PMEs with sequence data 85 were from higher plants. Of these 75% had alkaline p*I*s (>pH 7.5), 20% were acidic (<pH 6.5) and 5% had a neutral p*I* (pH 6.5–7.5). Of the 66 *Arabidopsis* PMEs included 73% were alkaline, 23% acidic and 4% neutral.

The acidic *Fa*PME is somewhat thermally tolerant (up to 60 °C) and maintains its enzymatic activity over a broad pH range. The *Fa*PME was also successfully expressed and secreted into the cultured medium of *Pichia pastoris* X-33 transformants and possessed equivalent enzymatic properties and thermo-tolerance in comparison with native *Fa*PME (Hsiao et al., 2009; Peng, Hsiao, Ding, & Tzen, 2005). This recombinant technique enhances the possibility of industrial utilization of the enzyme since direct purification of the enzyme from jelly fig achenes is not economically effective. Even though the stability and enzymatic properties afford this recombinant *Fa*PME's potential industrial utility (Komae, Sone, Kakuta, & Misaki, 1990) there is no information related to its, or any acidic plant PME, mode of action or the effect of its use on the resulting functionality of modified pectins.

The objectives of this study were to characterize the nanostructural features introduced during demethylesterification of a model homogalacturonan with the recombinant *Fa*PME and to thereby elucidate something of the enzyme's mode of action (p) at pH 4.5 and 7.5. We do this by analyzing the results from