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EVALUATION OF PECTIN METHYLESTERASE AND *LACTOBACILLUS* SPP. ISOLATED FROM TABASCO PEPPERS (*CAPSICUM FRUTESCENS* L.)

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by
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December, 2009

This work is dedicated to my family: Wil Jones, Sr., Zorla Jones, Ulandera J. Robertson, Wil Jones, II, and Lonja Jones. I am grateful for their support, encouragement, and understanding.

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ABSTRACT

One of the major concerns of the hot sauce manufacturers is separation of sediments and layering of the sauce. Separation is an undesirable production condition because consumers view this as a defect which consequently increases manufacturing costs. Pectins play an important role in pepper processing since it forms gels and influences the final viscosity of the sauce. Hence, its integrity during processing influences the quality of the final product. Failures in pectin stability can sometimes be related to the presence of enzymes. Understanding how the pectin enzymes function during fermentation of pepper sauce is a necessary step in controlling the enzymatic activity, and thereby reducing production cost and the risk of having a poor quality food product.

Given the lack of information in the enzymatic activity of pectin enzymes during the processing of hot sauce, our objectives were to partially purify pectin methylesterase (PME) from hot peppers, evaluate the properties of the enzyme, and investigate lactic acid bacteria isolated from pepper mash during fermentation that could have the potential of producing and excreting pectinolytic enzymes. In this study, PME was separated by weak anion-exchange and affinity chromatography. Based on our SDS-PAGE results, two major bands were present at 22 kDa and 36 kDa.

Our research revealed under acidic conditions, high salt concentrations, and temperatures above 50°C, PME can be effectively inactivated. We collected thirty mash samples (1-month aged and 36-months aged) under normal processing conditions from a local processing plant. All samples showed the presence of *Lactobacillus* as indicated by PCR. Quantitative real-time polymerase chain reaction (QRT-PCR) was employed to quantify total bacteria and lactobacilli in the samples. The percentage share of *Lactobacillus* in the counts was higher in 36-month aged samples than 1-month aged samples. Based on the concentrations, this bacteria plays a major role over the course of the fermentation aging process of pepper sauce.

CHAPTER 1

INTRODUCTION

Hot sauce is an international sales success, particularly in Southeast Asia. Produced for 137 years at Avery Island, Louisiana, it is exported to more than 100 countries. Due to the increasing ethnic diversity and the influence of ethnic foods, the demand for red pepper (*Capsicum spp*.) products has greatly increased. The U.S. is the largest consumer of hot pepper sauce, followed by Japan, with Guam and Saipan having the largest per-capita consumption of Tabasco pepper sauce (Turcsik 1992).

One of the major concerns of the hot sauce manufacturers is separation of sediments and layering of the pepper sauce. Separation is an undesirable production condition because consumers view this as poor quality and consequently increases manufacturing costs (Koh 2005). Pectins play an important role in pepper processing since it forms gels and influences the final viscosity of the sauce. Hence, its integrity during processing influences the quality of the final product. This is especially important for products without added thickeners or stabilizers such as Tabasco pepper sauce. Pectins are found universally in the primary cell walls and intercellular layers of plants, specifically in *Capsicum frutescens* L.

Pectins are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules that are comprised of water soluble polygalacturonic acids of varying methyl ester contents and degrees of neutralization which show colloidal properties (Kertesz 1951; Jayani and others 2005). The complex macromolecules of pectin would seem to offer a number of possibilities for enzyme action. Pectin enzymes have received considerable attention regarding their involvement in ripening and softening of cell wall components. Among the well known pectinases are pectin lyase (PL), polygalacturonase (PG) and specifically pectin methylesterase (PME), (NOSB, 1995).

In the food industry, control of PME activity is important in processes concerning the production and storage of fruit juices and purees. It is well known that PME activity changes the

texture of fruit products. For this reason storage at very low temperature or a high temperature pasteurization process are required in order to inactivate the PME in industrial fruit products. Pectinesterase was discovered in 1840 by Frémy in vegetable juice (Frémy 1840). Furthermore, all higher plants seem to contain pectinesterase in all living tissues. PME is carboxylic acid esterase and belongs to the group of hydrolases. The hydrolysis of methylester groups catalyzed by the enzyme produces pectin with a lower methylation degree that can undergo further enzymatic cleavage by polygalacturonase (PG) and pectin lyase (PL) (Whitaker 1994). Also, bacteria and fungus have been shown to contain or to be able to produce pectin methylesterase (PME).

Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. Many foods such as Tabasco pepper sauce are preserved products in that their shelf life is extended considerably over that of the raw materials from which they are made (Campbell-Platt 2000). The purposes of fermentation are to increase pepper value, prevent pulp separation after sauce making, and produce a shelf stable sauce. Therefore, the mash fermentation process is a very critical step in final hot sauce quality.

Failures in pectin stability can sometimes be related to the presence of enzymes. Understanding how the pectin enzymes function within Tabasco pepper sauce fermentation process is a necessary step in controlling the enzymatic activity, and thereby reducing production cost and the risk of having a poor quality food product. Given the lack of information for the enzymatic activity of pectin enzymes in the processing of hot sauce, our objective was to examine the enzyme, pectin methylesterase from Tabasco pepper.

In order to evaluate the presence of pectin methylesterase from Tabasco peppers, our objectives were to partially purify pectin methylesterase from mature red-ripe Tabasco peppers

and evaluate the properties of the enzyme. In order to achieve these objectives, our study was divided into three distinct phases:

(i) To determine whether pectin methylesterase can be consistently partially purified from mature red-ripe Tabasco peppers and evaluate the general properties of the enzyme

(ii) To evaluate the kinetic parameters of partially purified pectin methylesterase thereby providing a means of controlling the enzymatic activity

(iii) Investigate lactic acid bacteria isolated from pepper mash during fermentation that could have the potential of producing and excreting pectinolytic enzymes

1.1 References

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CHAPTER 2

LITERATURE REVIEW

2.1 *Capsicum frutescens* L.

The *Capsicum* genus represents a diverse plant group, from the well known Tabasco pepper and sweet bell pepper to the recently popular habanero pepper. According to Bosland (1992), the genus *Capsicum* is a member of the *Solanaceae* family that includes tomato (*Lycopersicon*), potato (*Solanum*), and tobacco (*Nicotiana*). The genus *Capsicum* consists of approximately 22 wild species and five domesticated species (Bosland 1994): *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. As stated by Heiser (1976), *Capsicum* is endemic to the western hemisphere and the pre-Columbian distribution extended from the southernmost border of the United States to the temperate zone of southern South America. It is a perennial small shrub in suitable climatic conditions, living for a decade or more in tropical South and Central America. *Capsicum* species are cold sensitive, typically hot and dry weather is desirable for fruit ripening. *Capsicum* probably evolved from an ancestral form in the Bolivia/Peru area (Heiser 1976). Chile fruits are considered vegetables, but are berries botanically. Chile types usually are classified by fruit characteristics, i.e. pungency, color, shape, flavor, size, and their use (Smith and Heiser 1957; Bosland 1992). Despite their vast trait differences most chile cultivars commercially cultivated in the world belong to the species, *C. annuum*. The Tabasco (*C. frutescens*) and habanero (*C. chinense*), are the best-known exceptions.

Enzymatic studies of *Capsicum* indicated by; Jensen and others (1979); McLeod and others (1979); Walsh and Hoot (2001), have demonstrated that species could be grouped into taxonomic categories that somewhat agreed with groups based on flower color. *C. frutescens* is grouped in the white flower complex. However, species delimitation within two *Capsicum* species complexes remains problematic: (i) the *C. annuum* complex, consisting of *C. annuum, C. frutescens*, and *C. chinense*, and (ii) the *C. eximium* complex, consisting of *C. eximium* and *C.*

cardenasii. Species of the *C. annuum* complex contain both domesticated and wild varieties, as well as a wide range of intermediates, which are all similar morphologically and indistinguishable based on enzyme profiles as stated by Jensen and others (1979). Some researchers have argued that *C. frutescens* and *C. chinense* should be combined into one species (Pickersgill 1966, 1971; McLeod and others 1979) because they interbreed fairly readily (Smith and Heiser 1957; Lippert and others 1966; Pickersgill 1966) and intergraded into a morphological continuum. *Capsicum frutescens* displays features considered typical of a wild species and is not cultivated on a large scale, except relatively recently on the Tabasco farms of Louisiana (Pickersgill 1971). *Capsicum chinense* does not have any true wild form and is cultivated extensively in South America. Several characteristics, such as non-dehiscent fruit, fruit shape, and gigantism of leaves, fruit, and flower structure, suggest it has been cultivated for a long time (Pickersgill 1966, 1971).

2.1.2 Tabasco Peppers

Hot sauce is an international sales success, particularly in Southeast Asia. Produced for 137 years at Avery Island and it is exported to more than 100 countries. Due to the increasing ethnic diversity and the influence of ethnic foods, the demand for red pepper (*Capsicum spp*.) products has greatly increased. The U.S. market for all peppers increased from 95 million pounds dry-weight basis in 1980 to 210 million pounds in 1993 (Buzzanell and others 1995; Koh 2005). The total value of combined capsicum imports averaged $44.6 million in 1990-94, compared with $24.7 million in 1985-89, and $15.5 million in 1980-84 (Buzzanell and others 1995; Koh 2005). In addition, the value of all pepper imports increased from $174 million in 1992 to $568 million in 2001 (FAOSTAT 2003). The US is the largest consumer of hot pepper sauce, followed by Japan, with Guam and Saipan having the largest per-capita consumption of hot pepper sauce (Turcsik 1992).

Hot sauce is widely available in Japan, where it is used primarily on pizza and spaghetti. Red peppers are used in pickling, relishes, ketchup, sauces, and processed meat and fish all around the world. Currently all Tabasco peppers (*C. frutescens*) that are grown in Central America, South America, and Southern Africa are processed either in Avery Island, Louisiana, or in Venezuela. One of the several factors that have initiated this shift in production areas is labor cost and availability. For both processing and fresh market, harvest of pepper fruit is still primarily conducted by hand and the greatest single production expense is often harvesting costs.

According to Koh (2005), the pepper sauce begins with whole peppers being crushed into mash using a hammer mill together with approximately 8% salt and fermented for three years before production. Peppers mash is usually fermented in wood barrels made out of oak which is often associated with a higher quality product. The McIlhenny Company prefers the charred Kentucky white oak barrels from whiskey distillers on which the wooden ends are secured with stainless steel hoops. Salt is added on top of the wood sealed lids to encourage an anaerobic condition and reduce contamination. Tiny holes present in the salt mound allow the gases of the peppers to escape during fermentation. The salt topping hardens in atmosphere humidity and naturally seals the barrel after the fermentation process ceases. The barrels are uncovered and oxidized mash is removed from the top of the barrels. Upon being accepted under certain requirements and standards (capsaicin by HPLC; pH; titratable acidity (TA) by titration; and % salt by titration), the mash is pumped into large blending vats and mixed with distilled, all-natural white vinegar to produce hot pepper sauce.

Koh (2005) concluded in her study that the dry weight of pepper mash significantly decreases during fermentation. Following the addition of 8% salt, the average pH was 4.7 and the average titratable acidity (TA) was 0.54% acidity (expressed as lactic acid). Titratable acidity in the pepper mash increases due to lactic acid production which leads to a slightly decreased pH.

According to the study, after fermentation, the pepper mash still contains residual sugars. Moreover, the fermentation process does not affect the capsaicinoids concentration which gives the pepper sauce its characteristic hot flavor. According to Arancibia (2003) the degree of pectin esterification (DPE) ranged between 55 % and 78 %.

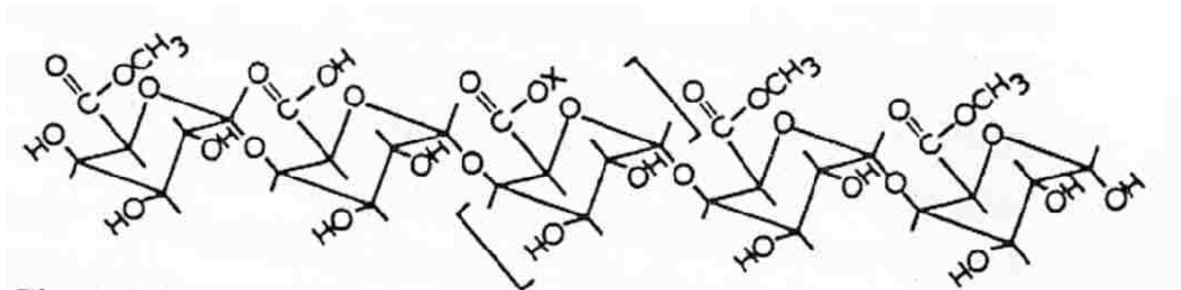
One of the major concerns of the hot sauce manufacturers is separation of sediments and layering of the sauce. Separation is an undesirable production condition because consumers view this as a defect which consequently increases manufacturing costs (Koh 2005). Pectins play an important role in pepper processing since it forms gels and influences the final viscosity of the sauce. Hence, its integrity during processing influences the quality of the final product. This is especially important for products without added thickeners or stabilizers such as hot sauce. Pectins or pectic substances are found universally in the primary cell walls and intercellular layers of plants, specifically in *Capsicum frutescens*.

2.1.3 Pectin

Pectin is used as a gelling and stabilizing agent in the food and cosmetic industries and has multiple positive effects on human health including lowering cholesterol and serum glucose levels, reducing cancer (Jackson and others 2007; Mohnen 2008), and stimulating the immune response (Inngjerdingen and others 2007; Mohnen 2008). Pectin is also used in the production of a variety of specialty products including edible and biodegradable films, adhesives, paper substitutes, foams and plasticizers, surface modifiers for medical devices, materials for biomedical implantation, and for drug delivery (Mohnen 2008). Previous studies have shown that pectin plays a role in plant growth, development, morphogenesis, defense, cell–cell adhesion, wall structure, signaling, cell expansion, wall porosity, binding of ions, growth factors and enzymes, pollen tube growth, seed hydration, leaf abscission, and fruit development (Willats and others 2000b; Ridley and others 2001; Willats and others 2001; Mohnen 2008). The amount of

pectin in fruits depends on the maturity, degree of ripeness, variety, and subsequent storage conditions of harvested fruit (Reed 1975).

Pectin is a generic name for the mixture of widely differing compositions. It is a component of all higher plant walls and walls of gymnosperms, pteridophytes, bryophytes and Chara (O'Neil and others 2004; Mohnen 2008). The compound is structurally and functionally the most complex polysaccharide in plant cell walls. Pectins are the predominant anionic polymer where they make up 35% of the primary cell wall in dicotyledonous plants and non-graminaceous (non-grass) monocots, 2–10% of grass primary walls, and up to 5% of wood tissues (Mohnen 2008). Pectins are highly complex polysaccharides that might contain as many as 17 different monosaccharides, and rich in galacturonic acid (GalA) (Albersheim and others 1996; Pelloux and others 2007). Pectin is a family of galacturonic acid-rich polysaccharides that includes four pectic polysaccharide domains homogalacturonan: (HG), rhamnogalacturonan I (RG-I), and the substituted galacturonans rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA), which differ in both structure of the macromolecule backbone and the presence and diversity of side chains; Figure 2.1. illustrates the pectin structure (Pelloux and others 2007; Mohnen, 2008).



The most abundant pectic polysaccharide is homogalacturonan (HG), a linear homopolymer of α-1,4-linked galacturonic acid that comprises ~65% of pectin (O'Neil and others 1990; MacKinnon and others 2002; Nakamura and others 2002; Jackson and others 2007; Yapo and others 2007; Mohen 2008). The most structurally complex pectin, RG-II, makes up ~10% of pectin (O'Neil and others 1990; Mohnen 2008). Its structure is largely conserved across plant species and consists of an HG backbone of at least 8 (and most likely more) 1,4-linked a-D-GalA residues decorated with side branches consisting of 12 different types of sugars in over 20 different linkages. Two other substituted galacturonans, xylogalacturonan (XGA) and apiogalacturonan (AP) are more restricted in their expression. XGA is a HG substituted at O-3 with a β-linked xylose (Zandleven and others 2006; Mohnen 2008). XGA is most prevalent in reproductive tissues; although XGA has also been detected in *Arabidopsis* stems and leaves (Zandleven and others 2007; Jensen and others 2008; Mohnen, 2008). RG-I represents 20–35% of pectin. It contains a backbone of the disaccharide repeat $[\text{-}\alpha\text{-D-GalA-1,2-}\alpha\text{-L-Rha-1-4-}]_n$ and exhibits a high degree of cell type and develop-dependent expression in the type and number of sugars, oligosaccharides, and branched oligosaccharides attached to its backbone (Ridley and others 2001; Wallats and others 2000a; Guillemin and others 2005; Mohnen 2008).

In an unripe fruit, pectin is bound to cellulose microfibrils in the cell wall. Such pectin is insoluble and hence confers rigidity to cell walls. However, during ripening the structure of pectin is altered by naturally occurring enzymes in the fruits. These alterations involve the breakdown of the pectin chain or of side chains attached to the units, which make up the main chain. In either case, the result is that the pectin becomes more soluble and its grip on the surrounding cells walls is loosened and the plant tissue softens. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) (Pifferi and others 1989; Kashyap and others 2001). Pectin or pectinic acids are water soluble polygalacturonic acids of varying methyl ester

contents and degrees of neutralization which show colloidal properties (Kertesz 1951). Galacturonic acid (GalA) comprises approximately 70% of pectin, and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position. Pectic materials that are made up of chains of 1,4-linked galacturonic acid (GalA) units are usually esterified to varying degrees with methanol (Lamikanra 2002). Galacturonic acid is a sugar acid and the oxidized form of D-galactose. GalA has the capability of forming gels with sugar and acid under certain conditions (Girouard 1962).

Based on the type of modifications of the backbone chain, pectic substances are classified into protopectin, pectic acid, and pectinic acid, and pectin (Be Miller 1986; Kashyap and others 2001). Pectic acids are polygalacturonic acids of colloidal nature, but essentially free of methyl ester groups. They are susceptible to precipitation with salts, alcohol, acid, and alkaline earth or heavy metals. Pectates are the salts of these acids. Protopectin is the water insoluble precursor of polysaccharides from which are derived pectins; they occur in a variety of plants, in the fruits, roots, leaves, and stems. Protopectin is solubilized by restricted hydrolysis resulting in the liberation of water-soluble pectin (Salvador and others 2002).

Anthon and Barrett (2008) developed a simple procedure for determining the galacturonic acid and methanol contents of soluble and insoluble pectins, relying on enzymatic pectin hydrolysis and colorimetric quantification. In the study, the combined enzymatic and colorimetric procedure correctly determined the galacturonic acid and methanol content of purified, soluble citrus pectin. Also, the utilization of antibodies that recognize specific structural features of primary cell wall pectin are powerful tools for determining the location and distribution of these polysaccharides within a single wall and for following changes in pectin structure during cell and tissue development (Knox, 1997; Vicre and others 1998; Willats and others 2000b). Antibodies have been described that are believed to recognize HG with different

degrees of esterification including JIM 5 (Knox and others 1990; VandenBosch and others 1989); JIM 7 (Knox and others 1990) and PAM1 (Willats and others 1999). Other anti-pectin antibodies are 2F4 that recognizes calcium cross-linked pectin (Liners and others 1989), and LM5 and LM6 that recognize, respectively, 1,4-linked β-D-galactan (Jones and others 1997) and 1,5-linked α-L-arabinan (Willats and others 1998) in the side chains of RG-I. Another series of antibodies includes CCRC-M1 that recognizes polysaccharides containing a terminal non-reducing α-L-fucosyl residue (Puhlmann and others 1994), CCRC-M2 that recognizes RG-I (Puhlmann and others 1994), CCRC-M7 that recognizes arabinosylated 1,6-linked β- D-galactan (Steffan and others 1995), and CCRC-R1 that recognizes RG-II (Williams and others 1996).

The epitopes recognized by LM5 and LM6 are known with some certainty since these antibodies were generated against specific oligosaccharides coupled to proteins (Jones and others 1997; Willats and others 1998). The epitope recognized by CCRC-M1 has been shown, using a series of structurally related oligosaccharides, to contain a terminal 1,2 linked α-L-fucosyl residue (Puhlmann and others 1994). The binding characteristics of PAM1, JIM5, and JIM7 have been compared using pectin of varying degrees and patterns of methyl esterification (Willats and others 1999, 2000a). PAM1 binds most effectively to fully de-esterified pectin whereas JIM5 and JIM7 bind to pectin with a wide range of degrees of methyl esterification. Some published studies, however, have been interpreted based on the assumption that JIM5 recognizes de-esterified HG and JIM7 recognize methylesterified HG. Thus, the results of immunolocalization studies using JIM5 and JIM7 must be interpreted with caution (Willats and others 2000a).

Immunolocalization studies have provided evidence that the localization and structure of pectin may have an impact on plant cell growth and development. For example, the presence of a pectin sheath on the primary walls of developing cotton fibers is correlated with the ability of epidermal cells to differentiate into elongating fiber cells (Vaughn and Turley 1999), while

epidermal cells that do not have a pectin sheath do not elongate. The role of the external pectin-rich layer in cell elongation, however, remains to be determined since epidermal cells in maize coleoptiles (Schindler and others 1995), flax (Jauneau and others 1997), and pea stems (Fujino and Itoh, 1998) have an asymmetric distribution of pectin but do not elongate (Ridley and others 2001).

The complex macromolecules of pectic substances would seem to offer a number of possibilities for enzyme action. Pectic enzymes have received considerable attention regarding their involvement in ripening and softening of cell wall components. Among the well known pectinases are pectin lyase (PL), polygalacturonase (PG) and specifically pectin methylesterase (PME), (NOSB 1995).

2.2 Pectinases

Pectinases were some of the first enzymes to be used in homes. Their commercial application was first observed in 1930 for the preparation of wines and fruit juices. Only in the 1960s did the chemical nature of plant tissues become apparent and with this knowledge, scientists began to use a greater range of enzymes more efficiently. As a result, pectinases are today one of the upcoming enzymes of the commercial sector. Pectinases are now an integral part of fruit juice and textile industries as well as having various biotechnological applications. The estimated value of sales of all industrial enzymes in 1995 was $1 billion, of which some $75 million was assessed for pectinases. As indicated by Kashyap and others (2001) by 2005, the whole world market for industrial enzymes was expected to be $1.7±2 billion (Godfrey and West 1996).

In particular, pectinase is used primarily to depolymerize and esterify plant pectins in fruits such as apples, lemons, cranberries, oranges, cherries, grapes, and tomatoes, to name a few. The application of pectinase enables the entire fruit to be liquefied. This has the effect of

improving saccharification and thus sweetness, reducing waste and energy use per unit of juice produced, improving aroma and color; enhancing clarity, removing haze, preventing gel formation, and increasing fruit juice yield (Nielsen and others 1994; White and White 1997). However, pectinase activity generally results in a loss of textural integrity; it's used to break down pectin for example in the juice industry. In determining the standard of identity of natural juice, juice extracted using pectinase is usually considered minimally processed (Haight and Gump, 1995). Commonly recognized pectinases are shown in Table 2.1.

The action of PME also makes the pectin susceptible to further degradation by PG; thereby cleaving the polygalacturonic acid backbone of the pectin and reduces the average length of the pectin chains. This degradation of the pectin chains reduces the viscosity of the juice. For example, collectively, polysaccharides reduce juice extraction and are primarily responsible for fouling of filters during clarification steps. Wine quality also can be affected through changes in clarity, while an effect on viscosity may influence mouth feel and the perception of tastes and aromas. PME which is capable of degrading polysaccharides therefore has the potential to improve juice yields and wine processability through the removal of problem colloids, to increase wine quality via breakdown of grape cell walls to yield better extraction of color and aroma precursors, and alter the perception of wine components (Matthews and others 2004).
Several studies have demonstrated the role of pectin disarrangement in fruit tissue during softening (Seymour and others 1990; Martin-Cabrejas and others 1994; El Buluk and others 1995). Some researchers have attempted to identify the enzymes responsible for these cell wall changes during ripening (Huber 1983; Brady 1987; Tucker and Grierson 1987). The role of cell wall hydrolases in fruit ripening has also been investigated (Fischer and Bennett 1991).

Table 2.1 Classification of the pectolytic enzymes

Name	EC No.	Primary substrate	Products	Mechanism
Esterase				
Pectin methylesterases (pectin-esterases)	3.1.1.11	Pectin	Pectic acid + methanol	Hydrolysis
Polygalacturonases				
Protopectinases		Protopectin	Pectin	Hydrolysis
Endopolygalacturonases	3.2.1.15	Pectic acid	Oligogalacturonates	Hydrolysis
Exopolygalacturonases	3.2.1.82	Pectic acid	Monogalacturonate	Hydrolysis
Oligogalacturonate hydrolases		Trigalacturonate	Monogalacturonate	Hydrolysis
Δ4:5 Unsaturated oligogalact-uronate hydrolases		Δ4:5 $(\text{Galacturonate})_n$	Unsaturated monogalacturo-nate and saturated $(n-1)$	Hydrolysis
Endopolymethylgalacturonases		Pectin	Methyl oligogalacturonates	Hydrolysis
Lyases				
Endopolygalacturonate lyases (endopectate lyases)	4.2.2.2	Pectic acid	Unsaturated oligogalacturo-nates	Transelimination
Exopolygalacturonate lyases (exopectate lyases)	4.2.2.9	Pectic acid	Unsaturated digalacturonate	Transelimination
Oligogalacturonate lyases	4.2.2.6	Unsaturated digalacturonate	Unsaturated monogalactur-onate	Transelimination
Endopolymethylgalacturonate lyases (endopectin lyases)	4.2.2.10	Pectin	Unsaturated methyl oligogalact-uronates	Transelimination

Adapted from Whitaker (1994); Stutzenberger (2000)

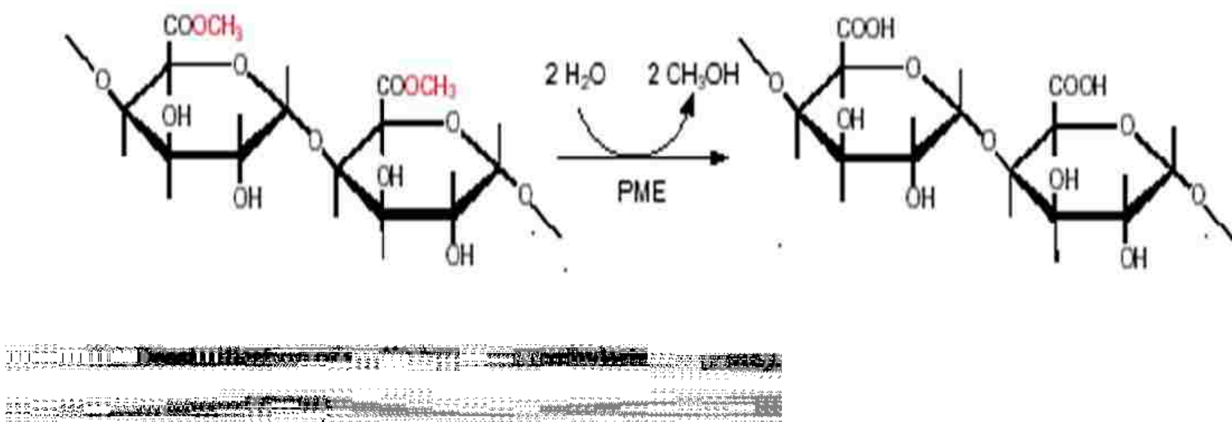
Nogata and others (1993) found low levels of PG activity in strawberry fruit. Ali and others (1995) also reported that changes in β-D-galactosidase (β-gal) activity, tissues softness, and increased pectin solubilization might play important roles in mango ripening. In addition, other enzymes, namely endo-glucanases (EGases) such as xylanases, glycosidases, and cellulases, play an important role in cell wall metabolism (Ahmed and Labavitch 1980; Awad and Young 1980). Pectin lyase (PL) carries out a reaction similar to that performed by PG, except it catalyzes a β -elimination reaction between the methylated residues (Collmer and Keen 1986). Ali and others (1995) also reported that changes in β-D-galactosidase (β-gal) activity, tissues softness, and increased pectin solubilization might play important roles in mango ripening. Pectin acetylesterase acts similar to PME in that it hydrolyzes the ester bond between a glycosyl carbon and an acetyl group (Williamson 1991). Moreover, protopectinases (PPases) are a heterogeneous group of enzymes that release water soluble pectin from insoluble protopectin in plant tissues by restricted degradation of the substrate (Salvador and others 2002). Peroxidase can generate hydroxyl radicals *in situ* within the cell wall (Everse and others 1991).

2.2.1 Plant Pectin Methylesterase (PME)

In the food industry, control of PME activity is important in processes concerning the production and storage of fruit juices and purees. It is well known that PME activity changes the texture of fruit products. For this reason storage at very low temperature or a high temperature pasteurization process are required in order to inactivate the PME in industrial fruit products. The complex macromolecules of pectin would seem to offer a number of possibilities for enzyme action. Pectin enzymes have received considerable attention regarding their involvement in ripening and softening of cell wall components.

According to Giovane and others (2004) pectinesterase was discovered in 1840 by Frémy in vegetable juice (Frémy 1840). Since then numerous higher plants and microorganisms have

been shown to contain or to be able to produce pectin methylesterase (PME). In addition, PME is particularly abundant in citrus fruits and vegetables. In fact all higher plants seem to contain pectin esterase in all living tissues. Pectin methylesterases (PME, EC 3.1.1.11) is carboxylic acid esterase and belongs to the group of hydrolases. It catalyzes the specific de-methylesterification of HGA within plant cell walls, releasing methanol and protons (and creating negatively charged carboxyl groups) in the process (Pelloux and others 2007) shown in Figure 2.2. Emerging evidence indicates that PME has been suggested to be involved in cell wall growth, extensibility, regeneration, in the separation of root border cells from the root cap, and in the formation of abscission zones, fruit ripening, parasitic plant haustorial formation and host invasion (Gaffe and others 1997).



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The hydrolysis of methyl ester groups catalyzed by the PME enzyme produces pectin with a lower methylation degree that can undergo further enzymatic cleavage by polygalacturonase (PG) and pectin lyase (PL) (Whitaker 1994). The two enzymes (PME and PG) have been shown to be synergically involved in the metabolism of plant cell wall. The enzymatic pectin de-esterification pattern shows plant PMEs are generally regarded to act in a block-wise pattern (Rexová-Benková and Markovi, 1976 and Bordenave, 1996). Block-acting PMEs proceed linearly along the pectin backbone, starting with a free carboxyl group at the reducing

end (Solms and Deuel, 1955; Voragen and others 2003) or non-reducing end (Limberg and others 2000), producing a block of de-esterified pectin. However, it is possible that a random-acting plant PME may exist (Markovic and Kohn 1984). The action of PME as stated above makes the pectin susceptible to further degradation by PG because this enzyme acts only on segments of the pectin chain that have been de-methylated by PME. PG cleaves the polygalacturonic acid backbone of the pectin and reduces the average length of the pectin chains. This degradation of the pectin chains reduces the viscosity of the juice (Konno and Yamasaki, 1982).

Most plant PMEs has a molecular weight in the range of 22.0-37.0 kDa (Seymour and Knox, 2002). The isoelectric points of plant PME are usually above pH 7.0 and the pH optimum ranges between 6.0 and 8.5 (Markovic and Kohn 1984; Voragen and others 2003). The kiwi PME enzyme is a monomer, with an apparent molecular mass (~50.0 kDa) higher than that of most plant PMEs. A molecular size higher than 35.0 kDa has been reported for PMEs such as thermally-tolerant isoenzyme PME (TT-PME) from orange (Cameron and others 2005; Ciardiello and others 2008), PMEα from mung bean hypocotyl (Goldberg and others 2001; Ciardiello and others 2008), and the acidic PME from jelly fig achene (Ding and others 2002; Peng and others 2005; Ciardiello and others 2008), for which glycosylation was suggested. Kiwi PME has been reported to be a glycoprotein since it is positively stained by the Schiff reagent after SDS-PAGE and binds to a concanavalin A-Sepharose column (Giovane and others 1990; Ciardiello and others 2008).

The recent heterologous expression of a functionally active jelly fig PME in a glycosylating system (*Pichia pastoris*) suggested that glycosylation is important for proper folding and stability of the enzyme (Ding and others 2002; Peng and others 2005; Ciardiello and others 2008). The neutral PMEα isolated from mung bean (*Vigna radiata*) hypocotyls has an

apparent molecular size of 45.0 kDa (Goldberg and others 2001; Ciardiello and others 2008). Its partial primary structure (deduced from the nucleotide sequence) revealed the presence of four putative N-glycosylation sites, indicating that the enzyme may be glycosylated. Kiwi fruit PME has the highest sequence identity with mung bean PMEα, with which it shares two N-glycosylation consensus sequences (Ciardiello and others 2008). Glycosylation does not seem to affect the catalysis of kiwi fruit PME. In fact, a comparative study carried out with the un-glycosylated kaki fruit PME and the kiwi fruit enzyme, showed similar specific activity values and similar salt and pH dependence of activity (Ciardiello and others 2004; Ciardiello and others 2008).

2.2.2 Bacteria and Fungus PME

PME is produced by a variety of microbes, including many fungal genera (*Aspergillus, Botrytis, Fusarium, Helminthosporium, Heterobasician, Lacknospira, Myrothecium, Penicillium, Phvtophthora, Saccharomyces,* and *Tubercularia* and variety of bacteria (*Bacillus, Clostridium, Corynebacterium, Lactobacillus, Pseudomonas, and Thermomonospora* species). Characteristics of PME from fungal species show some similarity to plant PME: (i) their molecular weight values are in the range of 26-45 kDa, (ii) their isoelectric points generally may fall in the pH range of 6.0 to 7.0, (iii) their pH optima fall in two ranges (pH 4.0 and 6.0), and (iv) their activity is stimulated by NaCl concentrations of 0.05-0.25 M (Stutzenberger 2000). However, there have been reports that the isoelectric points of PME's vary from as low as pH 3.8 for fungal PME (Christgau and others 1996) to pH 9.9 for *Erwinia* PME (Plastow 1988).

The microbial PMEs are medium sized enzymes (35.0-45.0 kDa) that are active as monomers (Seymour and Knox 2002). The pH optimum for microbial PME depends largely on the source of the enzyme, but usually tend to fall between pH 6.0-9.0 (Markovic and Kohn 1984; Voragen and others 2003). Many PMEs of fungal origin are glycoproteins and there is even a

report of a bacterial PME from *E. chrysanthemi*, which in fact is a lipoprotein (Shevchik and others 1996). Microbial PMEs differ in their mode of action; PMEs have been reported with both block-wise and random patterns (Rexová-Benková and Markovi 1976; Bordenave 1996). Fungal PME's attack the methyl groups on the pectin randomly, resulting in a random distribution of the non-methylated GalA residue (Versteeg 1979). It therefore appears, that the mode of de-esterification by PMEs is independent of the origin (microbial, fungal, or plant), but is rather determined by enzyme properties characterized by pI and pH optimum (Johansson and others 2002).

In *Corynebacterium* species isolated from yam soft rot, PME is induced during growth in plant tissue, but not in pectin media containing purified pectin. This suggests that additional factors other than substrate are necessary for induction. In *Clostridium multifermentans*, polygalacturonic acid is a better inducer of PME than is pectin because PME and PL activities reside in the same protein. It is interesting in this dual-function protein that PME activity was eliminated by heating for 30 min at pH 7.0 without loss of PL. Lack of PME in *Trichoderma reesei* and *Talaromyces emersonii* severely limits the ability of their pectolytic enzymes to solubilize agricultural residues. When their enzyme systems are supplemented with PME from another source, such plant materials are readily esterified (Stutzenberger 2000). The action of PME is obviously very important to the action of other pectolytic enzymes in nature. Because polygalacturonases (PG) and pectin lyase (PL) cannot act on highly methylated chains, the inability of a microbe to synthesize PME severely limits its ability to use pectin as a carbon and energy source.

2.2.3 Structure Determination of PME

The first PME primary structure was determined by direct protein sequencing for the main PME isoforms from tomato fruit. Several PME isoforms with different biochemical

properties are either constitutively or differentially expressed in specific plant tissues and at specific developmental stages (Giovane and others 1990; Gaffe and others 1997; Micheli and others 2000; Micheli and others 2001; Ciardiello and others 2008). Recently, sequencing of *Arabidopsis* genome allowed the identification of 67 PME-related genes in this species (*Arabidopsis* Genome Initiative 2000; Ciardiello and others 2008). The sequencing of the entire genome of *Arabidopsis* revealed the presence of two types of PME genes: type I, having a long N-terminal pro-region and type II, having a short or absent pro-region (Giovane and others 2004). PME proteins of bacteria and fungi do not contain any pro-region (Pelloux and others 2007). Most of plant PMEs are encoded as precursors, bearing at the N-terminus the signal peptide and a large pro-region (consisting of about 250 amino acid residues), which is cleaved off to yield the mature enzyme. The function of the pro-region is as yet unknown. Several possible roles, such as PME intramolecular chaperone, (Micheli and others 2001; Ciardiello and others 2008) correct targeting of PME toward the cell wall and inhibition of its activity before processing to mature protein have been suggested (Michel and others 2001; Bosch and others 2005; Ciardiello and others 2008).

Major advances facilitating attempts to elucidate the mechanism(s) of action of PMEs were achieved when 3-D crystallographic structures were obtained of first bacterial, (phytopathogenic bacteria *Erwinia chrysanthemi*, agent of the soft rot of plants, PME) and then plant PMEs. The structure of the bacterial PME from *E. chrysanthemi* showed convincingly that PME was a new type of hydrolase which contains neither α/β hydrolase fold nor a catalytic Ser-His-Asp triad. Instead, PMEs appear to be carboxylate hydrolases with two aspartic acid residues at the active site. This was not surprising since previous studies had already excluded tomato PME as a serine esterase and had also shown that there is no conserved histidine or serine residue as a potential catalytic residue in the PME primary structures. Furthermore, modification

of histidine residues in tomato and *Aspergillus niger* PMEs demonstrated that these residues do not have active site function, but contribute to overall structural stability (Johansson and others 2002).

Furthermore, the 3-D crystallographic structures of plant PMEs were obtained following the first large-scale purification of a ripe carrot root (*Daucus carota*) PME (Markovič 1978; Markovič and others 2002), which had previously been the limiting step for plant PME protein analysis, in part because of the difficulty of expressing plant PMEs in heterologous systems. However, an acidic PME from jelly fig (*Ficus awkeotsang*), which forms inclusion bodies when expressed in *E. coli*, was subsequently functionally expressed in *Pichia pastoris*, opening new possibilities for PME characterization. Analysis of the crystallized mature carrot PME at 1.75 Å resolution showed that the protein, as initially partially inferred from modeling, belongs to the family of right-handed parallel β-helix proteins. The long, aromatic residue-lined cleft across the molecule is characteristic of carbohydrate-binding sites and contains the active site. The active site contains two aspartic acid residues (D136, D157 in the carrot PME) at the center, which are distinguishing features of aspartyl esterases, two glutamines (Q113, Q135) and one arginine residue (R225). The importance of the glutamine and aspartic acid residues in the active site of a tobacco PME was recently confirmed in transgenic plants expressing a mutated isoform (Johansson and others 2002).

A mechanism of action has been proposed, according to which D157, stabilized by a hydrogen bond to R225, performs a nucleophilic attack on the ester bond of the carboxymethyl group of HGA. The negatively charged intermediate is stabilized by Q113, Q135 and D136; D136 acts as a proton donor and, thus, enables methanol to be released. The active site is restored via extraction of a proton from a water molecule by D136, and the HGA chain is held in place, awaiting the next de-methylesterification step, within the substrate cleft by its interaction

with the aromatic rings. The crystallographic structures of the carrot and tomato PMEs show striking similarities, and are almost entirely superimposable. In addition, both of these plant PMEs show similar folding topology to PME from *Erwinia chrysanthemi*, although one major difference lies in the form of the substrate cleft, which has higher walls in the bacterial PME. These findings suggest that the structure of PMEs has been strongly conserved and have profound implications for the interactions between PMEs and potential inhibitors (Giovane and others 2004; Pelloux and others 2007).

2.2.4 Inhibitors of PME

Recent advancement in the efficient purification of PMEs in a single step uses a proteinaceous inhibitor of PME (PMEI) which has been purified from kiwi fruit. The kiwi PMEI is active against plant PMEs, forming a 1:1 non-covalent complex. The sequence shows significant similarity with the N-terminal pro-peptides of plant PME, and with plant invertase inhibitors. PMEI has an interest in the food industry as inhibitor of endogenous PME, responsible for phase separation. The inhibitor is not species-specific since it has inhibited PME from several other plant sources. The inhibition has been observed in the pH range from 4.0 to 7.5. However, the PMEI does not seem to discriminate between PME isoenzymes, i.e. PME I and PME II (Giovane and others 2004). However plant PMEI has no inhibition on bacteria PME activity.

Kiwi fruit PME was initially overlooked because of the presence of PMEI in the tissue. It was then purified from the cell wall fraction of incompletely ripened fruit, where PMEI was present in lower amount (Giovane and others 1990; Giovane and others 1995; Ciardiello and others 2004; Ciardiello and others 2008). Kiwi PME activity is inhibited by PMEI (Ciardiello and others 2004; Ciardiello and others 2008). The discovery in kiwi fruit of a proteinaceous inhibitor (PMEI) of PME suggested a possible mechanism of regulation of the mature enzyme

activity (Balestrieri and others 1990; Ciardiello and others 2008). Kiwi PMEI is a monomeric protein of 152 amino acid residues, with a pI of pH 4.5.

Its amino acid sequence shows significant similarity with plant β-fructofuranosidase inhibitors (Scognamiglio and others 2003; Ciardiello and others 2008) and with the N terminal pro-region of plant PMEs (Giovane and others 2004). Recently, the structural determinants of the interaction between PME and its inhibitor have been investigated by crystallization and analysis of the complex formed by kiwi fruit PMEI and tomato fruit PME, chosen as a representative plant PME (Di Matteo and others 2005; Ciardiello and others 2008). The enzyme folds into a right-handed parallel β-helix, as already shown for PME from the bacterium *Erwinia chrysanthemi* (Jenkins and others 2001; Ciardiello and others 2008) and from carrot root (Johansson and others 2002; Markovič and others 2002; Ciardiello and others 2008)

This structure, described for the first time in pectin lyase (PL) (Yoder and others 1993; Ciardiello and others 2008) is common to several pectin modifying enzymes. The crystallographic structure shows that the arginine and the two aspartic acid residues, postulated to be involved in the catalytic activity, are located on the surface of the β-helix in a cleft formed by protruding loops, suitable for pectin binding. Kiwi PMEI folds as a four α-helix bundle bearing an unusual N-terminal hairpin-like helical extension, similar to the inhibitor of β–fructofuranosidase from tobacco (Hothorn and others 2004a; Ciardiello and others 2008) and PMEI from *Arabidopsis* (Hothorn and others 2004b; Ciardiello and others 2008). The structure of the PME/PMEI complex shows that the inhibitor binds PME covering the shallow cleft where the putative active site of the enzyme is located. The interaction interface is large and displays a high polar character, typical of non-obligate complexes of soluble proteins, which need to expose a hydrophilic surface in their uncomplexed form (Di Matteo and others 2005; Ciardiello and others 2008).

Girouard (1962) observed inactivation of PME by urea, glycine, and formaldehyde (Versteeg 1979). Moreover, Lewis and others (2008) reported epigallocatechin gallate (EGCG), a green tea component, as a natural inhibitor for PME. In a gel assay for PME activity, EGCG blocked esterase activity of pure PME as well as PME extracts from citrus and from parasitic plants. Molecular docking analysis of PME and EGCG suggests close interaction of EGCG with the catalytic cleft of PME. Ly-Nguyen and others (2004) showed types of inhibitions by kiwi fruit PMEI from purified banana, carrot, and strawberry PME. Banana and strawberry PMEs showed a noncompetitive inhibition, K_i (nM) 6.92 ±0.19 and 14.37 ±0.21 and K_M (mg/mL) 0.1458 ±0.0047 and 0.6010 ±0.0267, respectively. However, carrot PME showed competitive inhibition, K_i (nM) 0.91 ±0.17 and K_M (mg/mL) 0.2365 ±0.0478.

2.2.5 Stimulators/Activators of PME

The effect of salts on alfalfa PMEs was thoroughly studied by Lineweaver and Ballou (1945). It appeared that divalent cations and not anions stimulate PME at the acid side of its pH optimum. Castro and others (2004) purified PME form green pepper (Capsicum annuum) that required the presence of 0.13 M NaCl for optimum activity. Enzyme inactivation studies have shown that, under suitable high-pressure/temperature conditions, PG can be inactivated, whereas PME can remain active. At the same time, commercial tomato PME was found to be activated under lower pressure treatment at mild temperature (~300MPa, 60–65°C) (Castro and others 2006).

Activation of PME by metallic ions appears to be due mainly to interaction of the ions with the substrate rather than with the enzyme (Nari et al 1991). The metallic ions would release the enzyme molecules trapped in the blocks of free carboxyl groups, enabling them to reactivate. However, there is evidence against such a mechanism (Marcus and Schejter 1983; Alonso et al 1995); for instance, calcium and sodium, like other metallic ions, can bind enzymes, causing

changes to a more active conformation (Mildram 1970). In addition, *Aspergillus repens* PME activity increased with higher concentration of K^+ and Na^+. The concentration of 15 mM was highest for maximum PME activity when Mg^{2+} and Zn^{2+} were employed, while the highest PME of *Aspergillus repens* occurred at 20 mM Ca^{2+}. Further increase in their concentrations resulted in the decline of the PME activity (Arotupin and others 2008).

PME extracted from guava fruit also had high activity with Li^+, K^+ and Rb^+ monovalents ions at 0.15 M, but with slightly lower values than with Na+ ions (da Silva Cerqueira Leite and others 2006). Assis and others (2002), investigated PME from acerola in the presence of the Na^+, K^+, $Li^{+,}$ Ca^{2+} and Mg^{2+} which showed that Na^+ was the best choice and led to a higher catalytic action of PME. Nari and others (1991) suggested that the PME activation by cations could be due to interaction with carboxyl groups present in the pectin molecule. Alonso and others (1995, 1997) and Degraeve and others (2003) suggested an activation of PME by Ca^{2+} due to the binding of the ion to the enzyme itself as a co-factor rather than a direct effect of calcium on the product of the reaction.

2.3 Benefits and Importance of Fermented Foods

Fermented foods comprise about one-third of world consumption, and 20-40% of individual diets. Fermentation considerably increases variety in the human diet. Many interesting cuisines depend on regional fermented food products for their individuality and in this day and age international cuisines are popular (Campbell-Platt 2000). Many staple food crops cannot be readily or safely consumed prior to processing. Raw wheat, barley and maize are not very appetizing, but the products of their fermentation (breads and beer) are much more digestible and acceptable. People who cannot tolerate milk find that fermented milks, yogurts, and cheeses are more acceptable. Important flavor compounds, including diacetyl and acetaldehyde, are produced by lactic acid bacteria (LAB) during the fermentation of milk. Grapes are satisfying to

eat raw, but are highly perishable. Their fermentation produces a range of wines with different characteristics, depending on the cultivar of the grape. Wines are much safer to drink than contaminated water. Raw legume beans contain lectins, which are toxic; cooking and fermentation produce a variety of edible legume products.

Isoflavones, from soybeans, have been shown to be beneficial in the diet. Soy sauce is an essential component of many Eastern Asian dishes, more than 1 billion liters of soy sauce are produced every year in Japan alone. Fish sauces, which preserve the amino acids of highly perishable fish, have a similar role (Campbell-Platt 2000). Fermentation was the earliest form of food biotechnology. Fermented foods are the major group of functional foods which provide extra benefits to consumers' diets beyond those expected from the major nutrients present. Fermentation may result in particular desirable nutrients becoming more readily available, or in the amount of less desirable or toxic components being minimized. Knowledge of the mechanisms by which specific microorganisms and their enzymes change foods during fermentation facilitates control of the fermentation process.

It is known that diet is important for human well being, as well as being contributory or preventative in the development of a range of diseases. It is believed that several fermented foods help to improve the quality of human life and also delay or prevent the onset of some diseases, including cancer and some degenerative diseases. The importance of the polyphenolic antioxidants in tea and red wines has been recognized, and the role of certain lactic acid bacteria (LAB) and bifidobacteria as probiotics in fermented milks is an ongoing research and development in the food industry (Campbell-Platt 2000).

2.4 Lactic Acid Bacteria (LAB)

Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. Many foods such as ripened cheeses, pickles, sauerkraut, fermented

sausages, and Tabasco pepper sauce are preserved products in that their shelf life is extended considerably over that of the raw materials from which they are made (Campbell-Platt 2000). In addition to being made more shelf stable, all fermented foods have aroma and flavor characteristics that result directly or indirectly from the fermenting organisms. Therefore, the mash fermentation process is a very critical step in final hot sauce quality. The acidity of a plant product permits good bacterial growth and at the same time the product is high in simple sugars. Lactic acid bacteria (LAB) should be expected to grow, and the addition of low levels of NaCl will ensure their growth preferential to yeasts (Batt 2000).

Lactic acid bacteria (LAB) are widely used in the feed, food and dairy industries. LAB that are found in the Tabasco pepper mash fermenting in a wood barrel normally may come from the pepper itself or from natural inoculation during harvesting and grinding. Most fermentation is initiated by LAB, but during the initial fermentation stage, a great many unrelated bacteria, yeasts, and fungi can be isolated. All theses microorganisms are widespread in nature and, at the beginning of the fermentation, far outnumber the desirable LAB in uncontrolled fermentations. Therefore, the primary fermentation stage is the probably the most important phase in the Tabasco pepper fermentation process. During this period the number of LAB and the fermentative and oxidative yeasts increases rapidly, whereas the undesirable flora decreases rapidly and may even disappear. At the same time, there is a decrease in pH of the mash and an increase in total acidity. During the 3 year fermentation of the mash there are still a low number of yeasts while there is a further increase in total acidity. The remaining oxidative yeasts are held without further development by anaerobiosis (Campbell-Platt 2000).

LAB are a group of bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria includes a non-taxonomic group of Gram-positive non-sporeforming bacteria which ferment simple water soluble

carbohydrates (WSC) to produce lactic acid as the predominant end product. As fermenting organisms, the bacteria lack functional heme-linked electron transport systems or cytochromes, and obtain their energy by substrate-level phosphorylation; this group of bacteria does not have a functional Krebs cycle (Wood and Holzapfel, 1992). Lactic acid bacteria are mesophilic, although some can grow below 5°C and as high as 45°C. With respect to growth pH, the range is from pH 3.2 to pH 9.6. However, most of the bacteria grow in the pH range 4.0-4.5 (Battcock and Azam-Ali, 1998). LAB can tolerate high salt concentrations. However, the optimum salt concentration for growth is 6.5% (James, 2000). Moreover, Jones and Ethchells (1944) conducted extensive bacteriological studies on the fermentation of various vegetables in brines of different concentrations which stated that the salt concentration used, rather than the kind of vegetable, was the controlling factor on the character of the microbial flora and microbial activity. In fermentation at low salt content, 5 % or less, large populations of acid forming and other bacteria occurred. At or above 15 % salt, little or no growth of acid bacteria occurred.

The majority of LAB can ferment simple water soluble carbohydrates (WSC), such as monosaccharides, disaccharides and trisaccharides (e.g. glucose, arabinose, maltose, cellobiose, fructose, galactose, lactose, mannose, elizitose, melibiose, raffinose, rhamnose, ribose. sucrose, trehalose and xylose) and related compound such as mannitol, sorbitol, galacturonate and gluconate, which are part of the WSC of the pepper. In this regard, exogenous enzymes such as amylases, pectinases, cellulases and/or hemicellulases, can degrade polysaccharides (e.g., pectin, starch and cellulose) and/or oligosaccharides into monosaccharides, disaccharides, trisaccharides and/or other fermentable carbohydrates (Sneath and others 1986).

Given that LAB are described as microaerophilic as they do not utilize oxygen or need very little oxygen to function. Because of this, the changes that they effect do not cause drastic changes in the composition of the food (Sneath and others 1986). The LAB commonly used in

fermentation can be divided into two major categories: i) the homo-fermentative LAB which ferment predominantly hexoses to lactic acid; and ii) the heterofermentative LAB which ferment hexoses to lactic acid and other products, such as ethanol, acetic acid, CO_2, formate, or succinate (Wood and Holzapfel, 1999).A summary of the differentiation of LAB genera with classical phenotypic tests are shown in Table 2.2.

The boundaries of the group have been subject to some controversy, but historically the formation of the core group, mainly included species from the genera *Lactobacillus, Streptococcus, Leuconostoc* and *Pediococcus* (James 2000). Taxonomic revisions of these genera and the description of new genera suggest that the LAB comprise the following: *Aerococcus, Alloiococcus, Carobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella* species, which all carry out lactic acid fermentation. The taxonomy of these microorganisms has been described in *Bergey's Manual of Systematic Bacteriology* (Sneath and others 1986). The classification of LAB is largely based on phenotyical and biochemical characters which may not be enough to identify the bacteria strain. Today, with availability of rapid and automatic DNA sequencing technology, direct sequencing of the16S ribosomal RNA (rRNA) gene has emerged to resolve this identification problem. This method is used for determining phylogenetic positions of species and genera, rRNA; since the sequence contains both well-conserved and less conserved regions (Axelsson 2004).

The polymerase chain reaction (PCR) technique is a powerful and useful tool for identification purposes. Moreover, a number of techniques based on PCR have been developed. For example, the development of quantitative real-time polymerase chain reaction (QRT-PCR) which is useful in quantifying DNA.

Table 2.2 Arrangement of the genus *Lactobacillus*

Characteristic	Group I, Obligately homofermentative	Group II, Facultatively heterofermentative	Group III, Obligately heterofermentative
Pentose fermentation	−	+	−
CO_2 from glucose	−	−	+
CO_2 from gluconate	−	+[a]	+[a]
FDP aldolase present	+	+	−
Phosphoketolase present	−	+[b]	+
	Lb. acidophilus	*Lb. casei*	*Lb. brevis*
	Lb. delbrückii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantarum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sakei*	*Lb. reuteri*

[a]When fermented.
[b]Inducible by pentoses.

Adapted from Sharpe (1981); Kandler and Weiss (1986); Axelsson (2004)

Presently, there are two types of quantitative Real-Time PCR detection chemistries: (i) specific sequence detection; it distinguishes between a specific sequence of interest and non-specific products and (ii) non-specific detection; detects any double stranded dsDNA produced during the reaction. This method uses a double stranded (dsDNA) intercalating dye that that fluoresces once bound to DNA. The most common is SYBR green 1. This dye excites at 497 nm and emits at 520 nm. A melting curve is performed between 40°C-95°C. The fluorescence will decrease when the dsDNA disassociates. This depends on length and base pairs (bp) composition. Once the QRT-PCR reaction is optimized, the results will yield a single peak corresponding to the proper primer pair. This method yields high discriminatory power and is particularly valuable in optimizing a QRT-PCR reaction before developing more expensive specific tests.

The pectinolytic activities of LAB have been addressed in studies of fermentation processes like wine making, cassava roots, and silage. For example, PME and PG activities have been detected in the spontaneous fermentation of cassava roots (Brauman and others 1996; Ampe and Brauman 1995). Pectinolytic activities have been found in *Lactobacillus casei, Lactobacillus plantarum* and *Lactococcus lactis* isolated from raw milk in Algeria (Karam and Belarbi 1995).

2.4.1 *Lactobacillus* Genus

It is well known that LAB, in particular lactobacilli, plays an important role in fermentation processes (Fontana and others 2005). Their ability to lower the pH and produce bacteriocins prevent the growth of pathogenic and spoilage microorganisms, improving the hygienic safety and storage of pepper products. The genus *Lactobacillus* is a large, heterogeneous collection of genetically and physiologically diverse and highly acid-tolerant LAB species. A measure of their diversity can be estimated by the range in the G + C% of DNA of

species among lactobacilli. Members of the species have G + C% of 32-55%, which is a much wider range than is encountered with other LAB. Their common taxonomical features are restricted to their rod shape and their ability to produce lactic acid either as an exclusive or at least a major end product.

Lactobacillus are typically rod-shaped with a size range of 0.5-1.2 x 1-10 µm. Lactobacilli are facultative anaerobes that, in general, grow slowly in air, but their growth is sometimes enhanced by 5% carbon dioxide. Their optimum growth temperature is 30-40°C, but they can grow over a range of 5-53°C. They are aciduric with an optimum growth pH of 5.5-5.8, but in general they can grow at a pH <5.0. Approximately 135 species and 27 subspecies validly recognized currently exist (Bernardeau and others 2008). Table 2.3 summarizes the characters used to distinguish among the three groups and some of the more well-known species included in each group (Sharpe 1981; Kandler and Weiss 1986; Axelsson 2004).

In addition, certain bacteria in this genus are known to produce a group of low molecular weight substances, including antibodies, peptides, and peptidoglycans. *Lactobacillus* species are added to human and animal food stuffs to preserve them, enhance their flavors, and for probiotic purposes so that these bacteria will become available to the gastrointestinal tract. *Lactobacillus plantarum* strains, for example, are grown commercially in large amounts and used as starter cultures for the commercial preservation of a variety of human and animal foods. *Lactobacillus plantarum* strains are used to preserve meats, vegetables, and dairy products, as well as animal silage. *Lactobacillus acidophilus* strains are grown commercially in large amounts to be added to human (i.e., milk) or animal (feed stuffs) foods as a means of introducing these bacteria into the gastrointestinal tract for probiotic benefits (Axelsson 2004).

Table 2.3 Differential Characteristics of Lactic Acid Bacteria

	Rods		Cocci							
Character	*Carnob.*	*Lactob.*	*Aeroc.*	*Enteroc.*	*Lactoc.* *Vagoc.*	*Leucon.* *Oenoc.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*	*Weissella* [a]
Tetrad formation	−	−	+	−	−	−	+	−	+	−
CO_2 from glucose[b]	−[c]	±	−	−	−	+	−	−	−	+
Growth at 10°C	+	±	+	+	+	+	±	−	+	+
Growth at 45°C	−	±	−	+	−	−	±	±	−	−
Growth in 6.5% NaCl	ND[d]	±	+	+	−	±	±	−	+	±
Growth in 18% NaCl	−	−	−	−	−	−	−	−	+	−
Growth at pH 4.4	ND	±	−	+	±	±	+	−	−	±
Growth at pH 9.6	−	−	+	+	−	−	−	−	+	−
Lactic acid[e]	L	D, L, DL[f]	L	L	L	D	L, DL[f]	L	L	D, DL[f]

+, positive; −, negative; ±, response varies between species; ND, not determined.
[a]*Weissella* strains may also be rod-shaped.
[b]Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.
[c]Small amounts of CO_2 can be produced, depending on media.
[d]No growth in 8% NaCl has been reported.
[e]Configuration of lactic acid produced from glucose.
[f]Production of D-, L-, or DL-lactic acid varies between species.

Adapted from Axelsson (2004)

Reports on the beneficial effects of *Lactobacillus* therapy have increased in recent years with findings that dietary Lactobacillus therapy: (i) affords protection from colon cancer for human populations on Western diets, (ii) reduces the incidents of experimentally induced large bowel tumors in rats, (iii) reduces the fecal concentration of bacterial enzymes known to catalyze the conversion of procarcinogens to proximal carcinogens in humans, and (iv) reduces the serum cholesterol levels in swine (Fontana and others 2005). The purposes of fermentation are to increase pepper value, prevent pulp separation after sauce making, and produce a shelf stable sauce. The LAB are a diverse group of organisms with a diverse metabolic capacity. This diversity makes them very adaptable to a range of conditions and maybe largely responsible for their success in pepper sauce fermentations.

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CHAPTER 3

GENERAL PROPERTIES OF PECTIN METHYLESTERASE FROM TABSCO PEPPER, *CAPSICUM FRUTESCENS*

3.1 Introduction

Hot sauce is an international sales success, particularly in Southeast Asia. The product is produced from the Tabasco pepper (*Capsicum frutescens* L.). One of the major concerns of processing is separation of sediments and layering of the sauce. Separation is an undesirable production condition because consumers view this as a defect which consequently increases manufacturing costs (Koh 2005). Pectin plays an important role in pepper processing since it forms gels and influences the final viscosity of the sauce. Hence, its integrity during processing influences the quality of the final product. This is especially important for products without added thickeners or stabilizers such as Tabasco pepper sauce. Pectin is degraded by endogenous enzymes in fruits and vegetables which normally are compartmentalized before processing. In particular, pectin methylesterase (PME) is responsible for phase separation and cloud loss in fruit juice manufacturing (Giovane and others 2004). It plays a major role in either development or loss of textual characteristics as well (Vora and others 1999).

Pectin methylesterase has been isolated and characterized from many plant sources, such as fruits and vegetables from orange (Versteeg and others 1978), grapefruit (Seymour and others 1991), peach (Glover and Brady 1994), tomato (Warrilow and others 1994), carrot (Ly-Nguyen and others 2002b), green bell pepper (Castro and others 2004), and green beans (Laats and others 1997), and from vegetative tissue, e.g. hypocotyl from mung bean (Bordenave and Goldberg 1993) and potato tubers (McMillan and Pérombelon 1995). Multiple isoforms of plant PME have been isolated from the different species as well. They differ in various biochemical parameters such as relative molecular mass (Mr), isoelectric point (pI), pH optimum, substrate affinity, ion-requirement and location (Bordenave 1996). Different isoforms of PMEs have been shown to be expressed at different times and in different tissues during plant growth and fruit development

(Gaffe and others 1994). In this chapter, we describe the isolation, partial purification, and some general properties of PME from Tabasco peppers.

3.2 Materials and Methods

3.2.1 Raw Materials

Thirty mature Tabasco peppers (Capsicum frutescens) plants were obtained from the McIlhenny Company (Avery Island, Louisiana, N 29.91211; W 91.90619). The growing medium consisted of 90% aged pine bark, 5% peat moss and 5% perlite. The pH was adjusted to 5.7 with $CaCO_3$. An encapsulated slow release fertilizer (14-14-14 NPK) (14% nitrogen-14% phosphorus- 14% potassium) was thoroughly mixed in a soil mixer before depensing into the 19 L growing containers. Eighteen plants were re-potted in larger 19 L containers with 20-20-20 fertilizer (20% nitrogen-20% phosphorus- 20% potassium). The plants were placed in polyethylene covered greenhouse on Louisiana State University Hill Farm Facility Teaching Facility. The plants were randomly placed 30 cm apart in staggered double rows. The set up included a micro-irrigation system for potted plants (Mister Landscaper Micro Sprinkler and Drip Irrigation, Dundee, FL) with timers (Mister Landscaper, Dundee, FL) set for watering based on the seasonal temperatures. Fertilizing with Miracle-Gro® singles pre-measured packets (Scotts Miracle-Gro Products, Inc., Marysville, OH) was performed on a bi-weekly basis. Occasional treatment with Safer Insecticidal Soap (Safer Inc., Lititz, PA) and Merit Insecticide spray (Research Triangle Park, NC) was done to prevent infestation of aphids and fungus gnats. Mature red-ripe peppers were picked from the greenhouse grown plants and transported immediately to the laboratory for purification of PME. The peppers were air-dried on the laboratory bench for 4 h and stored in plastic bags in the walk-in -20°C freezer (Master Bilt Products, New Albany, MS) until ready for use. Then samples were placed at 4°C overnight, the evening before the extraction. The term “Tabasco pepper pectin methylesterase” should therefore

be taken simply to mean that the enzyme was isolated from extracts of the entire Tabasco pepper fruit.

3.2.2 Extraction Preparation

The extraction of PME was performed according to Macdonald and Evan (1996) and Ünal and Bellur (2009) with minor modifications. Preparations of the solutions were as follows. The dry chemicals obtained from Sigma Aldrich (St. Louis, MO) were weighed out using a balance (Sartorius TE 412, Edgewood, NY). Tris-HCl buffer (100 mM and 2 M NaCl), pH 7.0 and 25 mM Tris-HCl buffer, pH 7.5 were prepared prior to the extraction using a magnetic stirrer (Cimarec, Barnstead International, Dubuque, Iowa). The pH was adjusted using 1M NaOH or 1N HCl with a pH meter (Thermo Orion model 210, Boston, MA). The solutions were stored at 4°C (VWR International model # 2015, Suwanee, GA) until ready for use. Polyvinylpyrrolidone (PVPP) (5 % w/v) in 250 mL of 100 mM Tris-HCl and 2M NaCl, pH 7.0; PVPP (4g) in 20 mL of 100 mM Tris-HCl, pH 7.0; and PVPP (3g) in 15 mL of 25 mM Tris-HCl, pH 7.5 were soaked overnight at 4°C.

3.2.3 Tabasco Pepper Extraction

In an Erlenmeyer glass flask, 250 mL of buffer (100 mM Tris-HCl and 2 M NaCl, pH 7.0), 500 mg/L sodium metabisulfite, and 5% w/v PVPP were mixed together using a magnetic stirrer (Cimarec, Barnstead International, Dubuque, Iowa) for 2 min. This was considered as the extraction solution. PVPP and sodium metabisulfite prevents any loss of activity by phenolic inhibition and improves the enzyme solubilization from the cell walls (Denès and others 2000). The solution was added to a pre-chilled Waring blender (Waring model 51BL32, Torrington, CT) and 50 g of Tabasco peppers were weighed (Sartorius TE 412, Edgewood, NY) and added to the solution.

The mixture was homogenized at high speed in the Waring blender for (3) periods of 15 s with a 30 s interval between the periods. The homogenized solution was filtered through (4) layers of cheesecloth (Purewipe Cheese cloth, American Fiber and Finishing, Inc., Albemarle, NC) into a plastic beaker in an ice bath on a magnetic stirrer. The supernatant was retained after checking for PME activity and the pellet was discarded. The extraction was performed using an ice bath. The extracted solution was treated with further addition of 4g of PVPP and stirred gently for 2 h, then clarified by centrifugation (Avanti J-25, Beckman Coulter, Fullerton, CA) in large centrifuge bottles (VWR International, Suwanee, GA) at 10,000 x g for 30 min at 4°C to remove PVPP and cell residue. Attempts were made to remove a top layer of oil by pipetting (Weber Scientific, Hamilton, NJ), but was unsuccessful. The supernatant was filtered through a GD/X 25 (7-layer) syringe filter, polyethersulfone (PES), 0.45 um (Whatman, Piscataway, NJ) to separate debri. Samples were dialyzed using 12 kDa-14 kDa molecular weight cut off membrane (Spectrum Spectra/Por 4 Regenerated Cellulose (RC), Spectrum Laboratories, Rancho Dominguez, CA) in 100 mM Tris-HCl, pH 7.0 buffer. Buffers were changed after 1, 2, and 4 h and stored overnight at 4°C.

3.2.4 Ammonium Sulfate Fractionation

After dialysis, the extract was transferred into a glass flask and solid ammonium sulfate (made up to 35% saturation) was added in portions (at 5 min intervals) with continuous stirring until it was dissolved. The extract was transferred to large centrifuge bottles; and allowed to stand for 30 min in an ice bath, then centrifuged at 10,000 x g at 4°C for 45 min. The precipitate containing PVPP and inactive protein was discarded. The extract was filtered using a GD/X 25 (7-layer) syringe filter, polyethersulfone (PES), 0.45 um. The extract was treated with solid ammonium sulfate (made up to 85% saturation), in portions (at 5 min intervals) with continuous

stirring until it was dissolved. The extract was transferred to large centrifuge bottles and allowed to stand for 30 min in ice bath, then centrifuge at 10,000 x g for 45 min at 4°C.

This fraction was considered crude PME. This fraction was re-suspended in 25 mM Tris-HCl, pH 7.5 and treated for a third time with 3 g of PVPP. After allowing it to stand for 10 min with occasional stirring, the suspension was centrifuged at 10,000 x g at 4°C for 60 min. The sample was dialyzed against 25 mM Tris-HCl, pH 7.5, using a 12 kDa-14 kDa molecular weight cut off membrane (Spectrum Spectra/Por 4 Regenerated Cellulose (RC). Buffers were changed after 1, 2, 4 h and stored overnight at 4°C.The sample was concentrated using solid sucrose (Sigma Aldrich, St. Louis, MO) from 100 mL to 20 mL. The sample was further concentrated using an Amicon Ultra centrifugal filter unit, 10 kDa molecular weight cut off membrane (Millipore, Billerica, MA) centrifuged at 5,000 x g for 45 min. This concentrate was used as the starting material for enzyme purification.

3.3 Partial Purification of Enzyme

3.3.1 Weak Anion-Exchange Chromatography

The method for chromatography was that of Giovane and others (1996). Weak anion-exchange chromatography was performed on a fast protein liquid chromatography (FPLC) system, BioLogic LP system with BioLogic BioFrac fraction collector with ice bath test tube holder. The UV absorbance was read at 280 nm and visualized using LP Data View software version 1.03 ((Bio-Rad Laboratories, Inc, Hercules, CA). Buffers were filtered and degassed using filtering system with 0.45 um filter memberane (Millipore, Billerica, MA). The dialyzed enzyme solution was loaded onto a Bio-Scale™ Mini Macro-Prep® DEAE (diethylaminoethyl) column (1 mL column; 40 mm length x 5.6 mm inner diameter) (Bio-Rad Laboratories, Inc, Hercules, CA) previously equilibrated and washed with 25 mM Tris-HCl buffer (Sigma Aldrich, St. Louis, MO), pH 7.5. Then, a linear gradient was applied using 25 mM Tris-HCl and 1 M

NaCl buffer, pH 7.5 (0-100%) at a flow rate of 1.5 mL/min. The chromatography sequence is shown in Table A1 in Appendix A. The fractions of 2.5 mL each were collected and analyzed for protein concentration and PME activity. Active fractions were pooled together, concentrated using Amicon Ultra centrifugal filter units, 10 kDa molecular weight cut off membrane (Millipore, Billerica, MA) centrifuged at 5,000 x g at 4°C for 30 min. The enzyme concentrate solution was subjected to affinity chromatography.

3.3.2 Affinity Chromatography

The method for affinity chromatography was that of Giovane and others (1996). The concentrated enzyme was loaded onto a HiTrap™ Heparin-Sepharose High Performance column (1 mL column; 0.7 length x 2.5 cm inner diameter) (GE Healthcare, Piscataway, NJ). The heparin consists of alternating units of uronic acid and D-glucosamine. The column was equilibrated and washed with 25 mM Tris-HCl buffer (Sigma Aldrich, St. Louis, MO), pH 7.5. Then, a linear gradient was applied using 25 mM Tris-HCl and 1 M NaCl buffer, pH 7.5 (0-100%) at a flow rate of 1.0 mL/min. Fractions of 2.0 mL each were collected and analyzed for protein concentration and PME activity as later described. The chromatography sequence is shown in Table A2 in Appendix A. Active fractions were pooled together, concentrated using Amicon Ultra centrifugal filter units, 10 kDa molecular weight cut off membrane centrifuged at 5,000 x g at 4°C for 30 min. The enzyme was stored at -80°C (Revco, Kendro Laboratory Products, Asheville, NC) until needeed. Fold purification was calculated as the ratio of the specific activity at each step to that in crude extract; while percent yield was calculated as % of total activity in crude extract remaining at each step of purification.

3.4 Protein Determination

Protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) according to the manufactures' protocol. Bovine serum albumin, BSA

was used as the standard of calibration. Absorbance at 562 nm was measured in a Benchmark Plus microplate spectrophotometer reader with Microplate Manager 5.2 operating software (Bio-Rad Laboratories, Hercules, CA) after 30 min incubation (Hotpack model #352700, Philadelphia, Pennsylvania) at 37°C. Fractions, in which protein was detected by this method, were analyzed for pectin methylesterase activity. The enzyme solution was stored at -80°C until ready for use.

3.5 Pectin Methylesterase Activity

Pectin methylesterase activity was spectrophotometrically assessed using the adapted method of Anthon and Barrett (2004) with modifications made by Anthon (2009). This method indicates PME activity by measurement of methanol release through a highly sensitive colorimetric assay based on the condensation of aldehyde with methylbenzothiazolinone-2-hydrazone (MBTH) under neutral conditions using citrus pectin (DE= ≥85%) as the substrate. Absorbance was read at 620 nm using DU 550 Life Science UV/Vis Spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

3.6 Electrophoresis

The molecular mass of the partially purified enzymes was estimated using sodium dodecylsulfate (SDS-PAGE) by the method of Laemmli (1970). Gel electrophoresis was carried out using an 8 x 10 cm, and 1.0 mm thick Tris-HCl gel of 10% polyacrylamide. The procedure included 9 µL of sample, 9 µL Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), 1 µL reducing agent ((Invitrogen, Carlsbad, CA) and 1 µL Milli-Q water. Samples were solubilized in the SDS-sample buffer by heating to 85°C using a magnetic stirrer/hot plate (Cimarec, Barnstead International, Dubuque, Iowa) for 2 min. Tris/Glycine/SDS (10X) running buffer was diluted to 1X (50 µL of buffer and 450 µL of Milli-Q water) and added to Mini-

PROTEAN 3 cell chamber (Bio-Rad Laboratories, Hercules, CA). Twelve µL of sample and molecular marker was added to the wells. The voltage was set at 200 V and run time was 35 min.

The Precision Plus protein pre-stained standards (Bio-Rad Laboratories, Hercules, CA) contained, eight protein bands of 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa, and 250 kDa. The gels were stained with 1X Coomassie Blue R-250 (Bio-Rad Laboratories, Hercules, CA) following the procedure of the quick staining method by Costa (2000). The gel was stained with 1X Coomassie Blue R-250 in the microwave for 1 min or until boiling in clear plastic Sterilite's Ultra-Seal latching food storage containers (Sterilite Corporation, Townsend, MA). The gel was removed and placed under the hood (Class II A/B3 Biological Safety Cabinet, Forma Scientific Inc., Marietta, OH). It remained in the stain for 5 min. The gel was gently rinsed with Milli-Q water (Millipore Corporation, Bedford, MA). The stain was discarded. The 1X Destaining solution was added and placed in the microwave for 1 min or until boiling. The gel remained in the solution for 15 min, then it was pour off and more destaining solution was added for 20 min.

3.7 Isoelectrofocusing

Isoelectrofocusing (IEF) was performed according to Bio-Rad's instructions for mini vertical precast gels. The 1X cathode buffer (Bio-Rad Laboratories, Hercules, CA) was prepared using 20 mL of IEF 10X cathode buffer with 180 mL of Milli-Q water (Millipore Corporation, Bedford, MA). The 1X anode buffer was prepared by adding 40 mL with IEF 10X anode buffer to 360 mL Milli-Q water and thoroughly mixed on a magnetic stirrer (Cimarec, Barnstead International, Dubuque, Iowa). The IEF precast gel; pH 3-10 (Bio-Rad Rad Laboratories, Hercules, CA) was used for the analysis. The IEF sample loading buffer (Bio-Rad Laboratories, Hercules, CA) was added 1:1 to the protein sample. Twenty-five µL of sample were added to the well and then additionally, 5 µL of the IEF Standards were added. The molecular weight marker

proteins were phycosyanin (232,000, pI 4.45, 4.65, 4.75); human hemoglobin A (64,500, pI 7.1); human hemoglobin C (64,500, pI 7.5); lentil lectin (49,000, pI 7.80, 8.0, 8.20); bovine carbonic anhydrase (31,000, pI 6.0); human carbonic anhydrase (28,000, pI 6.5); ß-Lactoglobulin B (18,400, pI 5.1); equine myoglobin (17,500, pI 6.8, 7.0); cytochrome C (12,200, pI 9.6). Five of the nine proteins were naturally colored and provided continuous monitoring of the IEF.

The samples were loaded and approximately 200 mL of 1X cathode buffer and 400 mL of 1X anode buffer was added to the inner and outer chamber of the Mini-PROTEAN 3 cell, respectively (Bio-Rad Laboratories, Hercules, CA). The chamber was placed into an ice bath and a constant voltage of 100 V for 1 hr was applied. Next, the voltage was increased to 250 V for 1 hr. According to the procedure the voltage was suppose to be increased to 500 V for 30 minutes, but because of the limitations of our power supply (PowerPac Basic, Bio-Rad Laboratories, Hercules, CA) the voltage could only be increased to 300 V. Therefore, Bio-Rad technical support instructed the use of 300 V for 1 hr as a modification. After the electrofocusing, the protein bands were stained with Coomassie Brilliant Blue G-250 ((Bio-Rad Rad Laboratories, Hercules, CA) in trichloroacetic acid solution (Diezel and others 1972).

Determination of PME activity was investigated by colorimetric assessment of pH reduction directly onto the gel after electrophoresis. We followed the method by Arancibia (2003). This was considered a form of zymography. After the run, the gel was rinsed two times in 100 mL of cold 10 mM sodium phosphate buffer pH 7.0 for 20 min each. The gel was put in a petri dish (150 x 15 polystyrene disposable plates) (VWR International, Suwanee, GA) and a warm solution (1 % agarose, 0.3 % pectin, 2 mM sodium phosphate buffer at pH 7.0, and 0.05% bromothymol blue) [all of the products were obtained from Sigma Aldrich, St. Louis, MO] was poured to form a thin overlay. The gel was kept at room temperature in the dark for 24 h and the

appearance of a green-yellowish band indicating a localized pH reduction was considered to be PME activity.

We followed the method of Savary and others (2002) to detect enzyme activity of the bands using gels overlayed with 1% agarose and 1% pectin in 10 mM sodium phosphate buffer pH 7.0, [all of the products were obtained from Sigma Aldrich, St. Louis, MO] incubated at 30°C for 1 h. Activity bands were visualized by immersion of 0.05% ruthenium red (Sigma Aldrich, St. Louis, MO), followed by de-staining with Milli-Q water.

3.8 ELISA

The Enzyme Linked Immunosorbent Assay (ELISA) protocol was adapted from Pratt and others (1986) and Blumer and others (2000). All the chemicals were obtained from Sigma Aldrich (St. Louis, MO). The positive control PME was partially purified from *Lycopersicon lycopersicum* 'Better Boy' tomato as previously described and the negative control was the blocking buffer. Samples were tested in triplicate. The wells of a 96-well microtiter plate (2592 Costar, Corning, Inc., Corning, NY) were coated by overnight treatment at 4°C (VWR International model # 2015, Suwanee, GA) with 50 µL of PME extract diluted to a protein concentration of 20 µg/mL in 0.2 M sodium borate, 75 mM NaCl coupling buffer, pH 9.6. The pH was adjusted using 1M NaOH or 1N HCl with a pH meter (Thermo Orion model 210, Boston, MA).

The plate was tapped to evenly distribute antigen and covered with parafilm (VWR International, Suwanee, GA) to reduce evaporation. After the overnight incubation, the wells of the plate were flicked into the sink to remove residual solution several times. Following removal of coupling buffer, 200 µL of washing buffer (10 mM Tris (hydroxmethyl) amino methane-Cl, pH 8.0, 0.05% Tween 20, and 0.02% sodium azide) using a multi-channel pipette (VWR International, Suwanee, GA) was added to each well and washed three times at room

temperature. Following the removal of washing buffer, 200 µL of blocking buffer (1% bovine serum albumin in 0.067 M phosphate-buffered saline, pH 7.25) was added to the wells at room temperature and placed on a shaker (Red Rotor, Hoefor Pharamacia Biotech, Inc., San Francisco, CA) to block non-specific protein binding. The blocking buffer was removed after 30 min by overturning the plate in the sink and tapping it face down onto several paper towels and patted dry, removing liquid.

The monoclonal antibody PME-1 is specific for a 34.5 kDa PME from tomato; it was donated by Carl W. Bergmann from the Complex Carbohydrate Research Center, University of Georgia. Then 50 µL of PME-1 anti-serum was dispensed into each well, and the plates were incubated at 4°C (VWR International model # 2015, Suwanee, GA) for 3 h. After incubation, each well was washed three times with 200 µL of washing buffer (10 mM Tris (hydroxmethyl) amino methane-Cl, pH 8.0, 0.05% Tween 20, and 0.02% sodium azide). Then 50 µL of alkaline phosphatase-labeled rabbit anti-mouse immunoglobin G (IgG) (Sigma Aldrich, St. Louis, MO) diluted 1:500 (10 µL IgG) in 5 mL of diluent buffer (0.067 M phosphate buffered saline, pH 7.25, 1% bovine serum albumin, 0.05% Tween 20, and 0.02% sodium azide) was added to each well. The plate was incubated at 37°C (Hotpack model #352700, Philadelphia, Pennsylvania) for 2 h. Then wells were washed 3 times with 200 µL of washing buffer, overturned, tapped and patted dry, removing liquid as stated above.

A 50 µL aliquot of p-nitrophenyl phosphate (pNPP) at a concentration of 1 mg/mL in 9.6% (v/v) diethanaolamine buffer (pH 9.6) and 0.5 mM $MgCl_2$ was added to each well and incubated for 30 min at room temperature. Positive wells exhibited fluorescent yellow color. The reaction was stopped by adding 50 µL of 3 N NaOH. The absorbance was read at 414 nm in a Benchmark Plus microplate spectrophotometer reader with Microplate Manager 5.2 operating software (Bio-Rad Laboratories, Hercules, CA).

3.9 Results and Discussion

3.9.1 Purification of PME

Tabasco peppers were harvested for PME purification from grown plants greenhouse on Louisiana State University campus, until the plants became infected with the Mosaic Virus during the summer of 2009 and no longer produced any fruit. Plants are shown in Figure A1 in Appendix A. PME was extracted from mature red ripened Tabasco peppers. PME is located in the cell walls of higher plants and is ionically bound to the cell wall. Hence, a higher ionic strength buffer was required for extraction from the Tabasco pepper (0.1 M Tris-HCl buffer, 2 M NaCl, pH 7.0) as described by Balogh and others (2004) who found the carrot (*Daucus carrota*) PME was ionically bound to the cell wall and required use of a high ionic strength to extract it. The summary of the stepwise purification of Tabasco PME is shown in Table 3.1. The enzyme was partially purified about 40.1-fold with 26% recovery by crude extraction, ammonium sulfate precipitation, anion-exchange, and affinity chromatography. PME from crude extract with a total activity of 319 units and specific activity of 1.20 U/mg protein was further fractioned using 85% ammonium sulfate precipitation at 4°C.

Table 3.1. Summary of purification of PME from Tabasco pepper (*Capsicum frutescens* L.)

Purification Step	Volume (mL)	Total protein (mg)	Total Activity	Specific Activity (U/mg protein)[a]	Purification (fold)[b]	Yield (%)[c]
Crude extract	500	266	319	1.20	1	100
$(NH_4)_2SO_4$, 85% saturation	100	57	262	4.60	3.83	82.13
Weak anion exchange chromatography	20	2.1	89.1	42.4	35.33	27.93
Affinity chromatography	6	1.7	81.8	48.1	40.08	25.64

[a] Specific Activity: Total enzyme activity / Total protein

[b] Purification (fold): Specific activity / 1.20 (initial specific activity)

[c] Yield (%): Total enzyme activity / 319 (initial total activity) x 100

During the ammonium sulfate fractionation, approximately 18% activity was lost. After the dialysis, the PME solution was applied to DEAE weak anion exchange chromatography column which resulted in 35-fold purification with specific activity of 42.4 U/mg protein. The PME did not absorb on the DEAE column, and instead eluted out as a single peak before the NaCl gradient started (Figure 3.1). This suggests either Tabasco PME carried a net positive charge or did not carry any charge under the experimental conditions used (Mondal and others 2009).

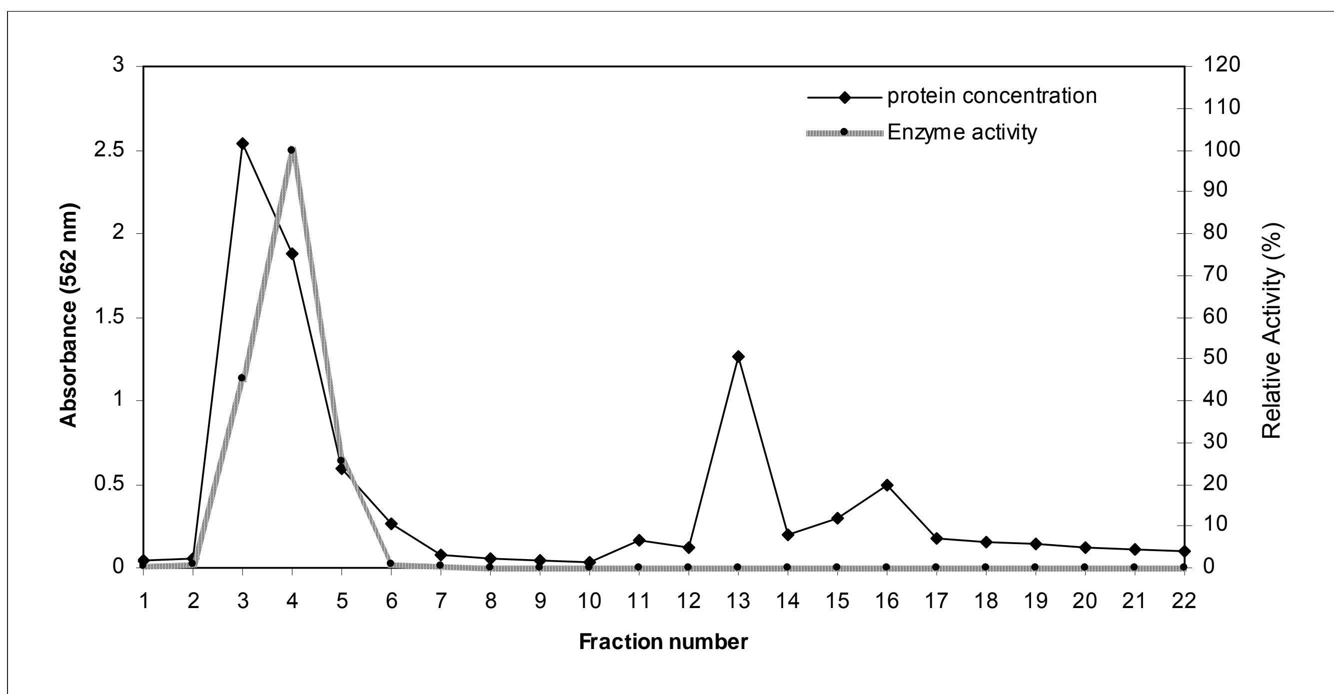

Figure 3.1 Elution profile of PME during DEAE anion exchange chromatography

This is considered a flow-through method where the bound contaminates eluted out between 570 mM and 750 mM NaCl. Our results are in agreement with Rillo and others (1992), Giovane and other (1996), Cameron and others (1998), and Cameron and others (2003) where PME did not bind to the column, instead inactive protein and contaminates at pH 7.5 were bound to the column. The fractions from anion exchange chromatography exhibiting PME activity were pooled together, concentrated and applied to a Heparin-Sepharose column. The PME eluted out

in a single peak at 0.05 M NaCl (Figure 3.2) which was a 40.1-fold purification with specific activity of 48.1 U/mg protein for a recovery of 26% activity of the original crude extract.

PME extracted and purified from green bell pepper (*Caspiscum annuum*) had a specific activity of 242.5 U/mg protein (Castro and others 2004). Ciardiello and others (2004) purified PME from kiwi (*A. chinensis*) and kaki *(Diospyros kaki)* fruits, approximately 100−110 µg of electrophoretically pure PME was obtained from 1 kg of kiwi or kaki fruits, with a purification factor of approximately 750-fold for both enzymes. The above mentioned results used the PME Inhibitor (PMEI)-Cyanogen bromide (CNBr)-activated-Sepharose 4B affinity chromatography to purifiy the enzymes.

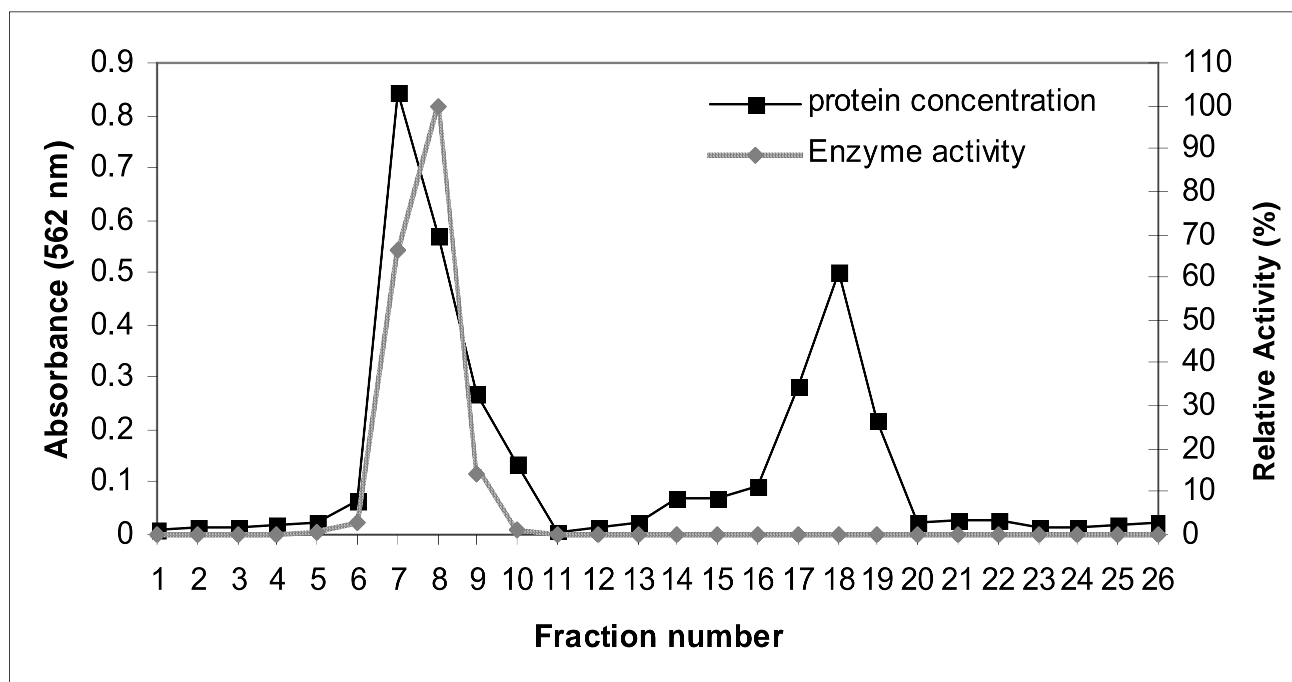

Figure 3.2 Elution profile of PME during Heparin-Sepharose affinity chromatography

Do Amaral and others (2005) performed partial purification of PME from orange (*Citrus sinensis* cv. Pera-Rio) where higher PME-specific activity was obtained with extracts containing 0.4 NaCl. The final step of purification yielded 23.0- fold purification and a specific activity of 26.6 U/mg protein. Purified strawberry PME was successfully eluted to give a single peak of proteins and PME activity. Purified strawberry PME had a maximum activity of 176.0 U/mg

protein corresponding to at least a 15.8-fold enrichment and an overall yield of at least 41.8%, based on the total enzymatic activity of 80% ammonium sulfate precipitate (Ly-Nguyen and others 2002a).

In our preliminary studies, we made an attempt to purify the PME crude extract using cation exchange chromatography (Hi-Prep 16/10 SP/XL, GE Healthcare) according to Arancibia (2003) and Duvetter and others (2006). Neither the protein or inactive protein and contaminants bound to the column in 25 mM sodium acetate at 4.5, 5.0 and 5.6 pH. Using the Jaffar and Oommen (1993) method with a hydroxyapatite column (donated from Bio-Rad) produced the same results. Finally, the procedure of Giovane and others (1996) using DEAE and Heparin-Sepharose columns was attempted. These chromatography methods were only able to partially purify the enzyme. Using a strong anion chromatography column, Bio-Scale Mini UNOsphere Q (donated from Bio-Rad in a starter kit), gave the same results as the DEAE column. To further purify the enzyme, using additional methods of Giovane and others (1996) and Ly-Nguyen (2002b) would require PME Inhibitor (PMEI)-Cyanogen bromide (CNBr)-activated-Sepharose 4B affinity chromatography which is a 1-step method. However, it was not possible for our laboratory to utilize this method because of the cost of the Cyanogen bromide (CNBr)-activated-Sepharose 4B resin (45 g), commercial PME from citrus peel (Sigma-Aldrich), column/flow adapter (Bio-Rad), and time limitations.

3.9.2 Molecular Weight Determination

On SDS-PAGE Tabasco pepper PME produced two major bands estimated at 22 kDa and 36 kDa (Figure 3.3), which are in range of other plant PMEs purified from various sources. The results compare favorably with Aranciba (2003), which revealed Tabasco pepper PME at 22.5 kDa and 36.7 kDa. However, along with the two major bands there were minor and faint bands indicating that the PME enzyme could not be purified to homogeneity. It was a partially purified

preparation. Castro and others (2004) purified PME from green bell pepper; the SDS-PAGE produced two bands with identical intensities. However, after comparison with the electrophoretic mobility of the standard proteins, the bands obtained indicated molecular masses of 33.0 and 37.0 kDa.

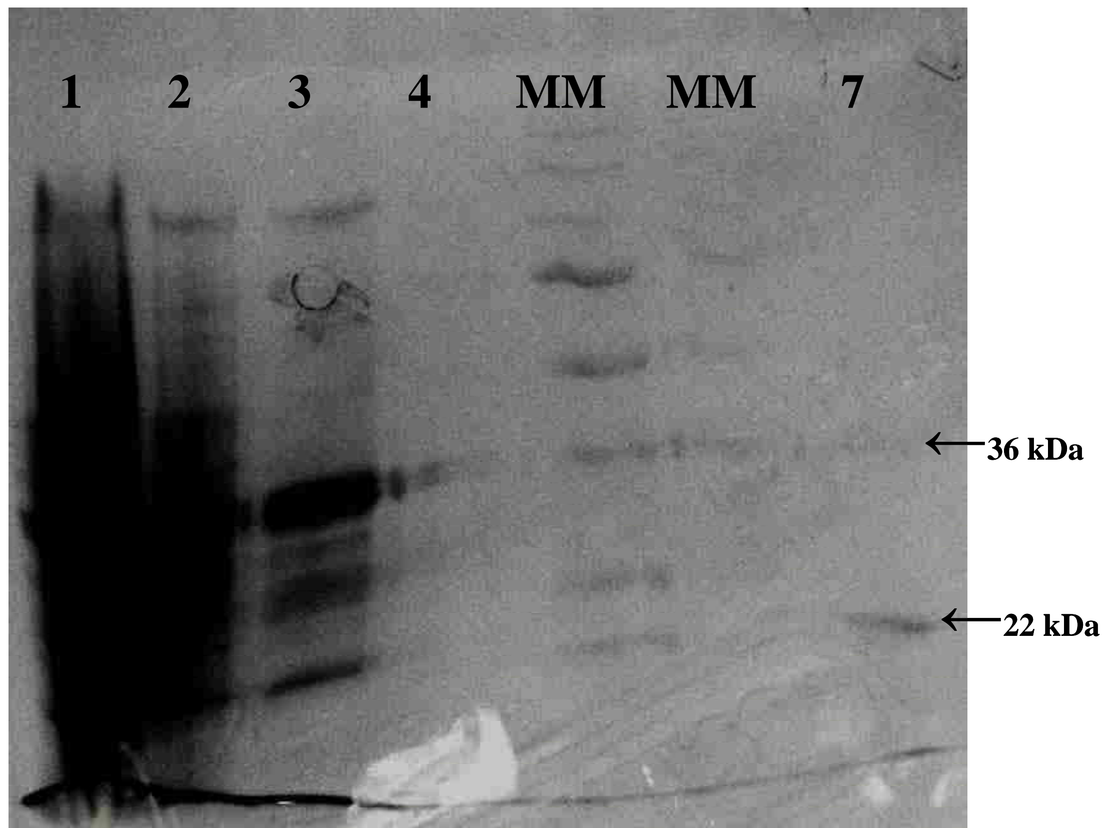

Figure 3.3 SDS-PAGE of purification steps of PME from Tabasco peppers. Lane 1: ammonium sulfate fraction (85%); Lane 2: ammonium sulfate fraction (85%); Lane 3: Weak anion exchange fraction; Lane 4: contamination from adjacent lane 3; Lane 5 and 6: Molecular markers; Lane 7: Heparin-Sepharose fraction

The molecular weight of PME from jelly fig achenes was estimated to be about 38.0 kDa based on SDS-PAGE and indicated that the jelly fig PME is a monomer (Lin and others 1989; Peng and others 2005). Partially purified pectin esterase (PE) present in green beans pods and seed hulls revealed two fractions, PE-1 and PE-2 which had molecular weights under native

conditions of 45.0 and 29.0 kDa, respectively, and under denaturating conditions at 42.0 and 33.5 kDa, respectively (Laats and others 1997).

PME extracted from Brazilian guava yielded a molecular mass of 43.9 kDa (da Silva Cerqueira Leite and others 2006). Yellow cedar seed PME isoforms both had molecular mass of 62.0 kDa (Ren and Kermode, 2000). Strawberry PME showed two bands on SDS-PAGE of apparent molecular masses of 33.5 and 43.0 kDa (Ly-Nguyen and others 2002a). PME isoforms purified from persimmon showed differences in molecular weight, PME I (51.0 kDa) and PME II (30 kDa) (Alonso and others 1997). Shen and others (1999) extracted PME from rice weevil insect where only one activity band was observed and only one band was visualized after silver staining within an estimated molecular mass of 38.0 kDa. PME purified from carrot indicated apparent molecular masses of 36.7 and 16.9 kDa (Balogh and others 2004).

Through further analysis, Tabasco PME can be purified based on the isoforms which we would be able to investigate thermostability of the enzyme. PME isoforms in various citrus fruits reportedly differ in molecular weight and heat stability, and are referred to as thermostable (TS-PE) or thermolabile (TL-PE). Versteeg and others (1980) reported that a high molecular weight (HMW, 54 kDa) pectin esterase (PE) in navel orange represented only 5% of the total activity, but was very heat stable and primarily responsible for cloud loss of citrus juice. Two navel orange TL-PE (PE I, PE II) with an approximate MW of 36.2 kDa were rapidly inactivated at temperatures of 4°C (Versteeg and others 1980). Two isoforms, PME 1 and a thermostable high-molecular weight PME, have been shown to clarify orange juice, and they are located in the juice and peel unlike the PME II isoforms which are found only in the peel and cannot clarify juice (Rombouts and others 1982). The heat stability of two TS-PE (54 kDa and 51 kDa) from Marsh grapefruit (Seymour and others 1991) was slightly lower than that of the HMW PE reported by

Versteeg and others (1980). Cameron and Grohmann (1995) partially purified one of the four PE isoforms (37.7 kDa) from red grapefruit.

3.9.3 PME Activity

Hagerman and Austin, (1986) proposed para-nitrophenyl acetate, as a specific substrate for esterase and a PME substrate, however Zocca and others (2007) tested para-nitrophenyl acetate substrate with grape PME. The results suggested the presence of esterases in the extracts. However, the trend of the para-nitrophenyl acetate activity was different from that obtained with pectin as a substrate and may be related to another enzyme. The question regarding the different behavior due to the two substrates was solved by electrophoretic analysis. To avoid problems with electrophoretic mobility and thus comparison of patterns, pectin was added when making the gels. Cut in two, gels were stained with ruthenium red and β-naphtylacetate Fast BB or β-naphthyl butyrate substrates, respectively. The β-napthyl acetate substrate showed two esterase isoforms which were different from those revealed with ruthenium red. This finding proved: (i) the aspecificity of β-napthyl acetate as a PME substrate and (ii) the presence of different enzymes hydrolyzing the ester bond when β-naphtyl butyrate was used as substrate. Additionally, an esterase activity band, completely different from the others, was detected and demonstrated by Lomolino and others (2005). These findings reveal that pectin is the specific substrate for PME from plant sources such as Tabasco pepper PME.

3.9.4 Isoelectrofocusing

The pI of Tabasco pepper PME could not be successfully determined. We were able to visualize bands in the mid-center of the gel but problems began to occur when we attempted to increase the voltage to 250 V. The power source Basic model (Bio-Rad) has an internal safety action when the milli-amp (mA) falls below 4 mA, the power is automatically shut off and an error message (E-1) appears. We contacted Bio-Rad technical support and were instructed to

deactivate the safety action. However, the problem still could not be resolved and functional equipment could not be obtained in the time frame needed. We performed an overlay of the gel to determine if the enzyme migrated to its pI. However, that was not successful as well. According to the literature, at acidic pH, proteins are positively charged. Additionally, at basic pH proteins are negatively charged. Based on the cation exchange chromatography results during our preliminary studies, neither the protein or inactive protein and contaminants bound to the column in 25 mM sodium acetate at 4.5, 5.0 and 5.6 pH. This suggests that Tabasco pepper PME is a negative charged protein.

Most known PME pI values are from pH 9.0 to 11.0 PME (Lin and others 1989; Peng and others 2005). Jelly fig achene PME has a pI about pH 3.5 as determined by isoelectric focusing (Lin and others 1989; Peng and others 2005). Green beans pectin methylesterase enzymes, PE-1 and PE-2 both contained two isoforms of PME activity with isoelectric points of pH 8.4, 9.8, 10.5, and >11, respectively (Laats and others 1997). Yellow cedar PME consisted of two isoforms, both basic with isoelectric points of pH 8.7 and 8.9 (Ren and Kermode 2000). PME purified from strawberry, did not show any protein band that was found between pH 3.0 and 9.0. Therefore, Ly-Nguyen and others (2002a) concluded that strawberry PME pI was higher than 9.0, as reported for many plant PMEs. Kiwi enzyme had a neutral-alkaline pI of pH 7.3; whereas the pI of the kaki enzyme was slightly acidic at pH 6.7 which is close to the pH of the pulp. This makes the kaki PME uncharged and unable to bind to the cell wall (Ciardiello and others 2004). On IEF gel, purified green bell pepper PME showed several bands between pH 6.0 and 9.3, of which two were cathodic (pI values of 7.9 and 7.5) and two were anodic (pI values of 6.3 and 6.1) (Castro and others 2004).

3.9.5 ELISA

Our results indicate that Tabasco pepper PME does not possess common epitopes with tomato PME. In the Blumer and others (2000) study, the 'Better Boy' cultivar exhibited an absorbance of 0.315. In our results tomato PME exhibited a positive color change and showed an absorbance of 0.296. However, the Tabasco PME did not exhibit any color change, showing no difference from the negative control (blocking buffer). The PME-1 antibody did not recognize the PME from Tabasco pepper. According to Blumer and others (2000) the PME-1antibody was specific to a 34.5-kDa tomato PME. In their study, it was suggested that multiple PME isoforms existed in the tomato, some of which are not recognized by PME-1, and are responsible for the balance of total detectable PME activity. Therefore, this may not exclude the possibility of common epitopes between Tabasco pepper PME and tomato PME.

3.10 Conclusions

We successfully isolated PME from Tabasco pepper. Studying the general properties of the enzymes will give us more insight on how PME affects processing of the peppers. PMEs from different sources have different characteristics, and it is not unusual to find in the same source two or more isoenzymes with different molecular weight (MW), isoelectric points (pIs) and/or kinetic properties.

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CHAPTER 4

KINETIC AND STABILITY STUDIES OF PECTIN METHYLYESTERASE FROM TABASCO PEPPER, *CAPSICUM FRUTESCENS*

4.1 Introduction

It is well-known that the presence of residual endogenous enzymes in both raw or processed fruit and vegetable products may cause a loss of quality during storage. These changes can affect the stability, texture, color, flavor, and nutritional quality of the food product (Adam 1991). For example, pectin methylesterase (PME) is involved in the degradation of pectin and therefore affects product viscosity and texture. PME activity has been shown to cause cloud loss in juices (Versteeg and others 1980). Also, the food is further degraded by other endogenous enzymes as well (Luh and Daouf 1971). To prevent unwanted changes during storage, fruit and vegetable products are generally subjected to some type of treatment during processing in order to inactivate these enzymes. A heat treatment, such as blanching, pasteurization, or commercial sterilization, is most commonly used. In the hot pepper sauce industry, high levels of salt are added to pepper mash to inactivate the enzymes.

PME is the first enzyme acting on pectin, a major component of plant cell wall. Pectin represents about 30-35% of polysaccharides constituting the cell wall in dicotyledonous plants (Grsic-Rausch and Rausch 2004). PME catalyzes the demethylation of galacturonic acid units of the pectin by hydrolysis, generating free carboxyl groups and releasing protons and methanol. As stated by Giovane and others (2004) the enzyme, PME was identified in carrots more than a century ago, in a study of the chemical processes occurring during fruit ripening, (Frémy 1840). It was observed that the protein (the "*albumine vegetale*") present in the carrot root juice transformed the pectin to pectic acid, which underwent gelation.

Most of the purified plant PMEs have neutral or alkaline isoelectric points, and are bound to the cell wall via electrostatic interactions (Lin and others 1989; Bordenave and Goldberg 1994; Ding and others 2000). In most cases, complete enzyme inactivation is the target. In the case of pepper products, there is very little information available in the literature on the effects of

active enzymes on stored fermented pepper mash quality. To the best of our knowledge, no published papers have investigated the characteristics of PME from Tabasco pepper in terms of varied pectin substrate solutions, salt concentration, pH, and temperature optima.

4.2 Materials and Methods

4.2.1 Standard Curve

The standard curve was constructed using various concentrations of spectrophotometric grade methanol (Sigma Aldrich, St. Louis, MO) ranging from 50, 40, 30, 20, and 10 nmoles.

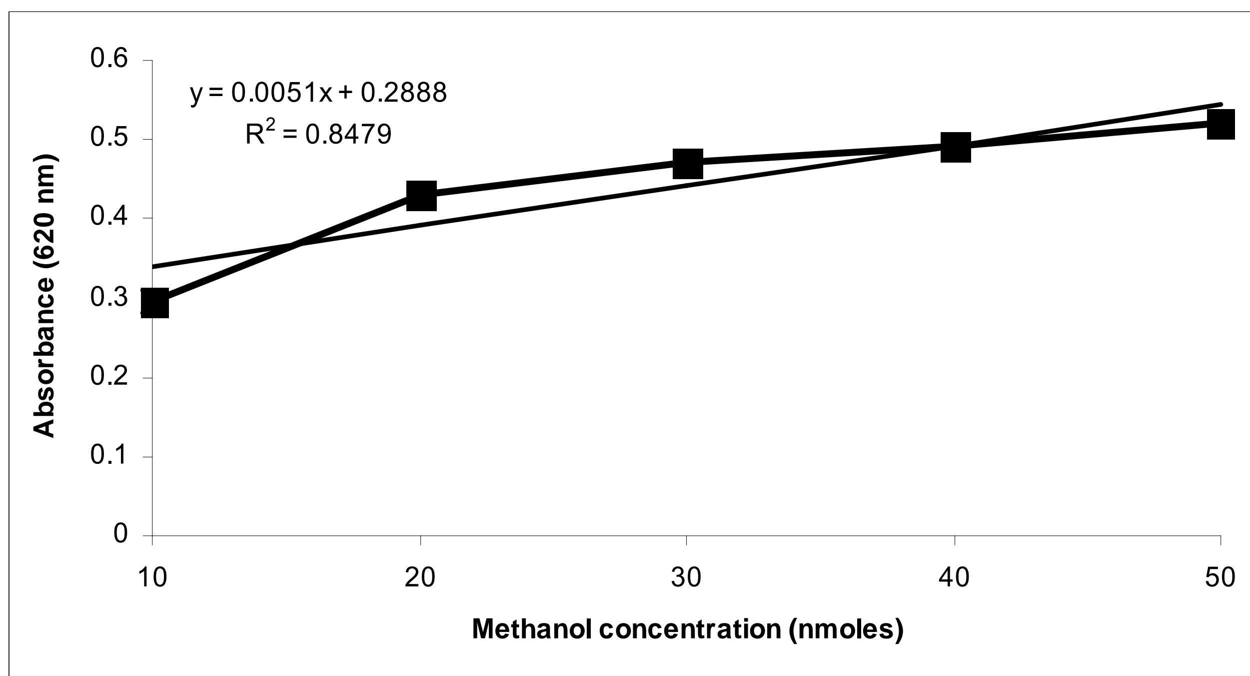

Figure 4.1 Methanol Standard Curve

The absorbance was read at 620 nm using DU 550 Life Science UV/Vis Spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

4.2.2 PME Activity

Pectin methylesterase activity was spectrophotometrically assessed using the adapted method of Anthon and Barrett (2004) with modifications made by Anthon (2009). This method indicates PME activity by measurement of methanol release through a highly sensitive colorimetric assay based on the condensation of aldehyde with methylbenzothiazolinone-2-

hydrazone (MBTH) under neutral conditions. It was included along with the alcohol oxidase to act as an aldehyde trap. This prevented further oxidation reactions by alcohol oxidase and allowed for extended incubations.

According Anthon and Barrett (2004) alcohol oxidase (AO) oxidizes the methanol to formaldehyde and then colorimetrically determines the formaldehyde. When the medium is acidified and an oxidant such as Fe^{3+} is added, this adduct then oxidatively couples with a second MBTH molecule to form a blue formazan dye. AO activity is not specific for methanol, actually its formaldehyde, the product of methanol oxidation, which is the substrate for AO. It is oxidized to produce formic acid and an additional equivalent of H_2O_2. While highly sensitive and specific for aldehydes, this reaction is not, however, specific for formaldehyde, and other aldehydes such as acetaldehyde react nearly as well as formaldehyde. Anthon and Barrett (2004) reported that Tris binds to aldehydes, which could potentially cause it to interfere with this assay. However, these authors showed that this effect is pH dependent and not significant at pH 7.5. Since this method is pH dependent; to determine optimum pH we used an automatic titration to perform the assay.

4.2.2.1 PME Assay

Alcohol oxidase (AO) (from Pichia pastoris, Lot # 087K1415), N-methylbenzothiazolinone-2-hydrazone (MBTH), Tris-HCl, citrus pectin (degree of methylesterification approximately ≥85%, Lot # 018K1650), NaCl, ferric ammonium sulfate, and sulfamic acid were all obtained from Sigma, St. Louis, MO. The preparation and stability of specific reagents and stock solutions were as follows. The dry chemicals were weighed out using a balance (Sartorius TE 412, Edgewood, NY). The 200 mM Tris-HCl (pH 7.5) with 200 mM NaCl solution was prepared with 4.72 g/L Tris base, 25.4 g/L Tris-Cl, and 11.6 g/L NaCl. The

pH was adjusted using 1M NaOH or 1N HCl with a pH meter (Thermo Orion model 210, Boston, MA).

MBTH was dissolved in Milli-Q water (Millipore Corporation, Bedford, MA) at 6 mg/mL on a magnetic stirrer (Cimarec, Barnstead International, Dubuque, Iowa). It is stable for at least a week at 4°C, after which it develops a brown color. The acidic iron solution used in the MBTH assay was prepared by dissolving 5 g of each ferric ammonium sulfate and sulfamic acid in 1000 mL of Milli-Q water (Millipore Corporation, Bedford, MA). This solution is stable indefinitely at room temperature. The pectin solution was prepared by heating 4 mg/mL in Milli-Q water (Millipore Corporation, Bedford, MA) using a magnetic stirrer (Cimarec, Barnstead International, Dubuque, Iowa).

The enzyme assay contained 1000 μL of 200 mM Tris−HCl (pH 7.5) and 200 mM NaCl, 700 μL of 4 mg/mL pectin, 200 μL of 6 mg/mL MBTH, 5 μL of AO at 5 U. This solution is enough for 20 assays. For each assay we added 95 uL of the assay mix, and then began the assay by adding 5 uL of the Tabasco pepper PME solution. The mixture was incubated at 30°C (VWR International model # 1525, Suwanee, GA), next we added 200 μL of the ferric ammonium sulfate and sulfamic acid solution after 30 min (if activity is low this time may be increased to many hours) at room temperature.

When activity was present the samples turned blue. After 30 minutes at room temperature we added 800 μL of Milli-Q water (Millipore Corporation, Bedford, MA) and read absorbance at 620 nm using DU 550 Life Science UV/Vis Spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The blank included 5 uL of buffer instead of sample. Also, phosphate buffers were incompatible with this assay. Each point on the figures is the mean of duplicated determinations. Standard errors are indicated by error bars; in most cases they are smaller than the symbol sizes.

4.2.3 Effect of Substrate Concentration on PME Activity

Performing activity assays at different pectin concentrations yields a plot of the velocity of reaction (methanol concentration), against the concentration of the substrate (pectin), which will give a rectangular hyperbola. The Michaelis-Menten equation includes the maximum velocity (V_{Max}), which was theoretically, attained when the enzyme was saturated by an infinite concentration of substrate; K_M, the Michaelis constant which was numerically equal to the concentration of substrate of half-maximal velocity; v_i was the initial velocity, and S was the substrate concentration as described by Dowd and Rigges (1965). K_M, reveals the affinity of the PME enzyme to citrus pectin substrate. The kinetic parameters were determined by non-linear regression analysis fitting the initial velocity versus substrate concentration to the Michaelis-Menten equation (equation 1) using the program of Cleland (1979).

$$v = V_{Max} \bullet S / (K_M + S) \quad (1)$$

The effect of substrate concentration on the enzyme activity was determined by incubating various concentrations of pectin ranging from 2.0, 1.5, 1.0, 0.5, 0.25, 0.125, 0.10 and 0.05 mg/mL for 30 min at 30°C (VWR International model # 1525, Suwanee, GA). The pectin methylesterase activity was determined as previously described. The K_M and maximum rate (V_{Max}) were determined using the Michaelis-Menten equation (Cleland 1979). Blanks lacking PME was made for each determination, and the amount of acid produced because of the spontaneous pectin demethylation was subtracted.

4.2.4 Effect of NaCl Concentration on PME Activity

The effect of NaCl concentration on Tabasco pepper PME was tested under standard assay conditions within the range of 0-14% NaCl. Blanks lacking PME were made for each determination, and the amount of acid produced because of the spontaneous pectin demethylation was subtracted.

4.2.5 Effect of pH on PME Activity

To investigate the effect of pH on PME activity, we followed the method by Castro and others (2004). The pH dependence of the purified pepper PME activity was assayed titrimetrically using 719 S Titrino and 703 Ti Stand titration unit (Brinkman/Metrohm, Herisau, Switzerland) at 25°C with 0.01 N NaOH (Sigma Aldrich, St. Louis, MO) after adjustment of the pH of the reaction solution to one of the pH values tested (4.5-9.5). The reaction mixture consisted of 200 uL of PME sample and 30 mL of 3.5 mg/mL of citrus pectin (DE= ≥85%) containing 0.117 M NaCl. The pectin solution was adjusted to the pH value of interest before the addition of the enzyme. The pH was maintained by continuously adding 0.01 N NaOH for 30 minutes. PME activity is proportional to the rate of consumption of NaOH ($\Delta V_{NaOH}/\Delta t$) Blanks lacking PME were made for each determination, and the amount of acid produced because of the spontaneous pectin demethylation was subtracted. One unit (1U) of PME activity was defined as the amount of enzyme that produces 1 μmol acid/min at the given pH.

4.2.6 Effect of Temperature on PME Activity

PME activity was tested under standard assay conditions with varying temperatures. The temperatures (30, 40, 50, 60, 70, 80, and 90°C) were controlled by means of a circulating water bath (Isotemp 105, Fisher Scientific and VWR International model # 1525, Suwanee, GA). Blanks without PME were made for each determination, and the amount of acid produced because of the spontaneous pectin demethylation was subtracted.

4.3 Results and Discussion

4.3.1 Effect of Pectin Concentrations on Activity of PME

The initial rates for Tabasco pepper PME activity were measured at various concentrations of pectin. The results are shown in Figure 4.2. The Tabasco pepper PME K_M value was 0.23 ±0.05 mg/mL and the maximum rate (V_{Max}) was 48.1 ±3.1 nmoles methanol/min.

Lineweaver–Burk analysis of Figure 4.2 was linear indicating the (2) bands at 36 kDa and 22 kDa from the previous chapter were not (2) separate forms of the enzyme catalyzing with different parameters. The green bell pepper PME kinetic parameters K_M and the maximum rate (V_{Max}) were determined by nonlinear regression analysis as 0.329 mg/mL and 0.272 mL/min at a neutral pH, respectively (Castro and others 2004). The green bell pepper PME kinetic parameters K_M and the maximum rate (V_{Max}) were determined at acidic pH (5.6) by nonlinear regression analysis as 1.614 ±0.065 mg mL^{-1} and 0.417 ±0.005 mL min^{-1}, respectively (Castro and others 2006). However, it is difficult to compare the K_M values found in this study with other K_M values cited in the literature because the K_M values are dependent on temperature, salt concentration, pectin source and pH of the reaction medium (Gaffe and others 1997; Laats and others 1997; Assis and others 2000; Goldberg and others 2001).

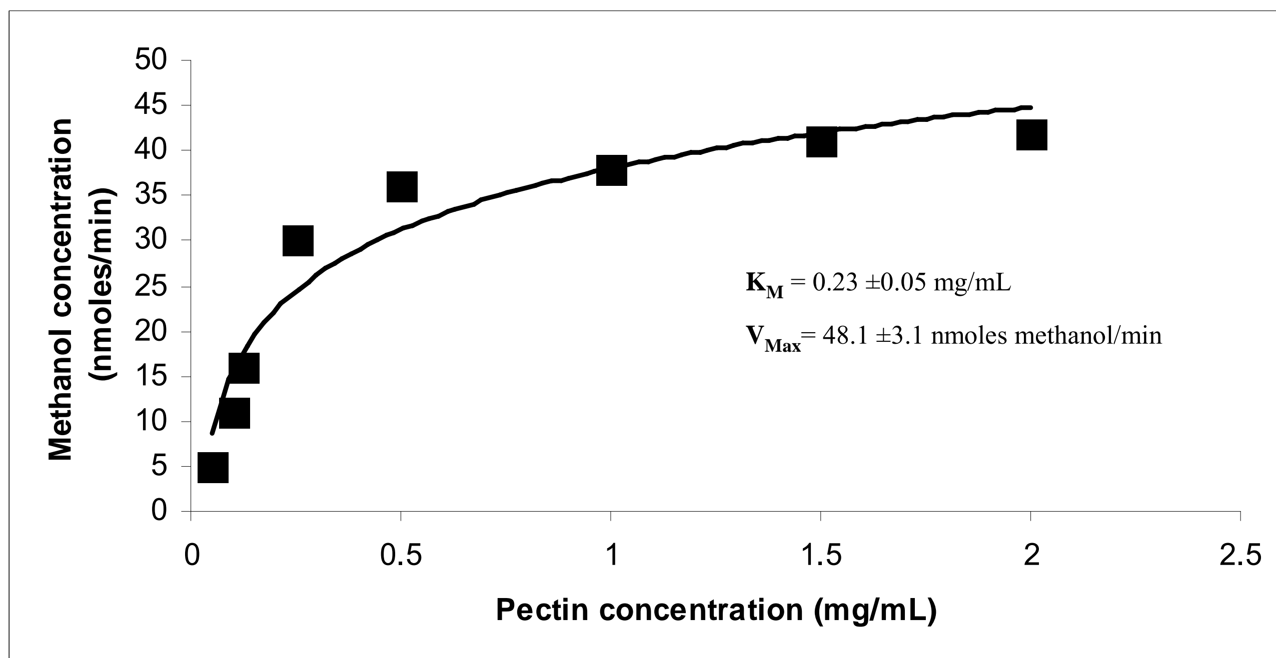

Figure 4.2 Activity of Tabasco pepper PME as a function of substrate concentration. Points represent experimental velocity. The line is the best fit of the data to Michaelis-Menten equation ($v = V_{Max} \cdot S/(K_M + S)$.

Alonso and others (1997) extracted two PME isoforms from persimmon, PME I accounted for 20% of the total activity and PME II for 80%. Both isoforms differed in their affinity for the apple pectin substrate with K_M values of 54 μg mL^{-1} and 31 μg mL^{-1} for PME I and PME II, respectively. In addition, Ly-Nguyen and others (2002b) obtained a K_M -value of 0.197 mg/ mL pectin solution for purified carrot. For PME from orange (*Citrus sinensis* L.) fruit peels, the K_M values determined for citrus and sugar beet pectins were 0.7 mg mL^{-1} and 8 mg mL^{-1}, respectively, indicating a stronger affinity for citrus pectin than for sugar beet pectin. The affinity declined significantly for citrus pectin as the degree of esterification (DE) % decreased. Christensen and others (1998) observed a 24-fold reduction as the DE % decreased from 70% to 25%. The result was in contrast to the effect found for grapefruit PME (Seymour and others 1991) and for mandarin PME (Baron and others 1991). The K_M increased with increased DE % for mandarin PME. For PME from both grapefruit and mandarin the activity increased with decreasing DE % and the initial rate of activity was highest at the lowest DE %. However, this was not reflected in a lower K_M value for pectin as it would have been expected, since the K_M increased.

The K_M was 0.274 mg mL^{-1} for grapefruit PME compared to 0.7 mg mL^{-1} for orange PME with pectin with DE=70%. The higher activity with lower DE % might have been due to an increase in the number of initiation sites (i.e. galacturonic acid residue adjacent to methyl galacturonic acid residues) in the pectin with lower DE % (Bordenave 1996). The K_M value for PME I from orange fruit determined by Versteeg and others (1978) was lower by a factor of 10 (K_M 0.083 mg mL^{-1}) than seen in the Christensen and others (1998) study. This difference in K_M could be due to the different pectin types used for analysis in the study. Zocca and others (2007) discovered that the pectin demethylation carried out by grape PME was incomplete. The reduced

rate of methanol production indicated a possible change in the PME enzyme, which has known to display end-product inhibition (Pilnik and Voragen 1991) or a change in its environment.

4.3.2 Effect of NaCl Concentrations on Activity of PME

Our results are in agreement with other studies in that there is an initial stimulatory effect of the salt on the Tabasco pepper PME, as compared to the control. We observed activity of PME up to 6% (1.03 M) NaCl shown in Figure 4.3; where the enzyme lost 50% activity. We did not observe minimal activity at 0% NaCl. Additionally, the enzyme appears to be inactivated at concentrations of 8% (1.4 M) and above the normal fermentation salt concentrations (8%) for Tabasco mash. The inactivation of this enzyme at that concentration is an important step because any concentration less than 8%, we can still observe PME activity. As a result the enzyme has the capacity of degrading pectin; it then becomes susceptible to other endogenous enzymes causing poor quality of the final product. Green beans pectin methylesterase enzymes, PE-1 and PE-2 both contained two isoforms of PME activity P1, P2, S1, and S2, respectively. The optimum salt concentration for P2 and S2 was 140 mM NaCl. The optimum for P1 was slightly lower. The P2 and S2 exhibited strong salt dependency as they were not active in the absence of NaCl (Laats and others1997).

We did observe maximum activity between 2% (0.34 M) to 4% (0.68 M) NaCl concentration which compares favorably with green bell pepper PME. Green bell pepper PME activity increased with the salt concentration up to 0.13 M, but when the NaCl level was further raised, the activity gradually decreased. At 0.13 M NaCl, ~60% of activity at optimal NaCl concentration (0.13 M) was observed (Castro and others 2004). Also, at pH 5.6, purified green bell pepper PME activity increased up to a maximum of 0.4 M of NaCl concentration, followed by decay in activity. For a 3.4-fold increase in salt concentration up to 0.4 M, there was a 4.3-fold increase in enzyme activity (Castro and others 2006).

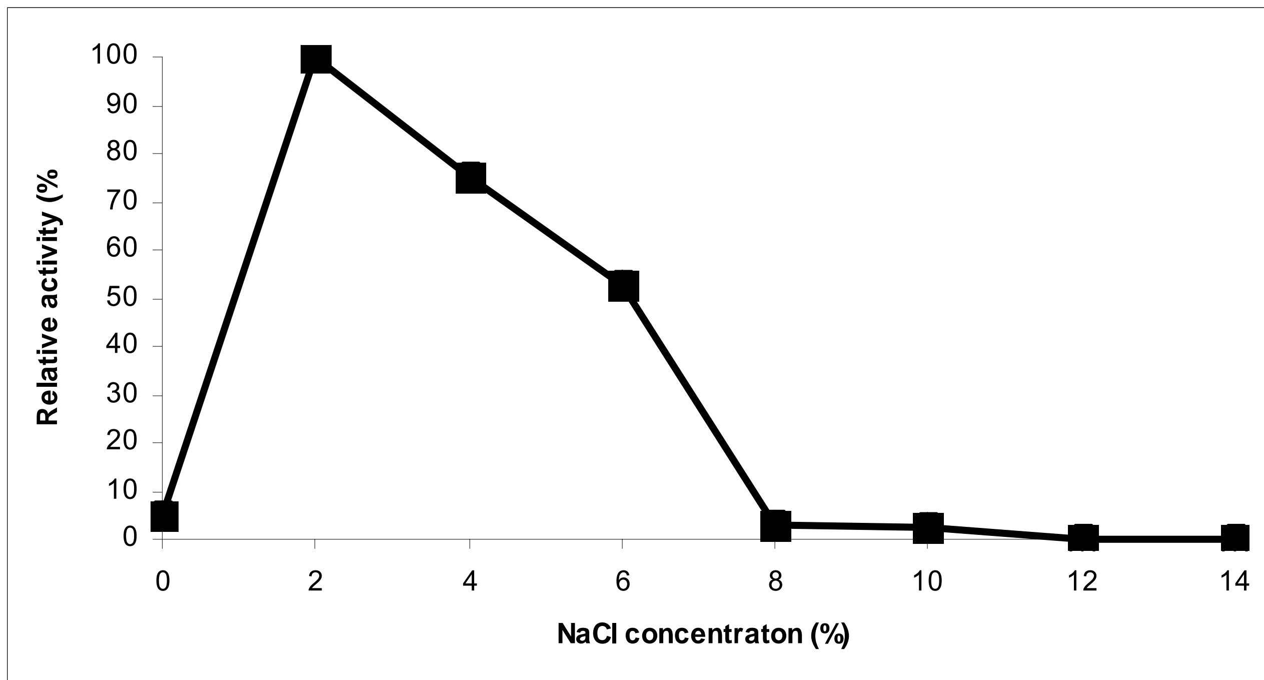

Figure 4.3 Effect of NaCl on PME activity from Tabasco pepper. Assays were performed with 4 mg/mL citrus pectin (DE= ≥85%) at pH 7.5.

PME extracted from guava fruit (*Psidium guajava L.*), cultivar 'Paluma', had optimum concentrations of 0.20 and 0.15 M NaCl, respectively, for PME and for Iso4-PME. On further increase of sodium ion concentration, the enzyme activity decreased (Leite and others 2006). Sodium ions are believed to bind to PME, inducing conformational modification, and favoring reaction of the enzyme with the substrate (Nari and others 1991). For the orange (*Citrus sinensis*) cultivar 'Pera-Rio' PME in the presence of NaCl the activity was higher in 0.025 M solution (Do Amaral and others 2005).

4.3.3 Effect of pH on Activity of PME

The study of the enzyme activity as a function of pH reveals that the optimum pH of Tabasco pepper PME activity was pH 7.5 shown in Figure 4.4 which compares favorably with green bell pepper PME at pH 7.5 (Castro and others 2006). At pH 6.5, the enzyme retained 74% of its activity. However, the enzyme lost most of its activity above pH 8.5. At low pH (4.5), the

enzyme retained more than 20% activity. The reduction of pH during the primary fermentation stage is an important phase in the fermentation process. During this period, there is a decrease in the pH and an increase in total acidity of the mash thereby decreasing PME activity.

The pH optimum for purified green bell pepper PME was pH 7.5. At the natural pH of green bell peppers (pH 5.6), the activity is only 45% of the optimum value. Above pH 7.5, the PME activity still remained relatively high over the range of alkaline pH (Castro and others 2006; Castro and others 2004). Alonso and others (1997) extracted two PME isoforms from persimmon, PME I and PME II optimum pH were 7.4 and 7.8, respectively. Specific activity of orange PME was optimum at pH 8.0. Optimum pH varies with PME fruits. Jelly fig achenes PME pH optimum is from pH 6.5 to 7.5 when the assay mixture contains no NaCl or low NaCl concentration.

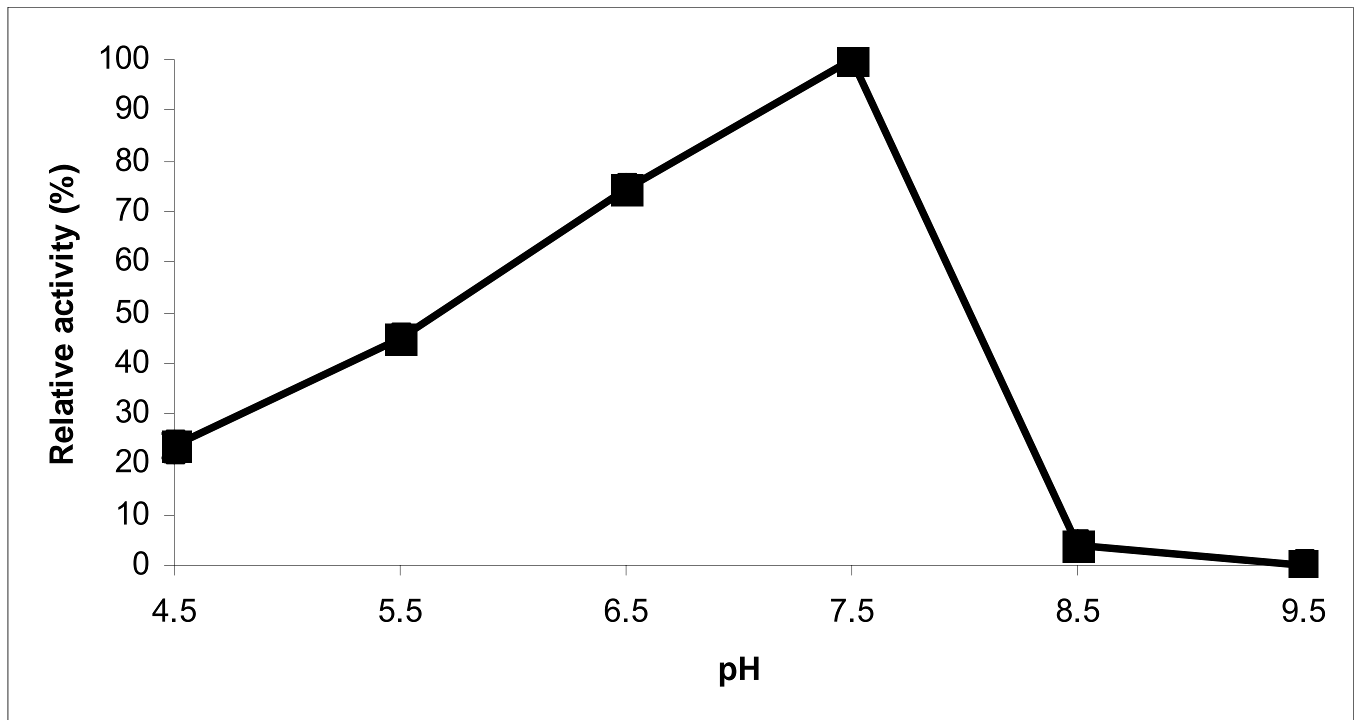

Figure 4.4 Relative activity of PME as a function of pH. Assays were titrimetrically performed with 3.5 mg/mL pectin (DE= ≥85%). Set point on Titrator was varied to achieve pH values.

Green beans PME isoform P1 had a pH optimum in the range of 6.5-9.0. In contrast, PME extracted from guava fruit (*Psidium guajava L.*), cultivar 'Paluma', presented an optimum pH between 8.0 and 9.0; also the PME Iso4 showed optimum pH at 8.5 (da Silva Cerqueira Leite and others 2006). Generally, optimum pH values for PME vary for fruits, and most are found to have pH values from 7.5-9.0 (Arbaisah, and others 1997; Lim and Chung 1997; Assis and others 2000). Concord grape crude PME activity was influenced by pH. The activity increased rapidly from pH 5.0 to a maximum at pH 7.5 and then decreased sharply with further increase of pH. The methanol content of Concord wines fermented without skins were very low compared with those of the same grapes fermented with the skins. The cause of the higher methanol content in the grape must with skins is the large portions of PME and pectin substances located skin (Lee and others 1979).

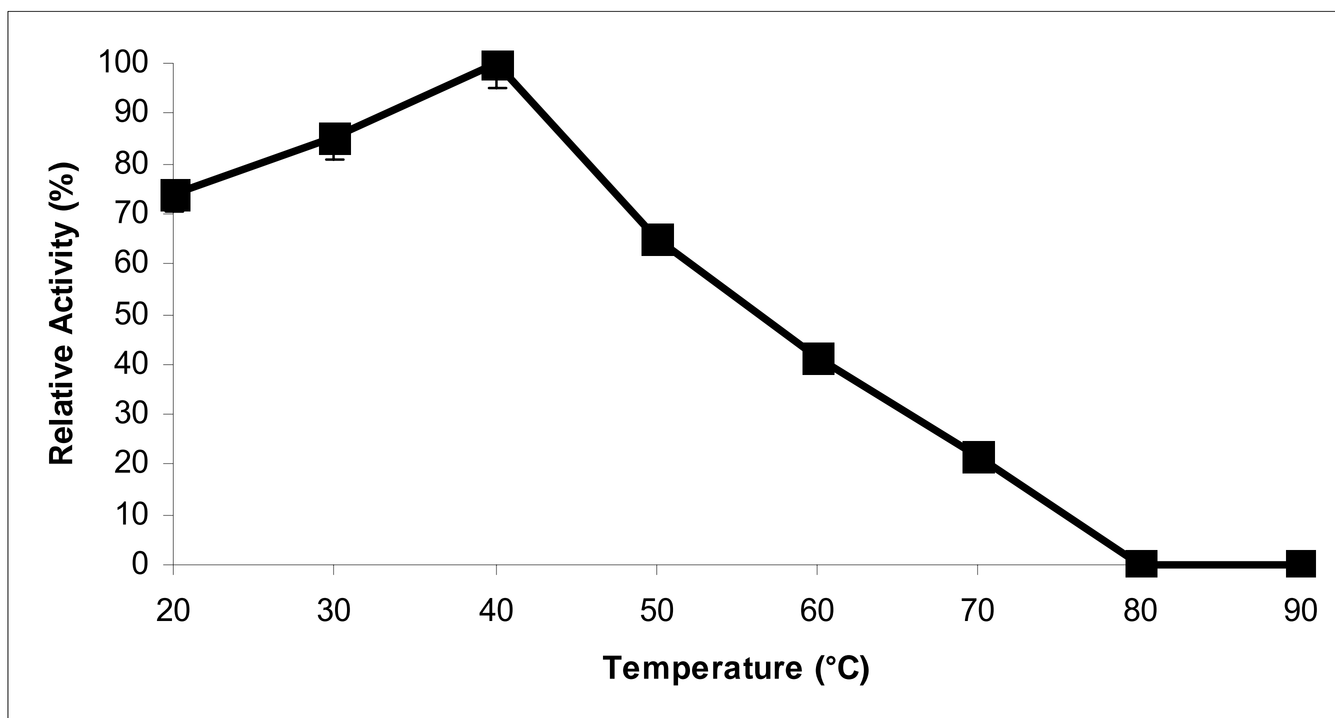

Figure 4.5 Temperature optimum of Tabasco pepper PME. All reactions were carried out at pH 7.5 with 4 mg/mL citrus pectin (DE= ≥85%).

4.3.4 Temperature Optimum for PME Activity

The temperature optimum for PME in Tabasco peppers was 40°C shown in Figure 4.5 which is similar to that of partially purified red grapefruit finisher pulp PME (Cameron and Grohmann 1995). The enzyme exhibited 85% activity at 30°C; moreover, there was a decline in activity at 50°C (65% activity) and higher. PME extracted from guava fruit (*Psidium guajava L.*), cultivar 'Paluma', showed optimum temperatures at 75°C for the PME and 85°C for the Iso4 PME partially purified enzymes (da Silva Cerqueira Leite and others 2006). Do Amaral and others (2005) purified PME from orange (*Citrus sinensis*) cultivar 'Pera-Rio', and found optimum specific activity of PME at 50°C. The temperature optimum for PME isolated from strawberry was between 59°C and 60°C (Ly-Nguyen and others 2002a).

4.4 Conclusions

The PME from Tabasco peppers (*Capsicum frutescens*) had an optimal temperature for activity at 40°C and was completely inactivated above 80°C temperatures. The pepper PME activity was optimum at pH 7.5 decreasing with pH, but still retained 20% activity at pH 4.5. Tabasco PME demonstrated activity in the 2% to 4% NaCl range, but the enzyme loss most of its activity at 8% and above concentrations. We suggest that under the combinatons of acidic conditions and high salt concentrations Tabasco pepper PME enzyme is capable of being inactivated.

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CHAPTER 5

QRT-PCR DETECTION OF *LACTOBACILLUS SPP*. ISOLATED FROM FERMENTED TABASCO PEPPER MASH

5.1 Introduction

Pectin methylesterase (PME) is an enzyme found in many plants walls. It is believed to be relatively inactive in most intact plant tissues, but when the tissues are macerated, the enzyme rapidly converts the pectin to pectic acid (Kertesz 1951; Lineweaver and Jensen 1951; Lee and others 1979). PME catalyzes the removal of the methyl groups from the polygalacturonic acid chain from the cell wall of the fruit and vegetable; leaving an increased number of free carboxyl groups which can be further hydrolyzed by polygalacturonase (PG), resulting in a decrease of the degree of polymerization of the pectin chains and a loss of firmness of the tissue as stated by Mohnen (2008), and Ünal and Bellur (2009). According to Sila and others (2007) and Ünal and Bellur (2009) the control of PME activity has been a common subject of study because of the implications in the modifications of the texture of fruit and vegetables and as a destabilizing agent of pectin materials in fruit juices and concentrates. In addition, PME has been detected in plants as well as bacteria and fungus.

Hot pepper sauce is the result of combined biochemical activities of microbial and vegetal origin. PME may be related to raw materials or released by microorganisms, specifically lactic acid bacteria (LAB) in Tabasco pepper mash. Moreover, there are many microorganisms that enter the Tabasco pepper mash from the immediate surroundings during the fermentation process (i.e. farm equipment or wood oak barrels). Adventitious non-starter lactic acid bacteria (NSLAB) may become the dominant bacterial population in the final stages of the mash fermentation. The pectinolytic activities of LAB have been addressed in studies of fermentation processes like wine making, cassava roots, and silage. For instance, PME and PG activities have been detected in the spontaneous fermentation of cassava roots (Ampe and Brauman 1995; Brauman and others 1996). Karam and Belarbi (1995) investigated excellular pectinolytic activities from raw milk in Algeria. The results showed several strains were able to grow in

medium using apple pectin as the sole energy source that was incubated at 30°C for 48 h, reaching 10^4 cells/mL. The strains *Lactobacillus casei* HNK10 and L1-8, *Lactobacillus plantarum* Lc5, and *Lactococcus lactis* NN01 exhibited pectin methylesterase and polygalacturase activity. The activity of the enzymes caused demethylation of pectin and the cleavage of polygalacturonic acid which causes defects in the food products. Such bacteria have developed the enzymatic machinery that allows them to use plant pectins because plant materials are their main habitat. These types of secreted enzymes are salt tolerant and are not susceptible to extreme salt conditions such as fermented pepper mash consisting of 8% salt as the normal fermentation conditions (G. Waldrop, personal communication, November 4, 2009).

It is well known that LAB, in particular lactobacilli, plays an important role in fermentation processes (Fontana and others 2005). Lactobacilli include a large number of non-pathogenic, non-toxic bacteria that play an important role in the health and well being of humans and animals. The metabolic end products of *Lactobacillus* metabolism include acetic acid, lactic acid and hydrogen peroxide. Their ability to lower the pH and produce bacteriocins prevents the growth of pathogenic and spoilage microorganisms, improving the hygienic safety and storage of Tabasco products.). In addition to being made more shelf stable, all fermented foods have aroma and flavor characteristics that result directly or indirectly from the fermenting organisms. Important flavor compounds, including diacetyl and acetaldehyde, are produced by LAB during the fermentation. Therefore, the mash fermentation process is a very critical step in final hot sauce quality. The genus *Lactobacillus* is a large, heterogeneous collection of genetically and physiologically diverse and highly acid-tolerant LAB species. A measure of their diversity can be estimated by the range in the guandide (G) + cytosine (C) % of DNA of species among lactobacilli (Gasser and Mandel 1968; Axelsson 2004).

According to Oneca and others (2003), molecular techniques offer a variety of advantages over conventional bacterial enumeration methods; most importantly, the high discriminating power. For example, clear identification of the genus *Lactobacillus* can be very complicated using sugar fermentation patterns due to an increasing number of LAB species which vary on a small number of characteristics (Quere and others 1997). Quantitative real-time polymerase chain reaction (QRT-PCR) has several advantages including enhanced speed and the absence of post-PCR steps. The incorporation of SYBR® Green into real-time PCR assays constitutes the simplest and least expensive reporter system. Also, the SYBR® Green method has the advantage of allowing a DNA melting curve of the amplicon to be obtained following the qPCR analysis, which allows distinction of the amplified product from primer dimmers as stated by Lee and Levin (2007).

The objective of this study was to improve the knowledge about the presence of *Lactobacillus* in aged fermented Tabasco mash; because this group of bacteria could have the potential to produce and excrete pectinases. *Lactobacillus* plays a central role in the fermentation process. The growth factors will allow us to better understand the bacteria community. In this study, qualitative PCR amplification of a 190-bp region of the 16S gene was used as a screening method to determine the presence of *Lactobacillus spp.* in fermented Tabasco pepper mash samples; then further evaluated using real-time PCR to quantify total bacteria and lactobacilli in the samples.

5.2 Materials and Methods

5.2.1. Parental Strains and Maintenance

Lactobacillus casei and *Lactobacillus plantarum* were used as the positive controls donated by the Department of Food Science at the University of Arkansas, Fayetteville. *Escherichia coli* ATCC 25947 was used as a positive control for enumeration of total bacteria

counts provided by Louisiana State University Department of Food Science, Food Microbiology Laboratory. Concentrated cell suspensions were maintained under cryoprotective conditions at -80°C (Revo, Kendro Laboratory Products, Asheville NC) as previously described (Darnell and others 1987) except in MRS broth and BHI broth for *Lactobacillus* spp. and *E. coli*, respectively with 15% glycerol (Difco, Laboratories, Detroit, MI). The primers were purchased from Intergrated DNA Technologies, Inc. (Coralville, IA).

5.2.2 de Man, Rogosa, and Sharpe (MRS) Growth Media Broth and Agar

MRS broth allows selective isolation, cultivation, and presumptive identification of lactic acid bacteria (LAB) (de Man and others 1960). The MRS broth (55 g MRS broth medium in 1 L of Milli-Q water) (Weber Scientific, Hamilton, NJ) and MRS agar (55 g MRS broth medium and 15 g agar technical solidifying media made up to 1 L of Milli-Q water) were prepared and autoclaved (AMSCO Eagle Series 3023 Vacamatic) at 121°C for 20 min. The MRS agar was disbursed as pour plates (100 x 15 polystyrene disposable pates) (VWR International, Suwanee, GA) to complete the preparation of the media.

5.2.3 Brain Heart Infusion (BHI) Growth Media Broth and Agar

The BHI broth (37 g BHI broth medium in 1 L of Milli-Q water) (Weber Scientific, Hamilton, NJ) and BHI agar (37 g BHI broth medium and 15 g agar technical solidifying media made up to 1 L of Milli-Q water) were prepared and autoclaved (AMSCO Eagle Series 3023 Vacamatic) at 121°C for 20 min. The BHI agar was disbursed as pour plates (100 x 15 polystyrene disposable pates) (VWR International, Suwanee, GA) to complete the preparation of the media.

5.2.4 Sample Collection

Thirty Tabasco Mash samples stored in 250 mL sterile plastic containers (Nalgene, Rochester, NY) were collected on three different processing days from randomly fermenting

barrels (aged 1 month or 36 months) located at a local processing plant under normal processing conditions. The mash samples were stored at refrigeration (4°C) (VWR International model # 2015, Suwanee, GA) until ready for use. The mash samples were collected from eight countries that included Brazil, Colombia, Ecuador, Honduras, Panama, Peru, Nicaragua, and Zimbabwe. Samples were weighed (Sartorius Corporation model # TE412, Edgewood, NY) and 5 mL of sterile 20 mM phosphate buffered saline (PBS); pH 7.2 and 5 g sample was added into a sterile filtered stomacher bag (VWR International, Suwanee, GA).

5.2.5 Pepper Mash Sample Preparation

The fermented mash samples were examined for *Lactobacillus* genomic DNA. The bags were stomached for 60 s in stomacher (Tekmar Company, Cincinnati, Ohio) and 5 mL of homogenate was added to 5 x 14 mm sterile plastic polystyrene test tubes (Weber Scientific, Hamilton, NJ). DNA Template was prepared by centrifuging (Centrifuge 5415C, Brinkman Instruments, Inc, Westbury, NY) the homogenate at 10,000 *x g* for 25 min. The supernant was decanted and the pellet was pippetted into sterile DNase, RNase, and endotoxin free Eppendorf tubes (VWR International, Suwanee, GA).

5.2.6 DNA Extraction from Fermented Tabasco Mash Samples

Approximately ~200 µL of lysozyme digestion buffer (25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 1% Triton X-100) was added to the Eppendorf tube (VWR International, Suwanee, GA) with fresh lysozyme (Sigma Aldrich, St. Louis, MO) to obtain a final concentration of 20 mg/mL and incubated at 37ºC (VWR International model # 1525, Suwanee, GA) for 30 min. The genomic DNA was purified according to the Gram-positive bacteria lysate protocol in the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The resulting supernatant was used as a purified DNA template for qualitative PCR and quantitative real-time PCR. *Detailed DNA purification is shown in Appendix C.*

5.2.7 PCR Amplification Conditions

Amplification of the target sequence was performed using S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Semi-universal primers were employed in the PCR mixture Lb1 primer (5′-AGAGTTTGATCATGGCTCAG-3′) and Lb2 primer (5′-CGGTATTAGCATCTGTTTCC-3′). These primers and thermal cycler conditions adapted from Klijn (1991), Quere and others (1997), and Oneca and others (2003), amplified a variable loop in 16S sequences of *Lactobacillus* species. The PCR mixture contained 0.2 μM of each primer; Platinum PCR Supermix {[22 U/ml complexed recombinant Taq DNA polymerase with Platinum® Taq Antibody, 22 mM Tris-HCl (pH 8.4), 55 mM, KCl, 1.65 mM $MgCl_2$, 220 μM dGTP, 220 μM dATP, 220 μM dTTP, 220 μM dCTP, and stabilizers] (Invitrogen, Carlsbad, CA)}; 2 μL of genomic DNA of sample, *Lactobacillus casei,* and *Lactobacillus plantarum* as positive controls; and sterile nuclease-free water as negative control (Ambion, Inc, Austin, TX); for the final volume of 25 μL in sterile thermal cycle tubes (Molcular BioProducts, San Diego, CA). The reaction mixture was subjected to the following thermal cycling conditions: 35 amplification cycles of 1 min at 94 ºC; 1 min at 54 ºC; and 1 min at 72 ºC, proceeded by 5 min at 95 ºC; followed by a final extension of 5 min at 72 ºC; and stored at 4 ºC until ready for further analysis.

5.2.8 Electrophoresis

The 10 μL of PCR products were subjected to gel electrophoresis with molecular markers (Bio-Rad Laboratories, Hercules, CA), (2.0 % w/v agarose in 1X Tris-borate ethylenediaminetetraacetic acid buffer, TBE) (Sigma Aldrich, St. Louis, MO), and run for 70 min at 90V. The samples were stained with ethidium bromide (4.5 μL/90 mL agarose solution) (Sigma Aldrich, St. Louis, MO), visualized with a UV transilluminator, 310 nm (Spectoline

Transilluminator, Model 7C-254R, New York), and photographed by Gel Doc XR, using Quantity One software, (Bio-Rad Laboratories, Hercules, CA).

5.3 DNA Concentration

The DNA concentration of each sample and standards was determined by using the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) at 260 nm. Each sample was diluted to 1 ng/ µL with sterile nuclease-free water (Ambion, Inc, Austin, TX) in MicroAmp® optical 96-well reaction plate (Applied Biosystems, Foster City, CA).

5.3.1 Quantitative Real-Time PCR

The analysis targeting a 92-base pair region of *Lactobacillus* genus and 200-base pair V3 region of *Escherichia coli* was adapted from Wise and Siragusa (2007) and Lee and others (1996), respectively. The sequences of the primers are shown in Table 5.1. Quantitative real–time PCR assay used the SYBR® Green method and was performed using an ABI Prism 7900HT Sequence Detection System (serial no. 100151) (Applied Biosystems, Foster City, CA). All reactions were performed in sterile MicroAmp® optical 384-well reaction plates with barcode sealed with MicroAmp® optical adhesive film (Applied Biosystems, Foster City, CA). Each reaction tube had a volume of 25 µL containing 10 µL of 2x SYBR® Green Master Mix {[SYBR Green I dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, and optimized buffer components]} (Applied Biosystems, Foster City, CA); 3 µL of forward and reverse primers at 10 µm; 3 µL of bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) at 2.5 mg/mL; 3 µL sterile nuclease-free water (Ambion, Inc, Austin, TX); and 3 µL of DNA template. The qPCR cycle conditions were performed at: 50ºC initial hold for 2 min in order to denature the DNA and activate the AmpliTaq Gold® DNA polymerase.

Table 5.1 Primers used in quantitative RT-PCR study

Specificity	Primers	Sequence	Reference
Lactobacillus	Forward	5′-TGGATG CCTTGGCACTAGGA-3′	Haarman and Knol (2006)
	Reverse	5′-AAATCTCCGGATCAAAGCTTACTTAT-3′	
Domain Bacteria	Forward	5′-CGGTCCAGACTCCTACGGG-3′	Lee and others (1996)
	Reverse	5′-TTACCGCGGCTGCTGGCAC-3′	

The run was set for 45 cycles of amplification with each consisting of denaturation at 95ºC for 15 s followed by primer annealing and extension step at 60ºC for 1 min, then 78ºC for 30 s. After the last cycle of each amplification, a dissociation step (60ºC to 95ºC) was included to analyze the melting profile of the amplicon. The threshold is the line whose intersection with the amplification plot defines the cycle threshold (C_T). This line is set above the baseline and within the exponential growth region of the amplification curve. The C_T is the fractional cycle number at which the fluorescence passes the threshold as indicated in the Applied Biosystems protocol manual (2006). Data was analyzed by Sequence Detection software version 1.6.3 supplied by Applied Biosystems.

5.3.2 Standard Curve for Quantitative Real-Time PCR Assay

A loopful (10 µL) of *Lactobacillus casei* and *Escherichia coli* were grown in 10 mL MRS broth and BHI broth, respectively for 24 h. The next day, each sample was serially ten-fold diluted with the highest dilution at 10^{-8} in sterile 20 mM PBS, pH 7.2. Duplicated agar plates of each dilution were spread on MRS for *Lactobacillus casei* and BHI agar plates for *E. coli* and incubated aerobically at 37ºC for 32 h and 24 h, resepectively. One mL culture from each dilution was taken and subjected to genomic DNA purification using a PureLink Genomic DNA Mini Kit as previously described for Gram-positive and Gram-negative cell lysates protocols,

respectively. The supernatant was considered as purified DNA template for quantitative real-time PCR.

To produce a standard curve, the quantity of the calculated number of cells added to each reaction tube (calculated from the plate counts, colony forming units, CFU) was plotted vs. the cycle threshold (C_T) value. The correlation coefficient (R^2) was provided.

5.4 Data Analyses

For statistical analysis, the software package JMP 7.0 (SAS Institute Inc, Cary, NC) was used. Differences were tested with ANOVA, and if P was < 0.05 the difference was considered statistically significant.

5.5 Results and Discussion

5.5.1 Qualitative PCR

The use of molecular characterization allowed the study of the complex microbial associations involved in the production of fermented mash from Tabasco pepper and the microbial population development during 1 month and 36 months fermentation process. The presence of LAB may play a relevant role in the fermentation of the mash. The decrease of pH as the result of the fermentation may have an influence on PME enzyme viability as well. In this study, qualitative polymerase chain reaction (PCR) amplification of a 190-bp region was used to determine the presence of *Lactobacillus spp.* in fermented Tabasco pepper mash samples.

The analysis of the lactic acid bacteria community began with the use of the Lb1/Lb2 primers that allowed a preliminary selection among the isolated DNA, indicating that these primers are able to discriminate this group at the genus level. These primers were also used previously by Quere and others (1997) and Oneca and others (2003) for differentiation of *Lactobacillus spp.* The PCR product was free from other by-products showing a distinct single band for purified product and at its correct size (190 bp) as judged by the agarose electrophoresis

gel in Figure 5.1. The results reveal that 100% of the fermented pepper samples were positive for *Lactobacillus*. However, the 36-months sample from Honduras (lane 11) shows a weak band. Thus, it is possible that there was less PCR product from this sample because of a lower number of cells in the well compared to the other samples.

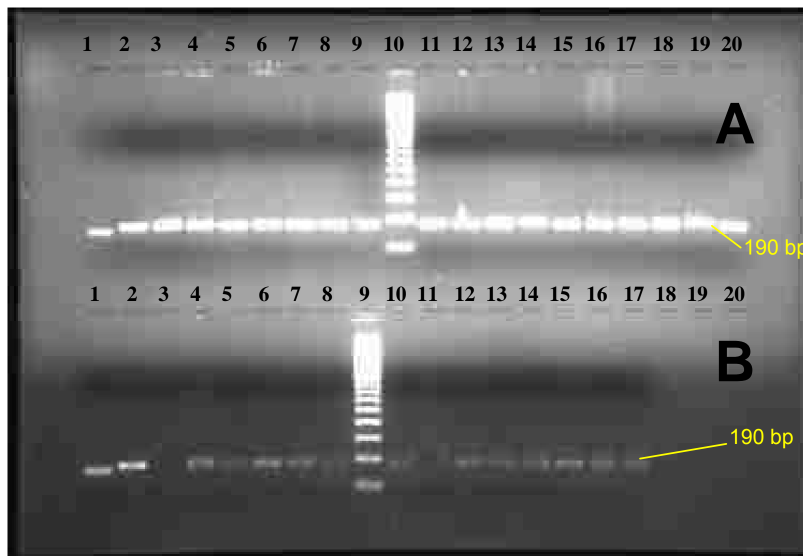

Figure 5.1 PCR specific to *Lactobacillus*, Lanes A10 and B9, molecular mass markers (2000 bp, 1500 bp, 1000 bp, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp).
A—1 month fermentation: (1) *Lb. casei*; (2) *Lb. plantarum*; (3) Zimbabwe; (4) Peru; (5) Panama; (6) Nicaragua; (7) Colombia; (8) Ecuador; (9) Honduras; (11) Zimbabwe; (12) Ecuador; (13) Honduras; (14) Nicaragua; (15) Peru; (16) Zimbabwe; (17) Ecuador; (18) Honduras; (19) Nicaragua; and (20) Peru.
B—36 months fermentation: (1) *Lb. casei*; (2) *Lb. plantarum*; (3) Sterile nuclease-free water (4) Zimbabwe; (5) Peru; (6) Brazil; (7) Colombia; (8) Honduras; (10) Ecuador; (11) Honduras; (12) Nicaragua; (13) Colombia; (14) Ecuador; (15) Honduras; (16) Nicaragua; and (17) Colombia.

5.5.2 Quantitative Real-Time PCR

Quantitative real time-PCR offers a sensitive, efficient, and reliable approach to quantitation of *Lactobacillus* in fermented pepper mash. Using the SYBR green system, we were able to determine both the amount of *Lactobacillus* and the total number of bacteria present in the mash samples directly without culturing. The data in Figure 5.4 shows the PCR products obtained following amplification of the positive mash samples. Furthermore, the cycle number increases consistently with decreasing bacteria concentrations shown in Table 5.2 of the cycle threshold (C_T) values. The lowest and highest C_T value for *Lactobacillus* (1-month) was 14.75

and 26.33, respectively. The lowest and highest C_T value for *Lactobacillus* (36-month) was 17.75 and 29.42, respectively. The lowest and highest C_T value for total bacteria (1-month) was 11.03 and 15.17, respectively. The lowest and highest C_T value for *Lactobacillus* (36-month) was 11.94 and 24.72, respectively.

The usefulness of our method is a very high analytical specificity and sensitivity in fermented mash samples, which permits a reliable identification of *Lactobacillus* when most of the bacteria are dead or damaged and therefore no longer detectable on agar plates. In addition, definitive, quantitative results are available within 1 d, in contrast to the classical bacteriological method, which requires 2 d of incubation.

Statistics for quantification of *Lactobacillus* (CFU/g) and Domain bacteria (total bacteria, CFU/g) results for the totality of DNA samples (n=30) are presented in Table 5.3. Bacteria cell values for 1-month and 36-months *Lactobacillus* and total bacteria (Domain), the efficiency of both reactions was very good (R^2=0.99 for *Lactobacillus;* R^2=0.97 for Domain bacteria) as calculated by the slope of a standard curve created by dilutions of a sample of known concentration. The percentage share of *Lactobacillus* in the counts were higher in 36-month aged samples than 1-month aged samples (prob>│0.0005│).

In the 36-month aged samples *Lactobacillus* (%) was the dominant bacteria population. Results revealed Brazil, Colombia, and Honduras had more than 50% *Lactobacillus* present. In contrast, the 1-month aged samples showed at least nine of the seventeen barrels had less than 1% *Lactobacillus* present. The comparison of the percentage of *Lactobacillus* by time based on country, Peru showed significant differences (prob>│0.0173│). However, when only comparing the percentage of *Lactobacillus* by country, we observed no significant differences (prob>│0.2988│).

Table 5. 2 Cycle threshold values of *Lactobacillus* and total bacterial cells in fermented mash samples

Group	Country	C_T Value [a, b]	
		Lactobacillus	Total bacteria
1-month	Zimbabwe	18.04 (± 0.430)	15.13 (± 0.090)
	Peru	16.77 (± 0.232)	13.47 (± 0.115)
	Panama	23.99 (± 0.014)	13.43 (± 0.132)
	Nicaragua	18.54 (± 0.173)	14.88 (± 0.062)
	Colombia	18.82 (± 0.378)	13.12 (± 0.042)
	Ecuador	22.37 (± 0.970)	15.01 (± 0.056)
	Honduras	17.45 (± 0.651)	15.17 (± 0.087)
	Zimbabwe	26.33 (± 0.275)	11.83 (± 0.084)
	Ecuador	16.17 (± 0.713)	11.55 (± 0.007)
	Honduras	15.60 (± 0.019)	12.35 (± 0.037)
	Nicaragua	20.40 (± 0.032)	12.53 (± 0.137)
	Peru	23.51 (± 0.203)	14.39 (± 0.029)
	Zimbabwe	17.74 (± 0.506)	12.37 (± 0.070)
	Ecuador	14.75 (± 0.044)	12.26 (± 0.059)
	Honduras	15.00 (± 0.537)	12.93 (± 0.031)
	Nicaragua	25.58 (± 0.032)	13.59 (± 0.016)
	Peru	16.75 (± 0.220)	11.03 (± 0.146)
36-months	Zimbabwe	27.69 (± 0.002)	23.28 (± 0.448)
	Peru	28.85 (± 0.188)	24.72 (± 0.032)
	Brazil	29.42 (± 0.184)	24.52 (± 0.042)
	Colombia	27.36 (± 0.355)	23.74 (± 0.057)
	Honduras	17.75 (± 0.116)	14.17 (± 0.010)
	Ecuador	20.13 (± 0.362)	16.60 (± 0.017)
	Honduras	27.63 (± 0.597)	23.46 (± 0.065)
	Nicaragua	23.81 (± 0.649)	20.00 (± 0.158)
	Colombia	25.61 (± 0.086)	20.84 (± 0.074)
	Ecuador	24.89 (± 0.263)	19.05 (± 0.034)
	Honduras	21.43 (± 0.045)	11.94 (± 0.051)
	Nicaragua	26.33 (± 0.980)	20.99 (± 0.420)
	Colombia	19.77 (± 0.106)	18.66 (± 0.090)

[a] Means ± standard error

[b] Cycle threshold (C_T) was the fractional cycle number at which the fluorescence passes the threshold

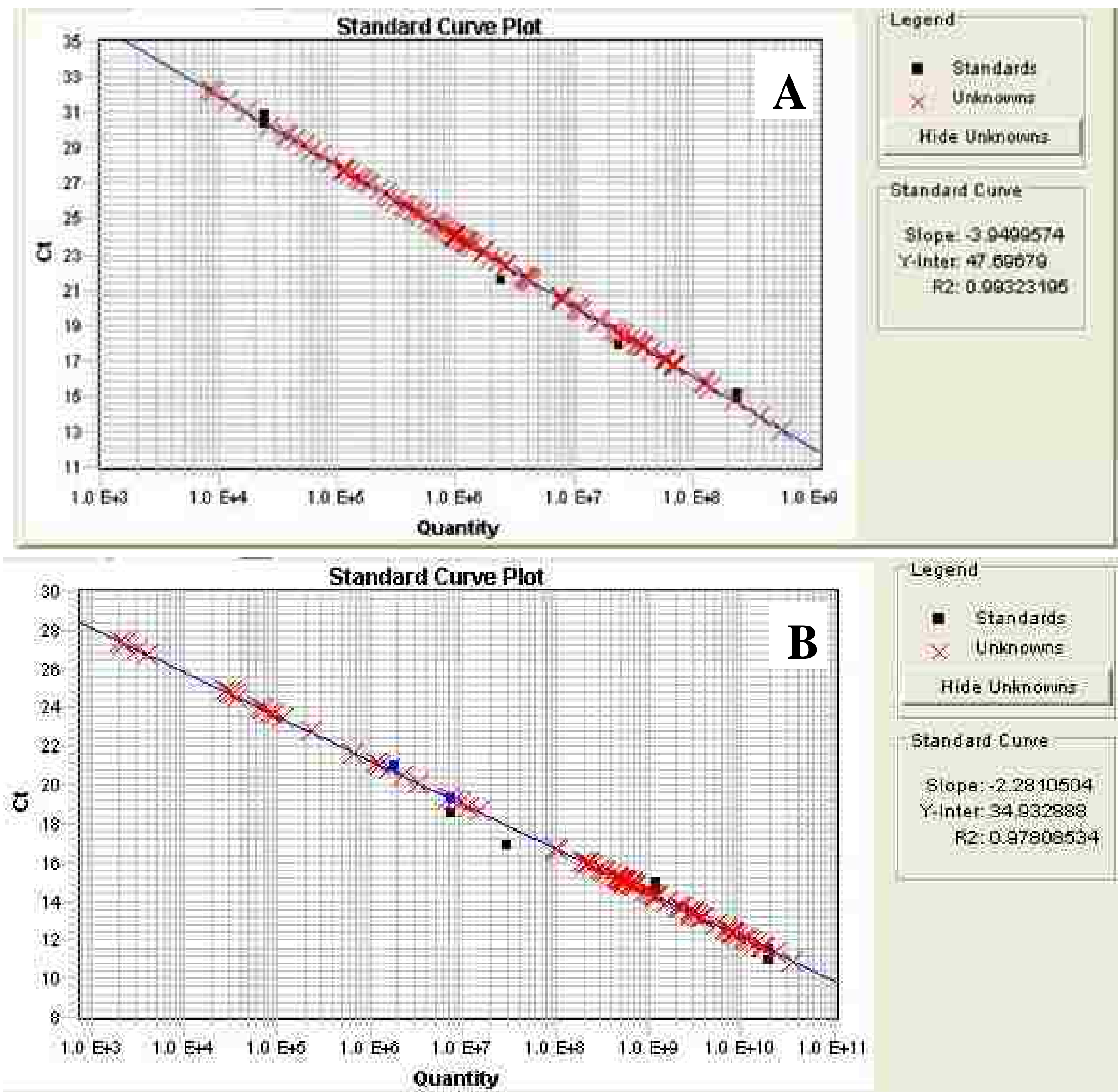

Figure 5.4 **(A)** Standard curve of *Lactobacillus casei* and unknown samples**;** **(B)** Standard curve of *total bacteria* and unknown samples. For the DNA standard curve, various dilutions of the DNA sample were directly quantified by real-time PCR indicating correlation coefficient R^2 of 0.993 and 0.978, respectively. The standard curve was performed using a DNA concentration of 1 ng/µL.

When *Lactobacillus* (CFU/g) was compared by time (1-month vs. 36-months) results showed slight significant differences (prob>│0.0731│). The 36-months range of *Lactobacillus* was 10^7-10^{11} CFU/g and the 1-month range of *Lactobacillus* was 10^6-10^{10} CFU/g. The 36-months range of total bacteria was 10^6-10^{12} CFU/g and the 1-month range of total bacteria was 10^{10}-10^{12} CFU/g. Our results are similar to a study of *Lactabacillus* present in Ronal cheese, it's the ripened uncooked cheese made from raw ewe's milk. The high concentration of *Lactobacillus* was shown in a study by Oneca and others (2003) which revealed Roncal cheese showed different species of *Lactobacillus* were already present in high numbers as much as 10^8 CFU/g even when not added to the cheese in the starter culture. However, the mean *Lactobacillus* counts in the raw ewe's milk were $10^{4-}10^6$ CFU/g and 10^7 CFU/g in the cheese.

Variations of *Lactobacillus* (CFU/g) counts were due to uncontrolled fermentations where the concentrations varied with the same country. Other possible causes of the variation in the concentration may be due from the normal flora differing among the countries or changes in the seasons affecting the bacteria growth. Based on the concentrations, the percentages of *Lactobacillus* present in the 36-month aged samples; this bacteria plays a major role over the course of the fermentation aging process.

5.5.3 The Melting Curve

In this study, the real-time PCR/melting curve analysis provided a rapid and precise method for *Lactobacillus* identification. Typically, the melting curve of a product is dependent on the G-C content, base pair length and sequence; PCR products can be distinguished by their melting curves (Kao and others 2007). The melting curve or dissociation curve was obtained by monitoring the fluorescence of the SYBR Green dye as the temperature passed through the product denaturation temperature.

Table 5.3 Number of *Lactobacillus* and total bacterial cells detected in fermented mash samples

Group	Country	Number of cells (CFU/g)		% *Lactobacillus*[a]
		Lactobacillus	Total bacteria	
1-month	Zimbabwe	9.18×10^{9}	4.82×10^{10}	19.1
	Peru	2.31×10^{10}	2.59×10^{11}	8.92
	Panama	1.10×10^{8}	2.70×10^{11}	0.04
	Nicaragua	6.19×10^{9}	6.18×10^{10}	10.0
	Colombia	5.11×10^{9}	3.63×10^{11}	1.41
	Ecuador	4.13×10^{8}	5.39×10^{10}	0.77
	Honduras	1.41×10^{10}	4.64×10^{10}	31.6
	Zimbabwe	1.97×10^{7}	1.35×10^{12}	0.001
	Ecuador	3.81×10^{10}	1.77×10^{12}	2.16
	Honduras	5.43×10^{10}	7.89×10^{11}	6.89
	Nicaragua	1.57×10^{9}	6.72×10^{11}	0.23
	Peru	1.58×10^{8}	1.01×10^{11}	0.16
	Zimbabwe	1.15×10^{10}	7.8×10^{11}	1.48
	Ecuador	2.14×10^{11}	8.77×10^{11}	24.4
	Honduras	8.76×10^{10}	4.86×10^{11}	18.0
	Nicaragua	6.55×10^{8}	2.27×10^{11}	0.29
	Peru	2.34×10^{10}	3.05×10^{12}	0.77
36-months	Zimbabwe	7.15×10^{6}	1.54×10^{7}	46.3
	Peru	1.37×10^{6}	2.99×10^{6}	45.7
	Brazil	2.00×10^{6}	3.65×10^{6}	54.6
	Colombia	5.49×10^{6}	8.09×10^{6}	67.8
	Honduras	1.11×10^{10}	1.26×10^{11}	8.82
	Ecuador	1.95×10^{9}	1.08×10^{10}	18.0
	Honduras	7.84×10^{6}	1.08×10^{7}	72.8
	Nicaragua	1.32×10^{8}	3.51×10^{8}	37.7
	Colombia	3.32×10^{7}	1.51×10^{8}	22.0
	Ecuador	5.66×10^{7}	9.15×10^{8}	6.21
	Honduras	7.32×10^{8}	1.20×10^{12}	0.06
	Nicaragua	2.20×10^{7}	1.52×10^{8}	14.5
	Colombia	1.31×10^{9}	1.36×10^{9}	96.7

[a] Estimated as *Lactobacillus* cells/total bacteria cells x 100, percentage of *Lactobacillus*

The characteristically shaped melt profile provided in evidence to identify genus; however, not only were the melting temperature (T_m) is important for genus identification, but the shapes of the curves can be useful as well (Skow and others 2005). The melting curves of *Lactobacillus* had different shapes and melting temperatures than other LAB (Renouf and others 2006). Additionally, other bacteria can be differentiated rapidly from *Lactobacillus* by genus-specific primers; therefore, it reduces the probability of false-positive judgment. All PCR products for a particular primer pair should have the same melting temperature.

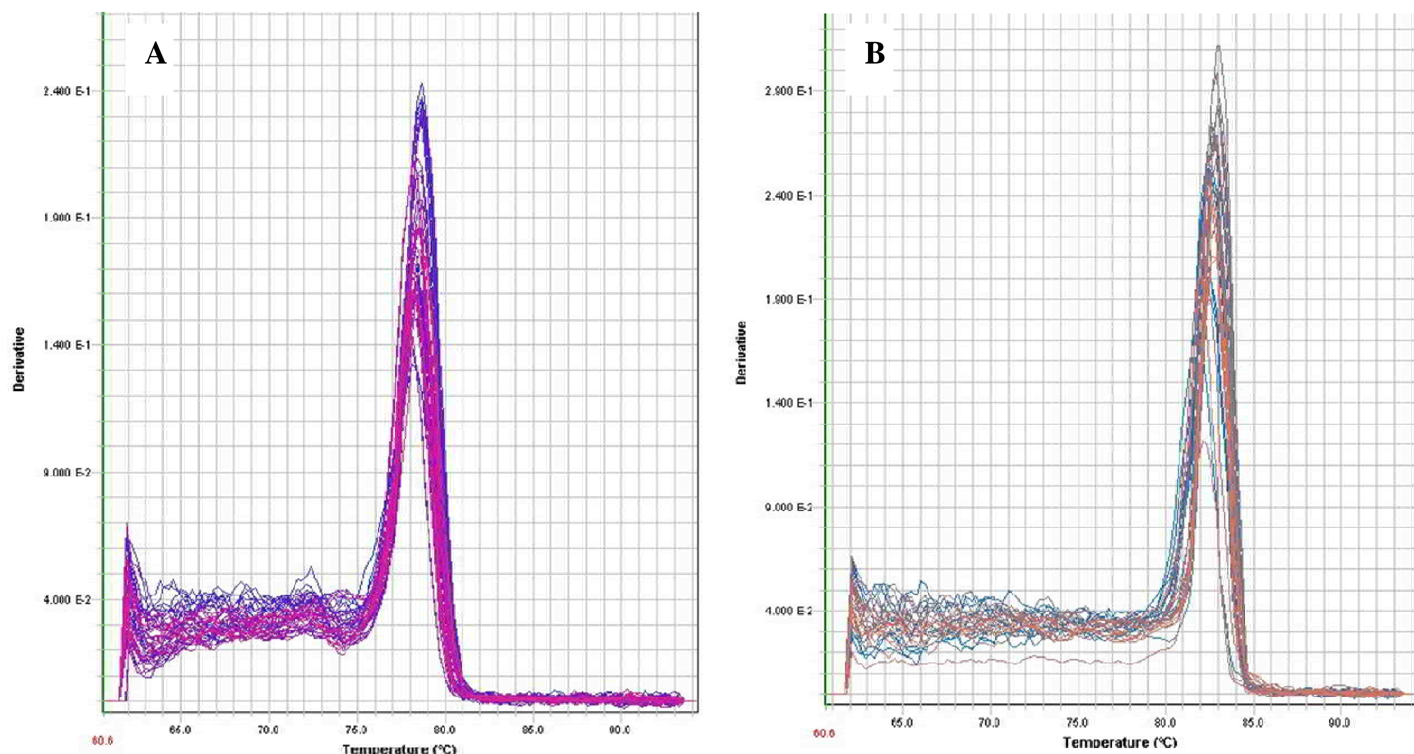

Figure 5.5 Melting curves acquired on a purified **(A)** 92-base pair region of *Lactobacillus* genus, **(B)** a V-3 region, 200 base pair fragment from total bacteria (Domain) extracted from fermented Tabasco pepper mash samples.

Melting peaks in fermented mash samples, revealed identical melting peaks for all DNA isolates. The melt curve is ranged from 60.6°C to 94.3°C, the temperature increased every 10 seconds. Results are shown in Figure 5.5 for the (A) 1-month and 36-months samples and (B) Domain (total) bacteria. *Lactobacillus* samples produced one high peak at 78.5°C and Domain

bacteria produced one high peak at 82.5°C. The results are different from *Lactobacillus* grown strictly anaerobically where a single peak was present at 83.5°C and total bacteria exhibited a melting temperature at 89°C (Fu and others 2006).

5.6 Conclusions

The molecular techniques were shown to be efficient tools for the study of the complex associations developed in food fermentations for the selection and determination and quantification of *Lactobacillus* spp. The presence of *Lactobacillus* was determined in 30 commercial fermented mash samples. This study demonstrates the utility of the PCR targeting a specific region to determine the presence of *Lactobacillus* spp. in the samples. The PCR assay evaluated in the current study was used as a screening test, since results would be available in less time than with the cultural method. PCR-positive results were further evaluated by QRT-PCR. This method can applied to determine other LAB present in the fermented Tabasco mash.

Such bacteria like *Lactobacillus* have developed the enzymatic machinery that allows them to use plant pectins because plant materials are their main habitat. These types of secreted enzymes are salt tolerant and are not susceptible to extreme salt conditions such as fermented pepper mash consisting of 8% salt as the normal fermentation conditions. Knowledge of the mechanisms by which specific microorganisms and their enzymes change foods during fermentation facilitates control of the fermentation process. Further reseach is essential to verify the presence of excellular pectinase enzymes.

5.7 References

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CHAPTER 6

SUMMARY AND FUTURE RECOMMENDATIONS

In summary, pectin methylesterase has grown in importance in the fruit/vegetable industry. The degradation of pectin in fermented pepper mash has become a major cause of concern. PME was isolated from mature red-ripened Tabasco peppers. In the literature, a number of papers stated PME can be purified using the DEAE and Heparin-Sepharose columns. We were able to partially purify the enzyme using DEAE and Heparin-Sepharose columns. The estimated molecular weight of PME was 22 kDa and 36 kDa, which is consistent with the literature for other vegetables. However, we did observe faint contaminating bands on the SDS-PAGE. In the literature, we found that several papers did not include their SDS-PAGE figures where we would be able to compare the purity of our enzyme to those research papers. To further purify the enzyme, using additional methods would require PME Inhibitor (PMEI)-Cyanogen bromide (CNBr)-activated-Sepharose 4B affinity chromatography which is 1-step method. Our study to purify PME extracted from Tabasco pepper will help characterize the enzyme further, to aid in addressing issues of the enzyme activity in the food industry.

The initial rates for Tabasco pepper PME activity were measured at various concentrations of pectin. The Tabasco pepper PME K_M value was 0.23 ±0.05 mg/mL and the maximum rate (V_{Max}) was 48.1 ±3.1 nmoles methanol/min. Our results are in agreement with other studies in that there is an initial stimulatory effect of the salt on the Tabasco pepper PME, as compared to the control. Additionally, the enzyme appears to be inactivated at concentrations of 8% (1.4 M) and above the normal fermentation salt concentrations for Tabasco mash. The inactivation of this enzyme at that concentration is an important step because any concentration less than 8%, PME retains activity. The enzyme lost most of its activity above pH 8.5. At low pH (4.5), the enzyme retained more than 20% activity. The reduction of pH during the primary fermentation stage is an important phase in the fermentation process. During this period, there is a decrease in the pH and an increase in total acidity of the mash thereby inactivating PME

activity. The enzyme exhibited 85% activity at 30°C; moreover, there was a decline in activity at 50°C (65% activity) and higher. Our research revealed the combination of acidic conditions, high salt concentrations, and temperatures above 50°C, PME can be effectively inactivated.

Our findings only add to other documentations of PME activity isolated from other food sources. Although a considerable amount of data have been generated over the past ten years regarding the structure and function of plant PMEs, exciting but difficult challenges remain to be addressed to improve our understanding of these proteins. The hot sauce manufacturers have suggested the cause of pectin degradation in hot sauce is from plant PME; however a recent study showed *Lactabacillus* has the ability to produce and excrete pectinases. The current study was able to detect the presence of lactobacilli in aged fermented pepper mash using DNA signatures. This dissertation supports that *Lactobacillus* species are adaptable organisms that can survive in low pH environments.

Future work should address the further purification of PME using PMEI affinity chromatography method, determination of the pI, confirmation of the molecular weight using mass spectrometry (MS), and protein sequencing of the enzyme to study similarities to other PME enzymes. Also, investigate the specific *Lactobacillus* species that have the potential to excrete and produce pectinases.

APPENDIX A

DATA, TABLES AND FIGURES FOR CHAPTER 3

A
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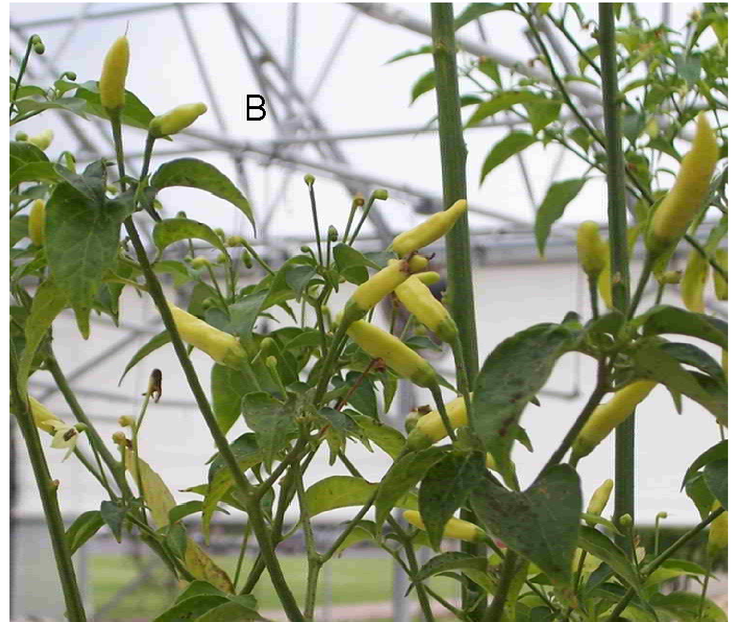
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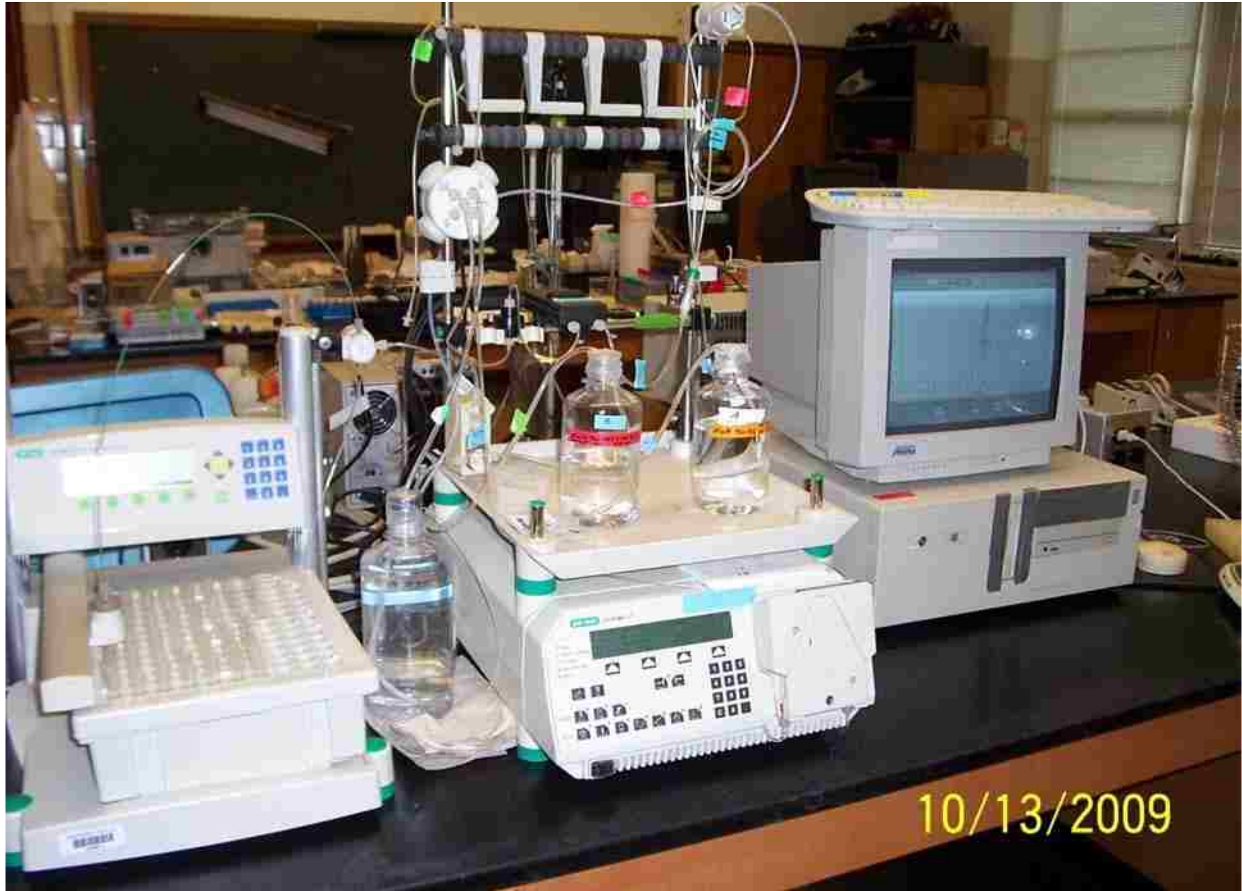


[illegible] 5. BioLogic [illegible]

- Performed using PME-1 from tomato as the primary antibody and PME from Tabasco pepper as the antigen
- PME-1 is specific a 34.5 kDa PME
- A1, A2, C1, and C2 wells coated with PME from tomato using a primary antibody (PME-1) to antigen
- E1, E2, G1, and G2 wells coated with Tabasco PME using a primary antibody (PME-1) to antigen
- The negative control - blocking buffer (1% bovine serum albumin in 0.067 M phosphate-buffered saline, pH 7.25) in wells B1, B2, D1, D2, F1, F2, H1, and H2
- Absorbance read at 414 nm

Figure A6. ELISA

Table A1. Ion-Exchange Purification Program
For analysis, the sample was applied to a Bio-Scale™ Mini Macro-Prep® DEAE (diethylaminoethyl) column (1 mL column; 40 mm length x 5.6 mm inner diameter) (Bio-Rad Laboratories, Inc, Hercules, CA). Buffer A: 25 mM Tris-HCl, pH 7.5 and Buffer B: 25 mM Tris-HCl and 1 M NaCl, pH 7.5.

Chromatography Program

Step	***Buffer***	***Volume***	***Flow rate***
Equilibrate column --5.00 mL Alarm --2.5 mL Collect fractions --inject sample into sample loop	Buffer A	5.00 mL	1.50 mL/min
Wash column	Buffer A	10 mL	1.0 mL/min
Gradient --55.00 mL Alarm --55.00 mL Divert to waste	0-100% B	40 mL	1.50 mL/min
Equilibrate 75.00 mL Alarm <End of program>	Buffer A	20 mL	1.0 mL/min

Table A2. Affinity Purification Program
For analysis, the sample was applied to a HiTrap™ Heparin-Sepharose High Performance column (1 mL column; 0.7 length x 2.5 cm inner diameter) (GE Healthcare, Piscataway, NJ). Buffer A: 25 mM Tris-HCl, pH 7.5 and Buffer B: 25 mM Tris-HCl and 1 M NaCl, pH 7.5.

Chromatography Program

Step	***Buffer***	***Volume***	***Flow rate***
Equilibrate column --5.00 mL Alarm --2.0 mL Collect fractions --inject sample into sample loop	Buffer A	5 mL	1.00 mL/min
Wash column	Buffer A	10 mL	0.5 mL/min
Gradient --55.00 mL Alarm --55.00 mL Divert to waste	0-100% B	40 mL	1.00 mL/min
Equilibrate 75.00 mL Alarm <End of program>	Buffer A	20 mL	1.0 mL/min

APPENDIX B

DATA, TABLES, AND FIGURES FOR CHAPTER 4

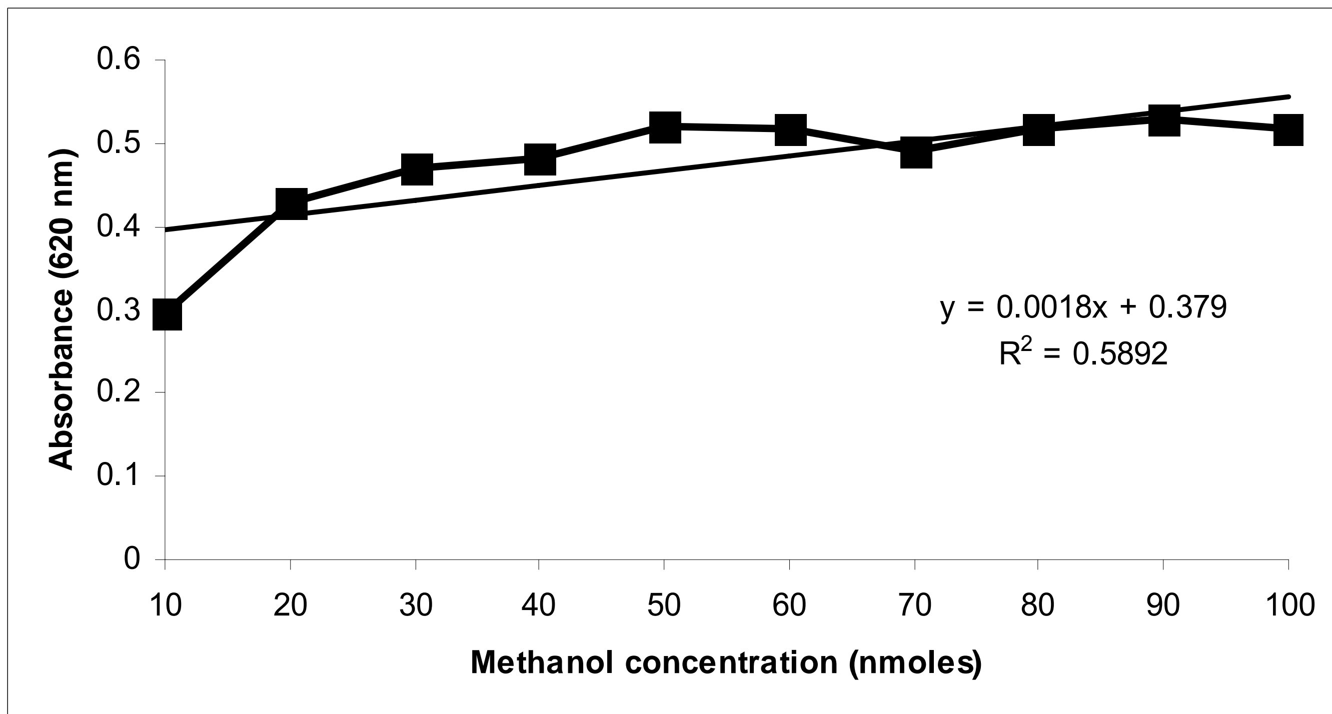


Figure B1. Methanol Standard Curve using concentrations on methanol ranging from 10 to 100 nmoles. Spectrophotometrically read at 620 nm.

Modifications made to PME activity assay Anthon GE and Barett DM from previous published reference:

Anthon GE and Barett DM. 2004. Comparison of three colorimetric reagents in the determination of methanol with alcohol oxidase. Application to the assay of pectin methylesterase. *Journal Agricultural and Food Chemistry* 52:3749-53.

MBTH methanol assay for PME Activity in Tomatoes (rev 1/09)

<u>**Sample preparation:**</u>

Homogenization buffer. 2 M NaCl
0.1 M Na Phosphate (pH 6.5)
(store at room temp.; may crystallize at 4°C)

Desalting buffer. 1:20 dilution of the PME assay buffer (10 mM Tris pH 7.5, 10 mM NaCl)

Procedure:

<u>Sample prep.</u> Exactly weigh about 2 grams of tomato tissue. Add an equal weight of homogenization buffer and grind in a small mortar and pestle. Transfer about 1 mL of homogenate to a 1.5 mL eppendorf centrifuge tube. Centrifuge full speed (16,100 xg) for 5 minutes. Remove a portion of the supernatant to a clean centrifuge tube (take only the cleanest portion of the supernatant, quality is more important than quantity here). If the removed supernatant isn't clean centrifugation it second time.

<u>Desalting.</u> Desalt supernatant on Bio-Rad mini desalting columns. To prepare, spin columns 30 sec at 1,000 xg and discard eluant. Add 0.5 mL desalting buffer and spin again 30 sec at 1,000 xg and discard eluant. Repeat this procedure 5 times. Columns may be prepared in advance and stored in the refrigerator. Just before desalting the sample, add 0.5 mL desalting buffer and spin for 2 minutes at 1,000 xg. Transfer the column to a clean, labeled 1.5 mL tube which has had the cap cut off. Apply 75 µL of the supernatant to the column. Spin 4 minutes at 1,000 xg. The material in the centrifuge tube is the desalted sample. Store on ice.

The desalting columns can be reused and should be washed promptly after use. For this add 0.5 mL of water and spin 30 seconds as before. Repeat at least 5 times.

<u>Alternative sample preparation procedure without desalting.</u> Homogenize the tomato with no additions. Take 1.0 mL of the tomato juice and centrifuge 5 min at 16,000 x g. Wash the pellet twice 5.0 mM acetate buffer (pH 4.5) then resuspended in 1.0 mL of 0.5 M NaCl, 0.1 M Tris-HCl (pH 7.5). Centrifuge again and collect the supernatant.

PME Assay:

Solutions:

(A) 200 mM Tris-HCl (pH 7.5) 4.72 g/L Tris base
25.4 g/L Tris-Cl
200 mM NaCl 11.6 g/L NaCl

(B) 4.0 mg/mL pectin (apple or citrus, dialyzed or passed through Sephadex)
(C) 6 mg/mL MBTH (store at 4C, discard if brown)
(D) Alcohol oxidase (in freezer, at concentration indicated)
(E) 5 g/L Ferric Ammonium Sulfate
5 g/L Sulfamic Acid (dissolve first)
(F) 3 mM Methanol standard

For 20 assays mix: 1 mL "A", 0.7 mL "B", 0.2 mL "C", and 5 U of AO (about 5 uL, depending on the particular batch)

For each assay take 95 uL of the assay mix then start the assay by adding 5 uL of the tomato extract. If activity is high (such as in an untreated raw tomato), the extract will need to be diluted many fold (try 50-fold) before assaying. The mixture is incubated at 30°C, then add to 0.1 mL of a solution "E"after 20 min (if activity is low this time may be increased to many hours). If activity is present the samples should turn blue. After 30 minutes at room temperature add 0.8 mL of water and read absorbance at 620 nm.

Run a blank (5 uL of buffer instead of sample) and a standard (5 uL of 3 mM methanol, equal to 15 nmoles of methanol).

Note: Phosphate buffers are incompatible with this assay.

APPENDIX C

DATA, TABLES, AND FIGURES FOR CHAPTER 5

Key Terms:
Ethidium bromide: is an intercalating dye. The planar molecule fits with high affinity between adjacent base pairs in the double helix of dsDNA. It is a popular fluorescent stain that is used for routine detection of nucleic acids in electrophoresis gels.

Melt curve: in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases.

Melting temperature (*T_m*): the temperature at which 50% of the DNA remains hybridized. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the T_m.

Mis-priming: PCR products made due to annealing of the primers to complementary or partially complementary sequences on non-target DNAs.

Primer-dimer artifacts: the primers can sometimes anneal to themselves and create small templates for PCR amplification.

SYBR Green: common intercalating dye that binds highly preferentially to dsDNA (not ssDNA), and exhibits strong fluorescence when bound and nearly zero fluorescence when unbound. Excitation wavelength: 498 nm and emission wavelength: 520 nm.

Prepare Gram positive bacterial cell lysate as described below.

1. Set two water baths or heat blocks at 37ºC and 55°C, respectively.
2. Prepare Lysozyme Digestion Buffer (supplied with the kit). To ~200 µL; (Lysozyme Digestion Buffer/sample, add fresh Lysozyme to obtain a final Lysozyme concentration of 20 mg/ml).
3. Harvest up to 2×10^9 Gram positive cells by centrifugation. If using a frozen cell pellet, proceed to Step 3.
4. Resuspend the cell pellet in 180 µL Lysozyme Digestion Buffer containing Lysozyme from Step 2. Mix well by brief vortexing.
5. Incubate at 37ºC for 30 min.
6. Add 20 µL Proteinase K and 20 µL RNase (supplied with the kit). Mix well by brief vortexing.
7. Add 200 µL PureLink™ Genomic Lysis/Binding Buffer and mix well by brief vortexing.
8. Incubate at 55°C for 30 min.
9. Add 200 µL 100% ethanol to the lysate. Mix well by vortexing for 5 s to yield a homogenous solution.
10. Remove a PureLink™ Spin Column in a Collection Tube from the package.
11. Add the lysate (~640 µL) prepared with PureLink™ Genomic Lysis/Binding Buffer and ethanol to the PureLink™ Spin Column.
12. Centrifuge the column at 10,000 × g for 6 min at room temperature.
13. Discard the collection tube and place the spin column into a clean PureLink™ Collection Tube supplied with the kit.
14. Add 500 µL Wash Buffer 1 prepared with ethanol to the column.
15. Discard the collection tube and place the spin column into a clean PureLink™ collection tube supplied with the kit.

16. Add 500 µL Wash Buffer 2 prepared with ethanol to the column.
17. Centrifuge the column at maximum speed for 4 min at room temperature. Discard collection tube.
18. Place the spin column in a sterile 1.5-mL microcentrifuge tube.
19. Add 100 µL of PureLink™ Genomic Elution Buffer to the column.
20. Incubate at room temperature for 1 minute. Centrifuge the column at 14,000 *x g* speed for 3 min
21. For storage, the purified DNA in PureLink™ Genomic Elution Buffer was store at -20°C as DNA stored in water is subject to acid hydrolysis.

Prepare Gram negative bacterial cell lysate as described below.

1. Set a water bath or heat block at 55°C.
2. Harvest up to 2 x 10^9 Gram negative (~1 ml of overnight *E. coli* culture) by centrifugation. If using a frozen cell pellet, proceed to Step 3.
3. Resuspend the cell pellet in 180 µl PureLink™ Genomic Digestion Buffer (supplied with the kit). Add 20 µL Proteinase K (supplied with the kit) to lyse the cells. Mix well by brief vortexing.
4. Incubate the tube at 55°C with occasional vortexing until lysis is complete (30 min).
5. Add 20 µL RNase A (supplied with the kit) to the lysate, mix well by brief vortexing, and incubate at room temperature for 2 min.
6. Add 200 µl µL PureLink™ Genomic Lysis/Binding Buffer and mix well by vortexing to obtain a homogenous solution.
7. Add 200 µL 100% ethanol to the lysate. Mix well by vortexing for 5 seconds to yield a homogenous solution.
8. Remove a PureLink™ Spin Column in a Collection Tube from the package.
9. Add the lysate (~640 µL) prepared with PureLink™ Genomic Lysis/Binding Buffer and ethanol to the PureLink™ Spin Column.
10. Centrifuge the column at 10,000 × g for 1 min at room temperature.
11. Discard the collection tube and place the spin column into a clean PureLink™ Collection Tube supplied with the kit.
12. Add 500 µL Wash Buffer 1 prepared with ethanol to the column.
13. Discard the collection tube and place the spin column into a clean PureLink™ collection tube supplied with the kit.
14. Add 500 µL Wash Buffer 2 prepared with ethanol to the column.
15. Centrifuge the column at maximum speed for 3 min at room temperature. Discard collection tube.
16. Place the spin column in a sterile 1.5-mL microcentrifuge tube.
17. Add 100 µL of PureLink™ Genomic Elution Buffer to the column.
18. Incubate at room temperature for 1 minute. Centrifuge the column at 14,000 *x g* speed for 1 min
19. For storage, the purified DNA in PureLink™ Genomic Elution Buffer was store at -20°C as DNA stored in water is subject to acid hydrolysis.

10 x TBE Buffer

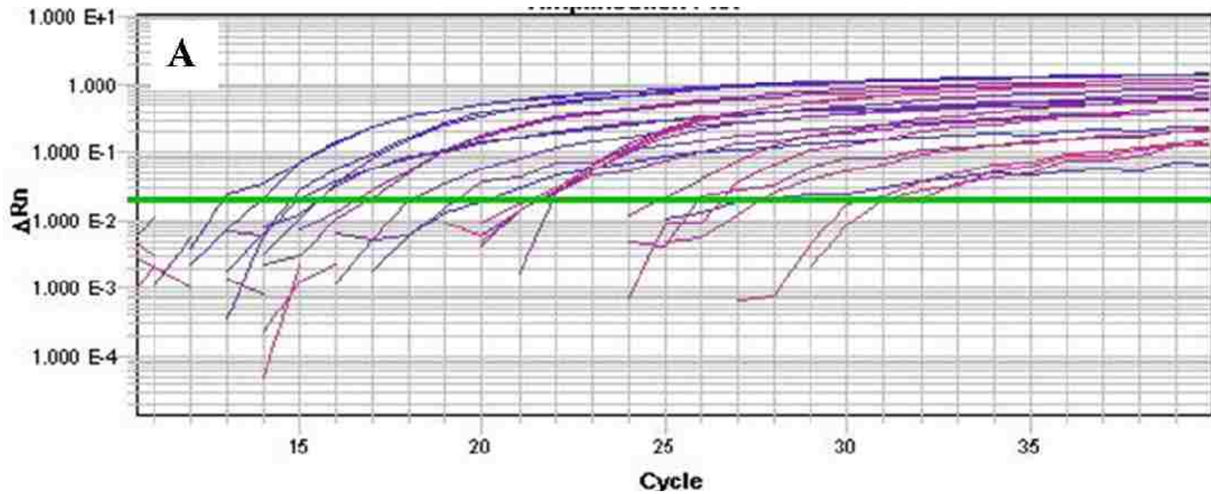
1. Dissolve 108 g Tris base and 55 g boric acid in 900 mL Milli-Q water
2. Add 40 mL 0.5 M Na_2EDTA (pH 8.0) and make up to 1 L
3. Store at room temperature

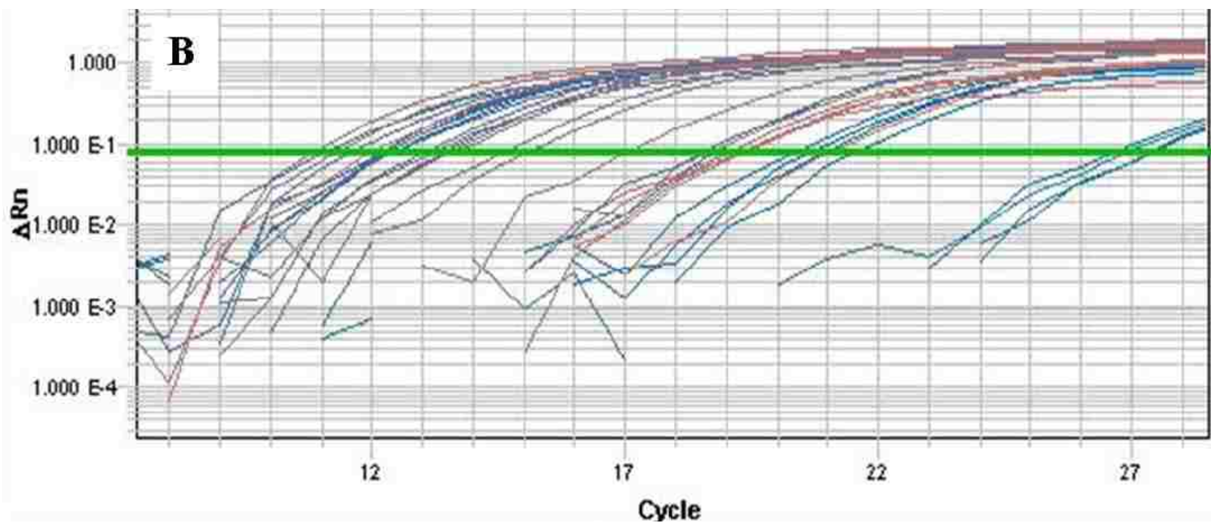
1 x TBE Buffer

1. Add 100 mL 10x TBE buffer in 900 mL Milli-Q water
2. Store at 4°C for up to seven days

20 mM Phosphate buffered saline

1. Dissolve 2.84 g sodium phosphate (dibasic) and 2.4 g sodium phosphate (monobasic)
2. Add 8.5 g of sodium chloride (0.85%)
3. Adjust pH to 7.2
4. Make up to 1 L in Milli-Q water
5. Autoclave to sterilize
6. Store at room temperature





VITA

Sonja T. Jones was born in August, 1977 along with her twin sister in Chicago, Illinois. She was raised in St. Tammany Parish in Louisiana. She earned her Bachelor of Science degree in microbiology in 2000 from Mississippi University for Women, Columbus, Mississippi. While pursuing her undergraduate degree, she was enlisted in the United States Army Reserves attached to the 4010th US Army Hospital in New Orleans, Louisiana. She then received her Master of Science degree in veterinary medical science in 2003 for Mississippi State University, Starkville, Mississippi. She joined as a doctoral student in the Department of Food Science at Louisiana State University, Baton Rouge, Louisiana, in the fall of 2003 and completed a Master of Public Administration in 2007.