Innovative Food Science and Emerging Technologies 29 (2015) 295–301

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Recombinant kiwi pectin methylesterase inhibitor: Purification and characterization of the interaction with plant pectin methylesterase during thermal and high-pressure processing

Xiaohong Mei [a,b], Avi Shpigelman [b], Tina A.J. Verrijssen [b], Clare Kyomugasho [b], Yunbo Luo [a], Ann M. Van Loey [b], Chris Michiels [c], Kunlun Huang [a], Marc E. Hendrickx [b,*]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Laboratory of Food Technology and Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Leuven 3001, Belgium
[c] Laboratory of Food Microbiology and Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Leuven 3001, Belgium

ARTICLE INFO

Article history:
Received 15 September 2014
Received in revised form 13 February 2015
Accepted 18 February 2015
Available online 7 March 2015

Keywords:
Pectin methylesterase
Pectin methylesterase inhibitor
Pichia pastoris
Protein purification
Complex formation
Thermal and high-pressure processing

ABSTRACT

Recombinant kiwi pectin methylesterase inhibitor (rPMEI) with a six-histidine tag at the C-terminus was successfully expressed in *Pichia pastoris* GS115 strains. rPMEI was purified using pectin methylesterase (PME)-CNBr-affinity chromatography or nickel affinity chromatography. The purified rPMEI had a slightly higher molecular weight than the kiwi PMEI probably due to the presence of added six histidines and some glycosylation. The expression in *P. pastoris* did not result in changes in the inhibitory activity of rPMEI, nor did it significantly change the complexation with PME under thermal and high pressure processing conditions compared to kiwi PMEI. While thermal treatment of an equimolar PME–rPMEI complex resulted in the aggregation of PME and rPMEI as an entity, high-pressure processing led to the dissociation of PME–rPMEI complex resulting in the presence of free rPMEI after processing. The obtained results presented a viable option for utilization of recombinant PMEI for control of PME activity.

Industrial relevance: The discovery of a pectin methylesterase inhibitor (PMEI) in kiwi fruit opened an additional way to control PME activity, although the industrial application is inhibited by the low yield obtainable from kiwi. For a possible utilization of PMEI in large scale production, recombinant PMEI (rPMEI) was modified with an additional six-histidine tag, expressed and studied. Our results showed that the expression of rPMEI with the additional six-histidine tag allows a feasible large scale purification of the inhibitor using nickel affinity chromatography without negatively affecting the inhibitory activity nor significantly changing the complexation with PME compared to kiwi PMEI. Thus the combination of rPMEI with high-pressure treatment can be exploited to process food systems where cloud stability is of importance but the high pressure itself is insufficient to completely inhibit the PME.

1. Introduction

Cloud stability of fruit and vegetable juices is important for the food industry. The cloud is a colloidal suspension composed of protein, pectin, lipids, cellulose and hemicelluloses (Baker & Cameron, 1999; Klavons, Bennett, & Vannier, 1991) existing in the form of solid particles having different sizes and shapes (Baker, 1977). It has a close relationship with the color, flavor, turbidity and aroma of vegetable and fruit juices (Baker & Cameron, 1999), therefore being an important quality factor for consumers. During processing and storage of juices, cloud loss often occurs. The main reason indicated for cloud loss is pectin methylesterase (PME, EC 3.1.1.11) activity that de-esterifies pectin, resulting in low methoxyl pectin that can interact with calcium ions forming insoluble pectates (Joslyn & Pilnik, 1961). The control of endogenous PME activity is a key in the production of cloud stable vegetable and fruit juices. Thermal treatment is the conventional method to inactivate PME, but complete thermal inactivation may lead to reduced nutritional and sensory attributes (Irwe & Olsson, 1994). High pressure treatment is a relatively novel food processing technology capable of PME inactivation (Jolie et al., 2012). Although high pressure treatment retains the nutritional and sensory attributes of fruit and vegetable

Abbreviations: PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; rPMEI, recombinant pectin methylesterase inhibitor; HPSEC, high performance size exclusion chromatography; RI, refractive index; MALLS, multi-angle laser light scattering.

* Correspondence to: M.E. Hendrickx, Kasteelpark Arenberg 22, Box 2457, 3001 Leuven, Belgium. Tel.: +32 16321572; fax: +32 16321960.
E-mail address: Marc.Hendrickx@biw.kuleuven.be (M.E. Hendrickx).

http://dx.doi.org/10.1016/j.ifset.2015.02.008
1466-8564/

juices, the currently used conditions at industrial scale (600 MPa for some minutes) often do not completely inactivate some PMEs and even sometimes can result in enhanced enzyme activity (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998).

The discovery of a pectin methylesterase inhibitor (PMEI) in kiwi fruit opened an additional way to control PME activity (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990). The complete sequence of kiwi PMEI is composed of 152 amino acid residues, yielding a molecular weight of 16.3 kDa and an acidic isoelectric pH (around 3.5) (Camardella et al., 2000; Giovane, Balestrieri, Quagliuolo, Castaldo, & Servillo, 1995). Kiwi PMEI is known to inhibit most plant PMEs but unable to inhibit microbial PMEs (Jolie, Duvetter, Van Loey, & Hendrickx, 2010). Studies using size exclusion chromatography and surface plasmon resonance revealed that kiwi PMEI selectively combines with active carrot PME (Jolie et al., 2009, 2010) in a reversible, non-covalent 1:1 complex (Giovane et al., 1995). The three-dimensional structure of the complex between tomato PME and kiwi PMEI indicates that PMEI covers the shallow cleft of PME where the putative active site is located (Di Matteo et al., 2005). Therefore, PMEI prevents PME from binding to the pectin substrate and thus inhibits the enzyme activity. The PME–PMEI complex is strongly affected by temperature and pH. The carrot PME–kiwi PMEI complex was shown to form large particles aggregating as a single entity during heat treatment (55–65 °C), whereas high pressure treatment led to complex dissociation prior to aggregation of PME alone (Jolie et al., 2009). Kiwi PMEI has a broad application prospect in food industry and research. It can be exploited to detect and inhibit residual PME activity in fruit and vegetable juices (Giovane et al., 1991, 1996), and can be used as a molecular probe for in situ detection of plant PMEs (Jolie et al., 2010). The low extract yield from kiwi fruit has already become a bottleneck to restrain further application of kiwi PMEI. The previously reported high expression (200 mg/L) of kiwi PMEI in *Pichia pastoris* seems to accelerate the possible utilization of this inhibitor for large scale production (Mei, Hao, Zhu, Gao, & Luo, 2007). Due to the fact that expression in *P. pastoris* can induce structural changes in recombinant proteins (for example due to glycosylation) that may result in enhanced or reduced bioactivity and variation in thermostability (Li et al., 2007), care has to be taken to verify the inhibitory activity (compared to kiwi PMEI) and to study the interaction of the recombinant inhibitor with PME under processing conditions. Additionally for a successful and feasible implementation an applicable method for the purification of the recombinant PMEI (rPMEI) is needed.

In this work, the production, purification and inhibitory activity of rPMEI containing a six-histidine tag for feasible large scale purification (Gaberc-Porekar & Menart, 2001) were studied. rPMEI was purified by PME-CNBr-affinity chromatography or by nickel affinity chromatography that utilizes the added six histidines. In addition, the effects of temperature and high-pressure on the complex of carrot PME–rPMEI were studied in order to confirm that the expression in *P. pastoris* did not result in significant modification of stability under processing conditions. This was achieved by utilization of an integrated high-performance size exclusion chromatography combined with refractive index (RI) and multi-angle laser light scattering (MALLS).

2. Materials and methods

2.1. Materials

Fresh Belgian orange carrots (*Daucus carota* cv. Nerac) and ripe kiwi fruits (*Actinidia deliciosa* cv. Hayward) were purchased from a local supermarket. Vector pPIC9K (Invitrogen, San Diego, CA) was used for gene cloning. *P. pastoris* GS115 (*his4*) (Invitrogen, San Diego, CA) was used for protein expression. Apple pectin (70–75% methyl esterified) was obtained from Fluka (Buchs, Switzerland). Demineralized water (18 MΩ·cm) was obtained from a water purification system (Elga, High Wycombe Buck, UK). Orange PME-CNBr-Sepharose matrix and kiwi PMEI-NHS-activated Sepharose matrix were previously produced in the lab of Food Technology (Jolie et al., 2009) (KU Leuven, Belgium). HIS-Select Nickel Affinity Gel was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and obtained from local commercial resources.

2.2. Construction of the recombinant plasmid pPIC9k-PMEI and transformation of P. pastoris GS115

The gene sequences (from base pair position 152 to base pair position 610) encoding the open reading frame of kiwi PMEI (GenBank, accession no. AB091088) were synthesized by AuGCT Biotechnology Company (Beijing AuGCT DNA-SYN Biotechnology Co. Ltd., Beijing, China). The fragment was introduced into the expression vector pPIC9k between the restriction enzyme sites of *Eco*R I and *Not* I to construct the recombinant plasmid pPIC9k-PMEI. Eighteen nucleotides (CATCATCACCATCATC AT) coding for a six-histidine tag were added to the C-terminal sequence of *PMEI*. The recombinant plasmid pPIC9k-PMEI was transformed into *P. pastoris* GS115 cells as previously described (Mei et al., 2007).

2.3. Production of rPMEI

rPMEI was produced according to the previous method (Mei et al., 2007). The cell culture supernatant was collected by centrifugation (15,000 × *g*, 5 min) and then stored in −80 °C.

2.4. Determination of protein concentration

The protein content of the purified carrot PME and rPMEI was measured using bicinchoninic acid (BCA) method (Smith et al., 1985). The protein concentration (mg/mL) was calculated by comparison with a standard curve of bovine serum albumin (Jolie et al., 2009).

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE and native PAGE were performed on a PhastSystem (GE Healthcare, Uppsala, Sweden). For native PAGE a homogeneous 20% phastgel and native buffer strips from GE Healthcare were used and the samples were dissolved in a PAGE buffer (50 mM Tris–HCl and 5 mM EDTA, pH 8.0). For SDS-PAGE a homogeneous 20% phastgel and SDS buffer strips from GE Healthcare were used. The samples were dissolved in a SDS-PAGE buffer (50 mM Tris–HCl and 5 mM EDTA, pH 8.0 containing 2.5% SDS and 5% mercaptoethanol). The samples were then heated at 100 °C for 5 min. The above gels were stained with the silver staining method according to Heukeshoven and Dernick (1985). The molecular weight of the protein samples was estimated by comparing the migration distance with those of standard protein markers (Low Molecular Weight SDS Marker Kit, ranging from 14.4 to 97.0 kDa).

2.6. Purification of rPMEI using nickel affinity chromatography

About 25 mL of His-Select Nickel Affinity Gel was suspended using 250 mL of 50 mM sodium phosphate, pH 8.0, containing 0.3 M NaCl. Then the gel was mixed with approximately 80 mL of the cell culture supernatant on an orbital shaker (175 rpm) for 15 min. The mixture was washed with 250 mL of 50 mM sodium phosphate, pH 8.0, containing 0.3 M NaCl for several times until the A_{280} of the elute remained stable. The affinity gel was washed with 50 mL of elution buffer (50 mM sodium phosphate, pH 8.0, with 0.3 M NaCl and 250 mM imidazole). The eluted fractions were combined to a single pool and concentrated using Vivacell 100 centrifugal filters (5 kDa MWCO, Sartorius, Goettingen, Germany). The concentrated purified rPMEI was dissolved in 10 mM Na-phosphate pH 6.5. Then it was immediately frozen in liquid nitrogen and stored at −80 °C.