

Analytical Methods for Pectin Methylesterase Activity Determination: a Review

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Abstract Pectin methylesterase is an enzyme with an important *in vivo* role in plants as well as in food industry. This enzyme catalyzes the hydrolysis of methyl ester bounds in pectin which is one of the main components of cell wall in plants, producing methanol and free carboxylic groups. The effect of pectin methylesterase in food quality has been extensively studied, producing desirable effects in texture improvement as well as undesirable effects in some beverages. Likewise, the low methoxyl pectin produced by this enzyme has characteristics that contribute to formulate best quality food products. Pectin methylesterase is a ubiquitously enzyme that presents multiple isoforms, but is not only present in plants; it is also found in fungi, bacteria, and yeast, which have specific chemical and physical characteristics. The latter makes the task of analyzing the wide variety of these enzymes with its specific characteristics difficult. Based on this enzyme relevance and the aforementioned, multiple methods have been developed in order to evaluate pectin methylesterase activity with different research objectives. In this paper, the importance of the enzyme as well as advantages and drawbacks of the different methods will be discussed besides applications and evolution of these will be mentioned. Additionally, this paper will improve the understanding of the systems used in pectin methylesterase activity analysis.

Keywords Pectin methylesterase (PME) · Alcohol oxidase (AO) · Pectin · Methanol · Carboxyl groups

Introduction

Pectin has many functions in plant growth and development, as well as plant defense. In food industry, it is used as a gelling and stabilizing agent. Pectins are a family of complex galacturonic acid-rich polysaccharides; these are considered as one of the three main components of primary cell wall and middle lamella in higher plants (Mohnen 2008). Pectins make up approximately 35% of dry weight of dicot cell walls (Micheli 2001), but in some fruits represent a higher amount, frequently more than 50% in cell wall composition (Castillejo et al. 2004). Pectic polymers are essentially composed by three main domains denominated: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II; nevertheless, other substituted galacturonans are recognized (e.g., xylogalacturonan and apiogalacturonan), which are found at lower extent (Harholt et al. 2010; Mohnen 2008; Somerville et al. 2004).

Homogalacturonan (HG) is the main pectic substance and composes between 50 and 70% of pectin in primary cell wall. It is composed by D-galacturonic acid residues (GalA) linked by glycosidic α (1,4) bonds (Verhertbruggen and Knox 2007; Yapo et al. 2007). The backbone of HG has been estimated as long as 100 residues of GalA (Wolf et al. 2009). HG is partially methyl-esterified on carboxyl group at the C6; however, depending on its source could be acetylated on hydroxyl groups at C2 and/or C3. Lineal units of HG in which more than 50% of GalA is methyl-esterified at the C6 are usually called “high methoxyl” pectins; meanwhile, those with lower percentage are denominated “low methoxyl” pectins (Ochoa-Villarreal et al. 2012).

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Moreover, other predominant domain in pectins is rhamnogalacturonan I (RG-I). This polysaccharide accounts for 20–35% of pectin. RG-I comprises a backbone constituted by a repeated disaccharide of GalA and rhamnose (L-Rha) [α -(1,2)-D-GalA- α -(1,4)-L-Rha] (Ridley et al. 2001). RG-I may be substituted in positions O-4 and/or O-3 of L-Rha residues with a single residue of a neutral glycoside and with lateral polymeric chains, mainly of α -(1,5)-L arabinans and β -(1,4)-D galactans, arabinogalactans-I, arabinogalactans-II, and likely galactoarabinans. Likewise, the backbone can be O-acetylated at the C2 and/or C3 in L-Rha residues (Jayani et al. 2005). Side chains mainly contain lineal or ramified residues of α -L-arabinose and/or β -D-galactose. The ramified chains also present high heterogeneity, which is related to the plant source. Additionally, other saccharides like α -L-fucose, β -D-glucuronic acid, and 4-O-methyl- β -D-galacturonic acid are present, such as ferulic and cumaric acids (Øbro et al. 2004; Ochoa-Villarreal et al. 2012). Finally, rhamnogalacturonan-II (RG-II) is the most complex and ramified domain in pectin. This is a minor component in plant cell wall; it comprises among a 0.5 and 8% in dicots. RG-II have a characteristic backbone structure of seven to nine α -D-GalA residues, with four ramifications which have between 11 and 12 different types of glycoside residues (Mohnen 2008).

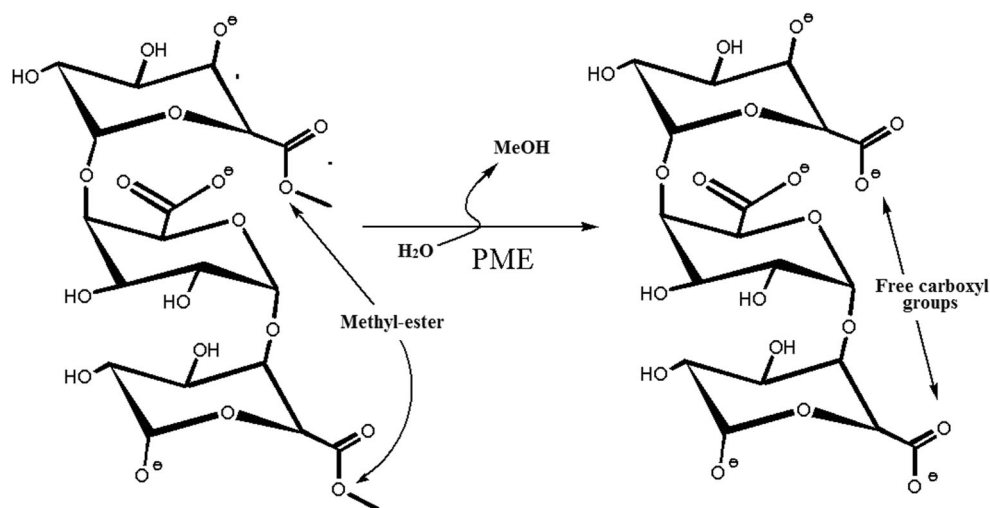
Pectin Methyltransferase

Pectin methyltransferase (PME) (E.C. 3.1.1.11), also known as pectin esterase, pectase, and pectin methoxylase, is an enzyme that catalyzes hydrolysis of methyl-ester bounds on HG in pectic substances, producing free carboxyl groups and methanol (Fig. 1) (Gummadi et al. 2007; Jayani et al. 2005). After performing the de-esterification, GalA residues in partially de-methoxylated pectins are negatively charged and are able to interact ionically with divalent cations (e.g., Ca^{++} , Mg^{++}), producing linkage and cross-linking of molecules forming

gels of pectates. The coordination of ten or more consecutive de-methoxylated GalA residues generates an “egg box structure” (Ochoa-Villarreal et al. 2012). Depending on presence or absence of an extension in the N-terminal region (also referred as the PRO region) which shows similarity with a PME inhibitor domain (PMEI, Pfam04043, IPR006501), PME is classified in two groups: PME type-I with PME domain (higher plants) and PME type-II without PME domain (present in bacteria and fungi) (El-Moneim et al. 2014). Furthermore, PMEs exhibit different action patterns, such as the single-chain mechanism, where enzyme-substrate complex is formed, and it is not dissociate until a blocking site is found or enzyme reaches the molecule end; meanwhile, a multiple-chain mechanism produces an enzyme-substrate complex, and after each catalytic event, it dissociates. Likewise, a third mechanism of multiple-chain attack has been described, where enzyme-substrate complex is formed and a determinate number of reactions are catalyzed, followed by enzyme-substrate complex dissociation. According to Cameron et al. (2008), in these three models, the same process is described, showing as variant the degree of multiple attacks in each one of them, the generation of a single transformation to hundreds or thousands of them being possible.

PMEs of fungi like *Aspergillus niger* preferably hydrolyze methyl esters randomly using a multiple-chain mechanism with limited degree of multiple attacks at pH 4.5 (Van Alebeek et al. 2003). This mechanism is similar to that observed in a recombinant PME from *Aspergillus aculeatus*, where it was also observed that temperature and high pressure stimulate its activity, but did not modify its pattern of de-esterification (Duvetter et al. 2006a, b). Meanwhile, plant and bacteria PMEs produce blocks of de-methylated GalA oligomers. Although its mechanism is still not completely understood, different studies have demonstrated the capability of plant PME to form long de-methylated blocks; this result is well described by a single-chain

Fig. 1 Pectin methyltransferase schematic reaction (Jolie et al. 2010; Ochoa-Villarreal et al. 2012)



mechanism, but in the same way is able to produce short oligomers of de-methoxylated GalA, which is related to a multiple-chain mechanism. This transition system in degree of processivity has not yet well established but seems to be very connected with pH (Cameron et al. 2008; Denès et al. 2000; Duvetter et al. 2006b; Kim et al. 2005, 2013). The degree of multiple attacks in plant PME is reported to be close to ten (Cameron et al. 2011); likewise, bacteria PMEs also have been reported with the multiple-chain mechanism, but with a lower degree of multiple attacks than plant PME (Fries et al. 2007; Remoroza et al. 2015). Nonetheless, there are exceptions like PME produced by *Trichoderma reesei* which produce blocks of free carboxyl groups in pectin, similarly functioning as plant PME and also exhibit an alkaline optimal pH (Markovič and Kohn 1984).

PME Importance in Food Industry

PME takes a significant role in food industry. Numerous studies indicate the capability of this enzyme in texture improvement of fruits and vegetables, through endogenous PME activation by low-temperature long-time (LT-LT) blanching treatments, as well as by infusion of calcium salts and/or exogenous PME (Javeri et al. 1991; Jensen et al. 2005; Ni et al. 2005; Sanjuán et al. 2005; Sirijariyawat et al. 2012; Villarreal-Alba et al. 2004). Besides PME plays a main function in juice industry, since pectin is one of the main components in juice cloud. PME action causes loss cloud stability through a de-methoxylation of pectin that interacts with divalent cations, producing insoluble pectates; the latter destabilize the suspension and produce precipitation of pulp particles (Schultz et al. 2014). In some cases, this PME activity for juice clarification (Bogra et al. 2013) is desirable. Meanwhile, in some industries, such as that of juice extraction, there is great interest in PME inactivation, in order to prevent loss of cloud (Riener et al. 2009; Terefe et al. 2009).

An important application of PME in food industry is formation of pectin gels; this has been used as gelling, stabilizer, and thickener agent in products like jams and jellies, dairy products, beverages, and pharmaceuticals (Kar and Arslan 1999; May 1990; Wicker et al. 2003). It is well known that pectin gelation could be induced in acid solutions with presence of sugars as well with use of calcium salts in combination with PME in order to produce pectins with a low degree of esterification (DE) and subsequently pectin cross-linking (Kastner et al. 2014; Slavov et al. 2009). Yoo et al. (2009a) mentioned that a combination system of fungal and plant PME produces a lower DE than any one individually, since both possess different patterns of action. Plant PME recognizes certain substrate sites; meanwhile, fungal PME can still lower the DE of pectin by catalyzing de-esterification of different methoxyl groups. Additionally, action pattern plays an important role in gel formation, since the plant blockwise de-

esterification promotes gelation process. Moreover, it is possible to induce gelation of pectin mediated by PME in combination with use of monovalent salts under acidic conditions (Yoo et al. 2003). In gel formation, molecular weight is important since at greater molecular weight of pectin, it produces more stable gels. A key factor in PME activity is that it does not modify significantly the molecular weight of pectin, because it does not hydrolyze HG; this allows interaction between pectin chains by presence of lengthier union sites (Yoo et al. 2009b).

PME Role in Plants

PME have been related with several physiological important process in plant development like cell wall expansion, seed germination, fruit maturation, tube pollen growth, ion partitioning, and leaf growth polarity (Bosch et al. 2005; Micheli 2001; Müller et al. 2013; Pelloux et al. 2007; Pilling et al. 2004; Tian et al. 2006). Furthermore, PME interferes in phytopathogenic microbial infections. Due to PME action, de-esterified GalA residues are released around the HG region in pectin; the latter can be de-polymerized by action of pectin lyase and polygalacturonase enzymes. Therefore, degradation of plant cell wall allows the pathogen to invade plant tissue (Fries et al. 2007). Additionally, activity of PME have an important role in phytopathogenic infections, through controlling methyl-esterification degree; this is based in the reported impossibility of necrotrophic fungus *Botrytis cinerea* to grow on methyl esterified pectins (Lionetti et al. 2007).

PME from Different Sources

Based on classification of presence of PME domain, the type-I who have the PME domain is present in higher plants. There are some characteristics that define this group of enzymes. Its optimal pH is found in a range of neutral to alkaline. Additionally, it has been proven that two varieties of PME could be found in function of cation effects; these varieties are salt-dependent and salt-independent PMEs. Cation effect has been demonstrated with the complete loss of activity of salt-dependent PME from Valencia orange peel at low concentrations of NaCl (pH 7.5). PME activity in salt-independent remains still without NaCl; in this case, pH is an important factor in relation to activity and salt effect (Cameron et al. 2003; Savary et al. 2002). These are proteins with molecular weights ranging from 30 to 50 kDa. Among these, it is possible to find different isoenzymes with variable isoelectric points (pI) (generally among 7 and 11), where some acidic forms are present (Castro et al. 2004). The optimal temperature for its operation is in an average of 55 and 60 °C.

The PME classified in type-II is structurally related to those identified in phytopathogenic organisms (bacteria and fungi). These are involved in cell softening process during plant

infections. Furthermore, PME in type-II have variable optimum pH, from acid in many fungi to alkaline in bacteria; however, there are some alkaline fungal PME (Markovič and Kohn 1984). Likewise, PME type-II has similar molar masses and optimal temperatures than PME in type-I (Table 1).

Methods Used for PME Activity Determination by Methanol Analysis

PME Activity Through Methanol Quantification

One of the products in PME reaction is methanol. Several studies have been developed in order to measure PME activity based in methanol quantification, either by direct quantification of methanol, besides by oxidation of the latter to formaldehyde and its subsequent analysis. Direct quantification of methanol is accomplished by gas liquid chromatography (GLC). This assay has been used to measure pectin DE in cell wall as well as quantification of PME activity. The released methanol can be quantified by GLC after a base hydrolysis of methyl esters in GalA (McFeeters and Armstrong 1984). Meanwhile, PME is measured after the reaction of released

methanol with nitrous acid to convert into methyl nitrite; the sensitivity estimated for this method is 3 ppm of methanol in a sample solution prior to nitrite conversion (Bartolome and Hoff 1972). Otherwise, oxidation of methanol mediated by KMnO_4 or alcohol oxidase (AO) exhibits an important alternative for PME activity.

Methanol Oxidation by KMnO_4

Quantification of PME activity by methanol oxidation through potassium permanganate was reported by Wood and Siddiqui (1971). This method consists in an oxidation (KMnO_4) and reduction step with sodium arsenite in order to oxidize methanol to formaldehyde. The produced formaldehyde reacts with acetylacetone and is measured at 412 nm, developing in this way a procedure for PME analysis; this applies oxidation mediated by KMnO_4 under acid conditions and use of NaAsO_4 . Moreover, Pifferi et al. (1985) developed a procedure for PME analysis which applies oxidation mediated by KMnO_4 under acid conditions and use of NaAsO_4 , but unlike Wood and Siddiqui method, in this procedure, analysis of produced formaldehyde is carried out by the 3-methyl-2-benzothiazolinone-hydrazone (MBTH or Besthorn's

Table 1 Characteristics of PMEs

Origin	pH	MW (kDa)	Temperature (°C)	NaCl (M)	Reference
PME in plants					
<i>Carica papaya</i> L.	6–9	37	70	0.15–0.3	Vasu et al. (2012)
<i>Capsicum annuum</i>	7.5	33 and 37	52.5–55	0.13	Castro et al. (2004)
<i>Citrus bergamia</i> R.	7 and 9	42	–	0.1	Laratta et al. (2008)
<i>Citrus sinensis</i> (L.) Osbeck	9	34	55–60	–	Savary et al. (2002)
<i>Datura stramonium</i>	9	–	60	0.3	Dixit et al. (2013)
<i>Daucus carota</i> L.	7.5	–	55	0.2	Ünal and Bellur (2009)
<i>Lycopersicon esculentum</i>	6.5	34	50	–	Kant and Gupta (2012)
<i>Prunus armeniaca</i> L.	7.5	–	60	0.15	Ünal and Şener (2015)
	9	–	60	0.1	Özler et al. (2008)
<i>Prunus domestica</i>	7.5	31	65	–	Nunes et al. (2006)
PME in fungi and bacteria					
<i>Aspergillus japonicus</i>	4.5	46 and 47	–	–	Semenova et al. (2003)
<i>Aspergillus niger</i>	–	38 and 40	–	–	Warren et al. (2002)
<i>Fusarium asiaticum</i>	6.5	31 and 42.5	–	–	Glinka and Liao (2011)
<i>Penicillium chrysogenum</i> F46	5	34.1	40	–	Pan et al. (2014)
<i>Bacillus licheniformis</i>	8	36.8	50	–	Remoroza et al. (2015)
<i>Erwinia chrysanthemi</i>	8–9	–	50	0.1	Laurent et al. (2000)

hydrazone) technique. Nevertheless, this method has disadvantages such as use of a strong oxidizing agent produces interferences with some ingredients (e.g., reducing sugars, carbohydrates) in samples like juices, which react with acetylacetone producing distortions in absorbance during methanol quantification (Wu et al. 2007), generating a problem of low specificity and accuracy of this method. However, posterior works lead to a method with various advantages.

Methanol Oxidation by Enzyme Alcohol Oxidase

Substitution of KMnO_4 in oxidation step of methanol was performed using AO (Fig. 2). This enzyme offers selectivity for produced methyl alcohol. Klavons and Bennett (1986) described this method by quantifying the methyl ester content in pectin. In this assay, reduction step is excluded and also the use of toxic arsenite salts. This method achieves an increase in sensitivity, beside more accuracy and speed. Formaldehyde produced by AO is later condensed with multiple specific reagents in order to produce a fluorometric or quantifiable colorimetric signal. Since the PME action produces methanol through methyl ester hydrolysis in pectin, several authors have reported the use of Klavons and Bennett's method for PME activity quantification, with interest in characterization of fruit development and PME inactivation (Castro et al. 2006; Deytieux-Belleau et al. 2008; Pires and Finardi-Filho 2005; Sila et al. 2007). Adaptation of the AO method has been carried out with a coupling reaction of peroxidase (POD) and formaldehyde dehydrogenase (FDH) enzymes. The coupled reaction of AO and POD has been employed on PME activity quantification and for determination of DE from pectin (Mangos and Haas 1996). This method consists a three-reaction-step mechanism, where hydrogen peroxide produced by AO is used in presence of POD to catalyze oxidation of reagent 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to form a colored ABTS radical cation which strongly absorbs 420 nm. With this method, an increase of 20-fold in sensitivity is achieved over the AO technique (Mangos and Haas 1997). A unit of activity was expressed as micromoles of methanol released per minute in base of the rate of absorbance change at 420 nm with a stoichiometric molar relation of 1:2

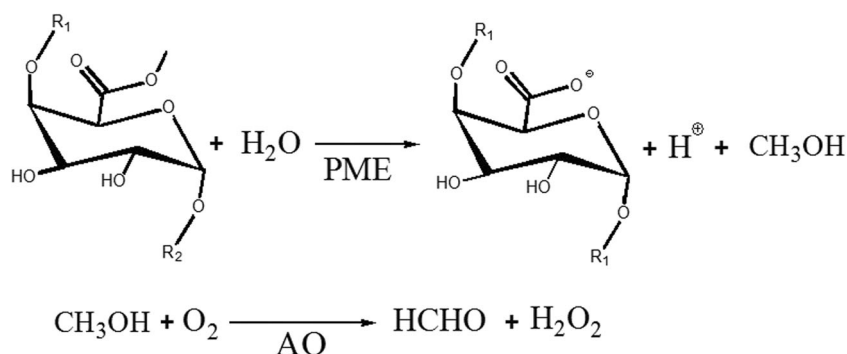
for methanol released and generation of the ABTS radical cation. Grsic-Rausch and Rausch (2004) described a coupled reaction of AO and FDH for PME activity and its inhibition by proteinaceous inhibitors. This reaction also consists of a three-step mechanism where methanol is oxidized by AO to formaldehyde with posterior oxidation of the latter to formic acid. In this method, produced NADH is monitored at 340 nm. A unit of PME activity is defined as 1 μmol of NADH formed per minute at 25 °C and pH 7.5. This assay shows a good efficiency for PME activities up to 8×10^{-3} units.

Method of Acetylacetone and Ammonium

There are several methods for PME activity determination based on methanol quantification through its oxidation to formaldehyde. One of the most important analytical techniques is the derivatization by means of the Hantzsch reaction, where formaldehyde reacts with diketone acetylacetone (2,4 pentanodione) in presence of ammonium (also referred as Nash reagent) (Fig. 3) (Li et al. 2008; Nash 1953). The final product of this reaction is 3,5-diacetyl-1,4-dihydrolutidine (DDL) which produces a yellow color in aqueous solution (Teramae and Maruo 2013).

This method that involves use of Nash reagent in combination with AO was introduced by Klavons and Bennett (1986), in order to determine DE of pectin. Since this methodology was established, it has become in a widely applied system for PME activity determination, based in the relation of released methanol by action of this enzyme. Houben et al. (2011) used this method in a comparative study of characteristics of cell wall polymers from different vegetables. Likewise, De Roeck et al. (2010) studied the texture degradation kinetics of carrots subjected to a high-pressure/high-temperature process, where PME plays an important role on pectin demethylation and subsequent texture loss. In the same way, other authors have employed this method to study PME stability and optimal activity parameters as well in fruit development (Castro et al. 2006; Deytieux-Belleau et al. 2008; Sila et al. 2007). A reagent that follows the same reaction mechanism than acetylacetone plus ammonium is Fluoral-P. This reagent is an intermediate in formation of

Fig. 2 Coupled reaction of pectin methylesterase and alcohol oxidase



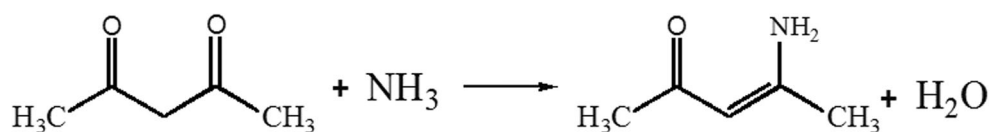


Fig. 3 Reaction of acetylacetone and ammonium (Teramae and Maruo 2013)

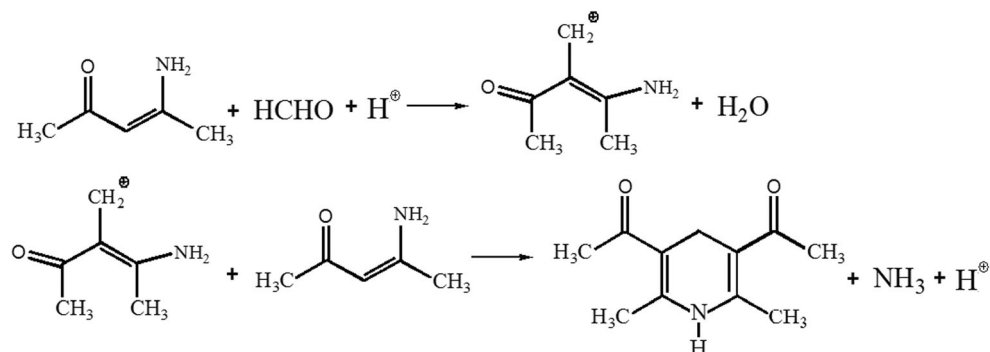
DDL, and this compound is the final product of both reactions (i.e., Nash reagent and Fluoral-P). After ammonium and acetylacetone react, the product is 4-amino-3-pentene-2-one (Fluoral-P) (Fig. 3); once this molecule is formed, the next steps of reaction are equal for both systems (Fig. 4) (Teramae and Maruo 2013; Wang et al. 2013). The Fluoral-P assay for PME activity analysis was proposed by Wojciechowski and Fall (1996). In this method, the methanol produced after hydrolysis of methylester bonds in pectin is later oxidized to formaldehyde by AO and continuously reacted with Fluoral-P. The fluorometric assay is carried out at 405 nm for excitation and 510 nm for emission. Substitution of Fluoral-P instead of Nash reagent allows a reaction at room temperature and at pH compatible with AO, which enables a one-step addition reaction, and with adaptation of the method to a 96-well plate, it becomes in a simple and faster analysis (Held et al. 2015). Likewise, another advantage is the increase in sensitivity with fluorometric analysis, which is 30 times more sensitive than common measuring of increase in absorbance at 412 nm (Wojciechowski and Fall 1996).

Method

of 4-Amino-3-Hydrazino-5-Mercapto-1,2,4-Triazole

The reagent 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) was employed by Jacobsen and Dickinson (1974) as basis of a method for determination of small quantities of aldehydes. In this method, aldehydes reacts with Purpald in a two-step process. First, an intermediate product of condensation of both (i.e., aldehyde and Purpald) is produced; the latter is then oxidized by oxygen in air to form a tetrazine (Fig. 5) (Quesenberry and Lee 1996). Under alkaline conditions, mercapto group is de-protonated, which produces an anion with a strong purple color, and absorbs in the visible spectral at 549 nm (Vogel et al. 2000; Zurek and Karst 1997).

Fig. 4 Reaction of Fluoral-P and formaldehyde (Wang et al. 2013)



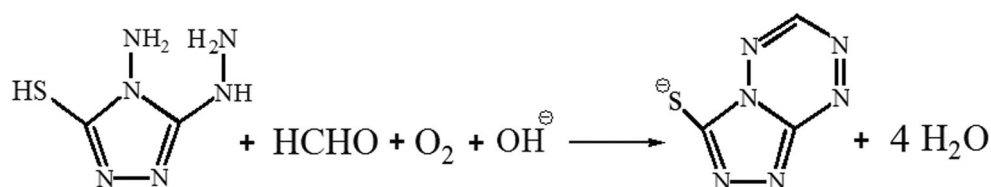
The requirement of alkaline conditions in Purpald reaction makes it an optimal alternative for plant PME analysis. Anthon and Barrett (2004) introduced this method as an effective way to follow methanol production related with PME activity and mentioned the advantages of its capability to selectively react with short aldehydes, analyzing demethylation of pectin from different vegetables under a treatment of LT-LT.

In PME assay, it has been used with different research objectives including allergen studies, textural quality maintenance, and in cell wall modification impact (Nguyen et al. 2010; Salamanca et al. 2010; Sun et al. 2016). Likewise, this method has been applied for evaluating release of methanol from different plant material by action of PME present in an enzymatic mixture (Anthon and Barrett 2006, 2008). Additionally, Purpald is 60-fold more reactive toward methanol than ethanol and it is 3-fold more sensitive to methanol than acetylacetone; nonetheless, selectivity of acetylacetone is higher for methanol (Anthon and Barrett 2004). Moreover, Purpald shows a great specificity toward formaldehyde, since response of this for other aldehydes is negligible (Zurek and Karst 1997).

Method of MBTH

There are numerous derivatizing agents for analysis of aldehydes and related compounds; a representative of this group is MBTH. The MBTH test is useful in aliphatic aldehyde determination and was introduced by Sawicki et al. (1961) and improved by Hauser and Cummins (1964). This analysis is carried out in a two-step process under acidic conditions; first, respective aldehyde reacts with a molecule of MBTH in order to form aldazine, and then in an oxidative coupling process, a second molecule of MBTH reacts to form blue cation tetra-aza-pentamethincyanine (Fig. 6) (Vogel et al. 2000; Zurek and Karst 1997).

Fig. 5 Reaction of Purpald and formaldehyde (adapted from Vogel et al. 2000)



The MBTH procedure for measuring the PME activity was introduced by Pifferi et al. (1985); nevertheless, its procedure utilizes KMnO_4 instead of AO for methanol oxidation, which reduces its sensibility and selectivity. However, Anthon and Barrett (2004) improved this method by introducing AO to the technique. Different authors have reported use of MBTH method, either for investigating the influence of PME during gelling process of pectin as well in cultivar resistance studies (Videcoq et al. 2011; Yang et al. 2008). Since this analysis is carried out under acidic conditions, it could be useful for analyses that require this specific condition; an example of this is orange juice that has a pH close to 3.6. Spagna et al. (2003) developed a method that is specific for orange juice conditions using MBTH reaction; this procedure was also later applied to thermal PME inactivation studies (Ingallinera et al. 2005). MBTH offers an entrapment mechanism which prevents posterior oxidation of produced formaldehyde to formic acid. Nonetheless, MBTH is not specific to formaldehyde; however, its selectivity is improved by specificity of the AO and greater response of MBTH toward methanol. In general, at greater lengths of alkyl chain, the response is smaller, with exception of acetaldehyde (Anthon and Barrett 2004; Zurek and Karst 1997).

Analysis of PME Activity Through Free H^+ Quantification

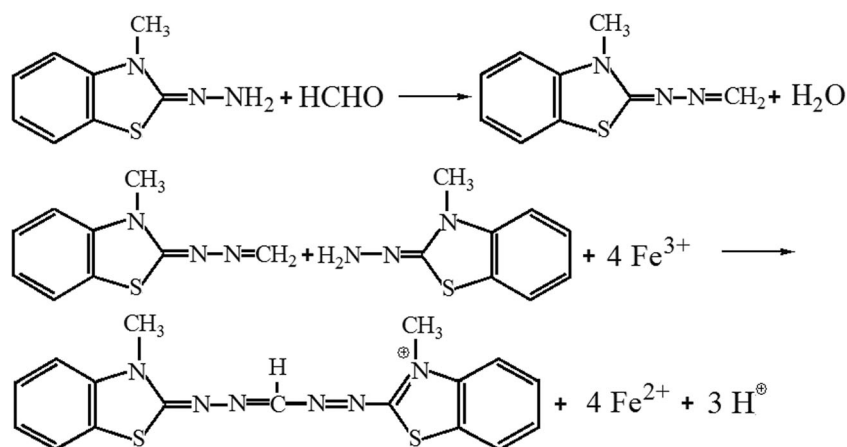
In an activity enzymatic assay, color variations are an efficient way to follow an enzymatic reaction; however, catalytic activity does not ever produce a color change. An alternative is the use of chromogenic substrates, which produce a color change into reaction, with the disadvantage of using an artificial

substrate that perhaps does not behave just as the natural substrate (Kazlauskas 2006). Zimmerman (1978) described use of a derivative of *p*-nitrophenyl for PME activity analysis; in this study, hydrolysis of *p*-nitrophenyl acetate (which is a common substrate in esterases analysis) was followed spectrophotometrically. Like the aforementioned, use of chromogenic synthetic substrates poses some drawbacks. Another possibility is detecting pH changes produced by esterases; this may be carried out by two methods, titrimetrically or with a pH indicator. PME activity could be easily monitored in solution with use of a pH indicator; since PME hydrolyzes methylester groups in pectin, released protons into solution induce a color change (Bordenave 1996; Kazlauskas 2006).

Titrimetrical Determination

Titration method is one of the preferred methods by researches because of its simplicity. In this analysis, an estimation of free carboxyl groups formed by PME action in pectin is made. Originally, this assay was performed using methyl red as pH indicator; the pH of reaction mixture containing pectin needs to be adjusted until the last pinkness disappears from the mixture (Kertesz 1937). Anywise, this method has gone through multiple modifications (Fish and Dustman 1945; Lee and Macmillan 1968; Rouse and Atkins 1955), and it is well described by Kertesz (1955); actually, measurements are made by an automatic titrator. A routine assay is performed using a pectin solution (0.25–1 %) supplemented with a determined concentration of NaCl (0.15–1.0 M) under constant conditions of pH (usually 7–7.5 for plant material) and temperature (30 °C). A unit of activity is defined as the amount of enzyme that releases 1 μmol

Fig. 6 Mechanism reaction of MBTH and formaldehyde (adapted from Jacobsen and Dickinson 1974)



of carboxyl groups/min under aforementioned conditions. Activity is calculated using the following formula:

$$\text{PME} \left(\frac{\text{units}}{\text{mL}} \right) = \frac{(\text{mL NaOH})(\text{Molarity of NaOH})(1000)}{(\text{time})(\text{mL sample})}$$

This assay is reported by several authors, varying slightly the aforementioned conditions (de Assis et al. 2001; Ly-Nguyen et al. 2002; Mejía-Córdova et al. 2005; Ortuño et al. 2013; Ünal and Şener 2015), with multiple applications in PME studies such as inactivation studies (Bermejo-Prada et al. 2015; Briongos et al. 2016). However, this method is limited by interferences in pH measurements by organic acids in non-purified samples and could be insensitive to pH changes because of the use of buffers during protein purification. Nonetheless, in many cases, sample volume is too low with respect to substrate volume and a great dilution factor is achieved. Due to the aforementioned, it is improbable that in a routine assay, buffers represent an interfering factor. Additionally, with low activity levels, pH changes could not be detected (Anthon and Barrett 2004, 2012; Wojciechowski and Fall 1996).

Colorimetric Analysis

The use of a pH indicator provides an easy, fast, and cost-effective alternative for PME activity analysis. Once PME hydrolyzes methyl esters, the produced protons could be detected by a pH indicator. Methyl red is an example of pH indicator that has been used in PME activity evaluation (Bordenave 1996; Bordenave and Goldberg 1993; Sajjaanantakul and Pitiifer 1991). Hagerman and Austin (1986) established a suitable method utilizing the pH indicator bromothymol blue, which acquires different conformations when pH varies among acid, neutral, and alkaline, becoming yellow, green, and blue, respectively, according to pH (Fig. 7). This assay is carried out at neutral to slightly alkaline conditions, and reaction is spectrophotometrically followed at 620 nm; meanwhile, quantification is made through a standard curve of GalA. Later, this procedure was taken to microplate

analysis, which improves its sensitivity plus a reduction in sample volume and possibility of a multiple sample assay (Cameron et al. 1992).

Since the Hagerman and Austin method is performed at neutral conditions based in pH range of color change of bromothymol blue (6–7.6), it is limited preferably to plant PME according to its optimal pH activity. However, Vilariño et al. (1993) modified this method for PME analysis choosing bromocresol green rather than bromothymol blue because of its pH range of change (3.8–5.4), producing a valuable assay for fungal PME evaluation which have an optimal pH activity average between 4.6 and 5.1 (Patidar et al. 2016).

The method of Hagerman and Austin has been widely applied in semi-quantitative measurements, particularly measuring increase in absorbance at 620 nm under defined conditions. This procedure is especially suitable in studies of PME inactivation, because of its fast performance in residual activity detection. However, one of its disadvantages is its low accuracy (Giner et al. 2000; Tiwari et al. 2009; Zocca et al. 2007). Additionally, it is possible to measure PME activity through a simple analysis such as the proposed by Zimmerman (1978). In this method, PME activity is assayed in agar plates containing a solution of pectin and bromothymol blue; activity is calculated by measuring hydrolysis area and quantified by comparison against a standard curve. Likewise, PME activity using a gel diffusion assay can be measured, where increasing binding of ruthenium red dye as pectin DE decrease is used. The stained zone is measured and quantified based in a standard curve of commercial enzyme activity versus stained zone diameter (Downie et al. 1998). However, Bourgault and Bewley (2002) describe a problem related with this assay, since it is performed for assay unpurified plant extracts; an underestimation of PME activity can befall because of action of proteolytic proteins. A solution for this problem is proposed by the addition of protecting proteins.

Considerations in PME Analysis

The selected method for PME activity evaluation depends on extent in the research proposes; methods based on pH

Fig. 7 Bromothymol blue protonated and de-protonated conformation

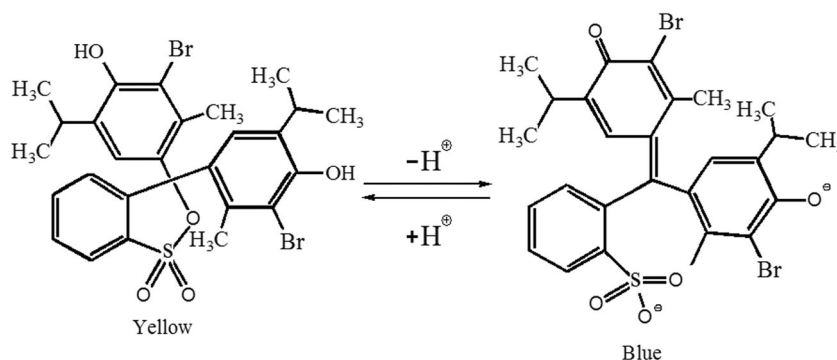


Table 2 Comparison of different methods for PME activity analysis

Factors	Titrimetrically	pH indicators	Nash reagent and Fluoral-P	MBTH	Purpald
Overall cost	Low	Low	Moderate	Moderate	Moderate
Relative sensibility	1×	2.2×	7.1×	50×	21.5×
Time	Low	Low	Moderate	Moderate	Moderate
Replicability	Moderate	Low	High	High	High
Multiple sample assay	No	Yes	Yes	Yes	Yes
Sample volume	High	Low	Low	Low	Low
Difficulty	Low	Low	Moderate	Moderate	Moderate

pH indicator relative sensitivity is based in microplate method

Relative sensitivities are based on data reported by Anthon and Barrett (2004), Cameron et al. (1992), and Klavons and Bennett (1986)

indicators are especially valuable in rapid evaluations like in fraction evaluation during purification, where the only proposed is evaluation of the presence of activity. Likewise, in studies of PME inactivation, researchers need to check presence of residual activity. Moreover, methanol quantifications are helpful when interference with released protons is present, as in process where a buffer with strong buffering capability is used. Because of trend of green chemistry, use of toxic reagents like arsenic salts is disuse and a major selectivity is achieved because of AO intervention in oxidation of methanol to formaldehyde. Although AO is not specific for methanol, other short-chain alcohols like ethanol have lower oxidation rates. AO would be used efficiently in combination with a reagent such as acetylacetone with a negligible or non-production of chromophore with other primary aldehydes than formaldehyde. Likewise, use of Fluoral-P produces the same final chromophore than acetylacetone but avoid use of ammonia. Another important consideration is if selected method is a continuous or end point assay. It is relevant since continuous assay allows to create a reaction progress curve regarding time. When activity is measured, this must correspond to linear part of progress curve from which velocity is derived from the slope. Meanwhile, in an end point assay, blank and one measure point are connected, and the slope of this line is which defines velocity of reaction. An erroneous result could be obtained in an end point assay, only if measure point is not within linear part of reaction curve (Bisswanger 2014). Wojciechowski and Fall's assay is proposed like a continuous fluorometric analysis, where reaction is linear for a time of 1 to 3 min, besides Klavons and Bennett analysis is typically evaluated as an end point assay (Deytieu-Belleau et al. 2008; Pires and Finardi-Filho 2005). Frequently optimal pH of AO and PME are close to neutral to slight alkaline, while pH of reaction between formaldehyde and Fluoral-P is among 2–3, and the rate of this reaction strongly decreases in pHs above 3. Likewise, the absorbance and fluorescence of the end product of both methods (i.e., 3,5-diacetyl-1,4-dihydrolutidine) vary

according to pH (Compton and Purdy 1980; Wojciechowski and Fall 1996). Other methods such as Purpald and MBTH imply steps that lead them to be performed as discontinuous enzymatic assays. However, this kind of assay is especially valuable when a great number of samples have to be analyzed, offering great replicability. Anthon and Barrett (2004) pointed out a disadvantage in use of Purpald or acetylacetone after AO step, since a further oxidation of formaldehyde to formic acid could occur. MBTH method offers a chemical trap that prevents further oxidation of formaldehyde and allows an accurate measurement of total produced methanol, in the same way is useful when an acidic PME is under study, since this system is performed under these conditions. Likewise, calorimetric or titrimetric methods allow to construct an activity curve through a continuous analysis of released protons with the aforementioned advantages of a continuous enzymatic assay.

These analysis provide advantages and drawbacks; when we refer to price of the assay in the particular case of titrimetric method, it results as a low-cost assay but without taking into consideration the price of the specific equipment required for this analysis. Most other methods use routine laboratory equipment (Table 2). Anyhow, there is a continuous production of new derivative reagents with potential for analysis of methanol and formaldehyde which perhaps become promising for accurate measurements of PME activity.

Conclusions

Study of food-modifying enzymes such as PME is kept to be an important target in food science due to its importance in food quality assurance. An enzymatic assay must ensure always its accuracy and replicability as well as have to be preferably as faster as can. Multiple sample analysis and in many cases small sample volumes are desired characteristics in the chosen assay. While it is true that any method accomplishes with the target, researchers must evaluate the interest factors in

its investigation, and based on this, the optimal method that meets the aims and scopes of the project should be selected.

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Compliance with Ethical Standards

Conflict of Interest J.A. Salas-Tovar declares that he has no conflict of interest. A.C. Flores-Gallegos declares that she has no conflict of interest. J.C. Contreras-Esquivel declares that he has no conflict of interest. S. Escobedo-García declares that she has no conflict of interest. J.A. Morlett-Chávez declares that he has no conflict of interest. R. Rodríguez-Herrera declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

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