PROGRESS TOWARDS DETERMINING THE STRUCTURE OF THE

SMALLEST ACTIVE PECTIN THAT INDUCES APOPTOSIS *IN VITRO*

By

CRYSTAL LYLES JACKSON

(Under the Direction of Debra Mohnen)

ABSTRACT

Pectin has been shown to reduce the incidence of prostate cancer (PC) growth and metastasis in animal and human studies and to induce apoptosis in human cultures *in vitro*. Due to pectin's complex structural heterogeneity, however, human PC cells respond in at least two, and likely more, distinct ways to specific types of pectins, indicating that more than one active pectin structure affects prostate cancer cell growth and longevity. To effectively utilize pectin as a reservoir of potential cancer-reducing agents, rigorous attention to the unique pectin structure(s) that reduces PC occurrence and longevity is required. We have identified a heat-modified citrus pectin (HTCP) that induces apoptosis in human prostate cancer cells *in vitro*.The identification and purification of the specific structure, in heat-treated citrus pectin (HTCP), that possesses this cancer cell-inhibiting and apoptosis-inducing ability is the subject of this dissertation.

INDEX WORDS: pectin, prostate cancer, apoptosis, structure, heat treat

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CRYSTAL LYLES JACKSON

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CRYSTAL LYLES JACKSON

Major Professor: Debra Mohnen

Committee: Lianchun Wang
      Alan Darvill

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this Doctoral dissertation to my parents for their unwavering support, profound wisdom and incessant encouragement and for devoting themselves to my purpose.

ACKNOWLEDGEMENTS

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INTRODUCTION

Scope of Study

Approximately 140 American men in every 100,000 will develop prostate cancer each year, making it the most common malignancy in men in the United States (U.S. Cancer Statistics Working Group. *United States Cancer Statistics: 1999–2009 Incidence and Mortality Web-based Report.* Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute; 2013). Prostate cancer is the second leading cause of death from cancer in American men (American Cancer Society Cancer Facts and Figures 2010). One in every six American men will likely develop prostate cancer in their lifetime (http://surveillance.cancer.gov/devcan/). Once the cancer has metastasized, the five year relative survival rate drops to less than 30% (Howlander *et al*. 2011). The goal of many cancer therapies, such as antihormonetherapy and chemotherapy, is to induce apoptosis in tumor cells. Androgen deprivation therapies induce cell death in androgen-sensitive cells (Colombel and Buttyan, 1995 *et al.*, 1999; Perlman *et al*. 1999) Bruckheimer, whereas androgen-insensitive cells remain unaffected (Kozlowski *et al.*, 1991; Santen, 1992; Kreis, 1995). Furthermore, because of the heterogenous population of prostate cancer cells, castration resistant prostate cancer (CRPC) often emerges (Rini *et al.* 2006; Small *et al*. 2006; De Bono *et al.* 2008) even after androgen ablation by surgical removal of the male gonads or treatment with androgen receptor antagonists. In such cases and with such treatment, the median increase in survival is only 4 months with combination docetaxel chemotherapy (Petrylak, *et al*., 2004). Thus, the identification of novel methods to induce apoptosis in prostate cancer cells irrespective of their

androgen response has significant therapeutic value. **We have demonstrated that pectin, a plant polysaccharide, induces apoptosis in both androgen-responsive and androgen-independent prostate cancer cells (Jackson *et al.* 2007). This is the first extensive analysis that attempts to identify fine structural features of pectin with apoptosis-inducing activity in cancer cells.**

The cancer inhibitory effects of modified pectin

Both the American Institute of Cancer Research and the World Cancer Research Fund estimate that 30–45% of worldwide cancer cases are preventable by dietary means. Pectin is a natural complex plant polysaccharide present in all higher plant primary cell walls (30%; $\leq$10% grasses) and, consequently, is a dietary component of all fruits and vegetables. Pectin has multiple roles in plant growth, development, and disease resistance (Ridley *et al.,* 2001) and is used as a gelling and stabilizing agent in the food industry (Thakur, *et al.*, 1997). Pectin has been shown to suppress colonic tumor incidence in rats (Heitman, *et al. 1992)* and inhibit cancer cell metastasis in mice and rats (Platt and Raz, 1992; Pienta *et al.*, 1995; Nangia-Makker *et al.*, 2002) and prior studies have shown that pectin binds to B16-F1 melanoma cells *in vitro* (Platt and Raz, 1992). Relatively large commercial pectin injected intravenously into mice increased homotypic cell–cell aggregation and metastasis to the lung while pH-modified, relatively small pectin inhibited lung metastasis (Platt and Raz, 1992), demonstrating a differential response depending upon the type of pectin used. Peinta *et al.* (1995) showed that oral administration of a pH-modified citrus pectin (CP) significantly reduced metastasis of rat prostate adenocarcinoma MATLyLu to the lung. It has been hypothesized that pectins antagonize β-galactoside-binding proteins like galectin-3 by binding to these cell surface galectins (galactose-binding lectins) and interfering thereby with cell–cell or cell–matrix adhesion, inhibiting metastatic lesions (Inohara

and Raz, 1994). While animal and cell studies are very encouraging, there is little information available about the efficacy of modified citrus pectins in humans. In one published clinical trial, 10 men with prostate cancer were treated with a pH-modified citrus pectin after standard treatment failed. While the study lacked a control group that did not receive the modified pectin, 7 of 10 men in the pectin-treated group had blood tests that were positive for prostate-specific antigen (PSA), a controversial but commonly utilized marker of prostate cancer growth that is present at low levels in healthy men. Despite considerable PSA levels, the PSA doubling time (a measure of how fast PSA goes up) improved in comparison with measurements done before taking MCP, indicating that MCP may slow prostate cancer growth (*Strum et al*., 1999).

Several studies have indicated that induce apoptosis in cancer cells. Azoxymethane-injected rats fed a citrus pectin or fish oil/pectin diet had a greater number of apoptotic cells per colon crypt column compared with rats fed corn oil and/or cellulose (Chang *et al.* 1997*)*. In addition, pectin/fish oil-fed rats had a lower incidence of adenocarcinoma (51.5%) than animals fed cellulose/corn oil (75.6%) (Chang *et al.* 1997; Chang *et al.* 1997). This was accompanied by reduced expression of the anti-apoptotic protein Bcl-2 and activation of caspase-1 and poly (ADP-ribose) polymerase (PARP), substrates of caspases (Avivi-Green *et al*. 2000; Avivi-Green *et al*. 2000b). Kossoy *et al*. (2001) showed that the administration of a pectin-rich 15% orange-pulp diet to dimethylhydrazine injected Sprague-Dawley rats resulted in a decreased number of endophytic tumors, an activation of caspase-3, and an increased activity of T-cell killers in the tumors. In human colon adenocarcinoma HT29 cells, caspase-3 activity was significantly higher in cells treated with 10 mg/mL low-methylated apple pectin (Olano-Martin *et al.*, 2003). Preclinical studies using a modified a citrus pectin, GCS-100, showed induction of apoptosis in human multiple myeloma cell lines that are resistant to conventional and bortezomib therapies

(Chauhan *et al*., 2005). While GCS-100 did not alter normal lymphocyte cell viability, in the myeloma lines it induced DNA fragmentation and the activation of caspase-8, caspase-3, and PARP. Interestingly, the GCS-100 also inhibited the growth of multiple myeloma cells directly purified from patients who had relapsed following multiple therapies with multiple chemotherapeutics (Chauhan *et al*., 2005), providing evidence that GCS-100 can induce apoptosis in chemo-resistant myeloma cells. Each of these oral pectic treatments was administered without any characterization of the pectic structure and without addressing the rate of absorption in the human gastrointestinal tract. An incomplete understanding of either could greatly limit the effectiveness of oral pectic treatments *in vivo*. Thus, purifying a specific active structure that is minimally processed in the gastrointestinal tract or that could be encapsulated and time-released could deliver a greater cancer payload than a heterogenous mixture wherein the active component represents a small fraction of the total pectin.

Taken together, these results suggest that exposure of malignant cells to pectin induces apoptosis and reduces tumor growth in multiple cancer types. While our focus is on the identification of the smallest pectin that induces apoptosis in human prostate cancer, it is possible that there are multiple anti-cancer pectic structures that are active against multiple cancer types.

SPECIFIC AIMS OF PROJECT

The main goal of this body of work was to determine the smallest pectin structure in heat modified citrus pectin (HTCP) that induces apoptosis in human prostate cancer cells *in vitro*. Multiple purification strategies were used to isolate the smallest pectic structure that retained the ability to induce apoptosis in human prostate cancer cell lines and to purify and characterize the apoptotic pectin. These studies also addressed how heat treatment of citrus pectin caused an increase in the amount of pectic apoptotic activity. **Purification strategy:** HTCP was generated from citrus pectin and human prostate cancer cells treated as described (Jackson *et al*., 2007). To identify the smallest active pectin that elicited an apoptotic response in human prostate cancer cells (i.e. LNCaP cells), the starting HTCP was enzymatically-digested with purified pectin methylesterase (PME) and endopolygalacturonase (EPG) from *Aspergillus niger* which we previously showed results in a fragmented pectin but does not destroy the apoptotic activity (Jackson *et al*., 2007). The resulting fragmented HTCP was subjected to additional enzymatic digests to remove glycan sidechains and to further fragment the backbone of the respective pectic substructures. Fractions that tested positive for uronic acid using an uronic acid assay developed by Blumenkrantz et al. (1973) were assayed for apoptotic activity. The glycosyl residue composition and linkage of the active fraction(s) was determined to reveal the smallest structure that retains the apoptosis-inducing activity.

Impact: A therapeutic that specifically kills human prostate cancer cells would have a dramatic impact on human prostate cancer occurrence and lethality in both recently diagnosed and preexisting cancer patients. Furthermore, the identification of the apoptotic pectin would allow assays for detecting its presence in any commercial pectin claiming to

contain the apoptotic activity. The need for this is demonstrated by the batch to batch variation that we have found in commercial pectin used as starting sources for generating pectins that contain both PC apoptosis-inducing activity and anti-metastatic activity. The complexity of pectin itself clearly demonstrates the need for a structure/function level understanding of a pectic activity that reduces PC (prostate cancer).

CHAPTER 1

PROGRESS TOWARDS DETERMINING THE STRUCTURE OF THE SMALLEST ACTIVE

PECTIN THAT CAN INDUCE APOPTOSIS IN HUMAN PROSTATE CANCER CELLS *IN*

VITRO[1]

[1]Crystal L. Jackson[A,B], Natalia Suarez[B], Manal Eid[C], Vijay Kumar[C], Debra Mohnen[A,B]
[A]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, GA 30602-4712
[B]Department of Biochemistry and Molecular Biology, UGA Cancer Center, The University of Georgia, Athens, GA 30602
[C]Medical College of Georgia and VA Medical Center, Augusta, GA 30912
To be submitted

Abstract

Pectin has been shown to reduce the incidence of prostate cancer (PC) growth and metastasis in animal and human studies (Glinsky and Raz 2009) and to induce apoptosis in human cultures *in vitro* (Jackson *et al* 2007). Due to pectin's complex structural heterogeneity, however, human PC cells respond in at least two, and likely more, distinct ways to specific types of pectins (Glinsky and Raz 2009) (Jackson *et al* 2007), indicating that more than one active pectin structure affects prostate cancer cell growth and longevity. To effectively utilize pectin as a reservoir of potential cancer-reducing agents, rigorous attention to the unique pectin structure(s) that reduces PC occurrence and longevity is required. We have identified a heat-modified citrus pectin (HTCP) that induces apoptosis in human prostate cancer cells *in vitro* (Jackson *et al* 2007). Purification efforts to identify the specific structure in heat-treated citrus pectin (HTCP) that has apoptotic activity have revealed that there are fine structural differences in apoptotically active and inactive pectin that confer its apoptosis-inducing activity. It appears that the smallest active pectin, HTCP treated with PME and EPG (HPE), remains cross-linked when viewed by OGA-PAGE and that this activity is lost when the pectin is hydrolyzed into mostly discretely-sized OGAs upon mild base deesterification. Furthermore, when separated over an anion exchange column, pooled uronic acid positive material in the PME+EPG treated HTCP (HPE) is still apoptotically-active, but this activity on a mg/mL dry weight (DW) basis is greatly reduced compared to the unfractionated pectin. The fine structural characteristics of the apoptotic pectins: heat-modified and commercially available FPP, heat modified pectin created in-house HTCP, and heat-treated citrus pectin that is hydrolyzed by treatment with endopolygalacturonase and pectinmethylesterase (HPE) were analyzed by glycosyl residue and linkage analyses and compared to inactive and unmodified CP (citrus pectin) and pH modified

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pectasol (PeS). Composition analysis reveals that galacturonic acid (GalA) was the major component in all pectins tested and that generally there is no correlation found between the apoptosis-inducing activity of FPP, HTCP and HPE and their glycosyl residue composition compared to inactive unmodified CP and pH-modified PeS, suggesting that fine structural differences are responsible for the apoptotic activity. To determine if RG-I, arabinan, galactan or arabinogalactan side branches of RG-I were important for the apoptosis-inducing activity of pectin, the smallest active pectin HPE was further digested with enzymes to degrade the RG-I backbone and side branches. Apoptosis analysis of the digested pectin revealed that terminal galactose residues in the galactan side branches of RG-I and/or a linkages involving terminal galactose residues in the arabinogalactan side chains of RG-I as well as the RG-I backbone appear to be important for apoptotic activity. In addition, there is limited evidence for a protein component associated with the apoptotic moeity; however this structure is also present in the inactive starting "mother" pectin, CP. Thus the possibility remains that a specific glycan moiety on a protein-containing pectic structure that is present in FPP and HTCP may be required for apoptotic activity.

1. Background

The structural complexity of the plant-derived cell wall polysaccharide pectin

The elucidation of the mechanism(s) of action of pectin and the nature of pectin's anticancer activity is complicated by (i) the structural complexity of this plant-derived cell wall polysaccharide, (ii) the modifications in pectin structure resulting from the process of its extraction from plants, and (iii) the additional modifications of pectin structure that result from the diverse fragmentation techniques used to produce specialized pectins e.g. high-pH (base) treatment (Platt and Raz, 1992; Pienta *et al*., 1995; Nangia-Makker *et al.*, 2002; Eliaz 2001). When pectin is isolated from the primary plant cell wall, a diverse mixture of enzymes is used, often including pectinases like polygalacturonases (Pattathail et al, 2010), along with mild base deesterification (Figure 1.1). The resulting carbohydrate chains consist of homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Albershiem et al., 1996; Ridley et al. 2001) (Figure 1.2).

Xylogalacturonan and HG have a (1-4)-α−D-Gal*p*A backbone that is partially methyl esterified at the C6 position. HG can also have acetyl esters at the O2 and/or O3 position (Ishii,, 1997; Komalavilas and Mort, 1989; Lerouge *et* al., 1993). In addition to an α−D-1,4-linked GalA backbone, XGA is substituted sporadically at the 3-position with β-D-Xyl*p* (Schols *et al.,* 1995). RG-II is a substituted galacturonan that accounts for 10–11% of pectin with a complex structure that is highly conserved across plant species. RG-II has an HG backbone of 8-10 GalA (Ishii and Matsunga 2001) residues with four side branches of complex structure, consisting of 12 different types of sugars connected by more than 20 different linkages (O'Neill et al., 1996; O'Neill et al., 2004). RGI polymers are repeats of -2)-α−L-Rha*p*-(1-4)-α−D-Gal*p*A-(1- that are branched at the O4 of rhamnose (Lau et al. 1985; McNeil et al., 1980). The side chains fall into several structural
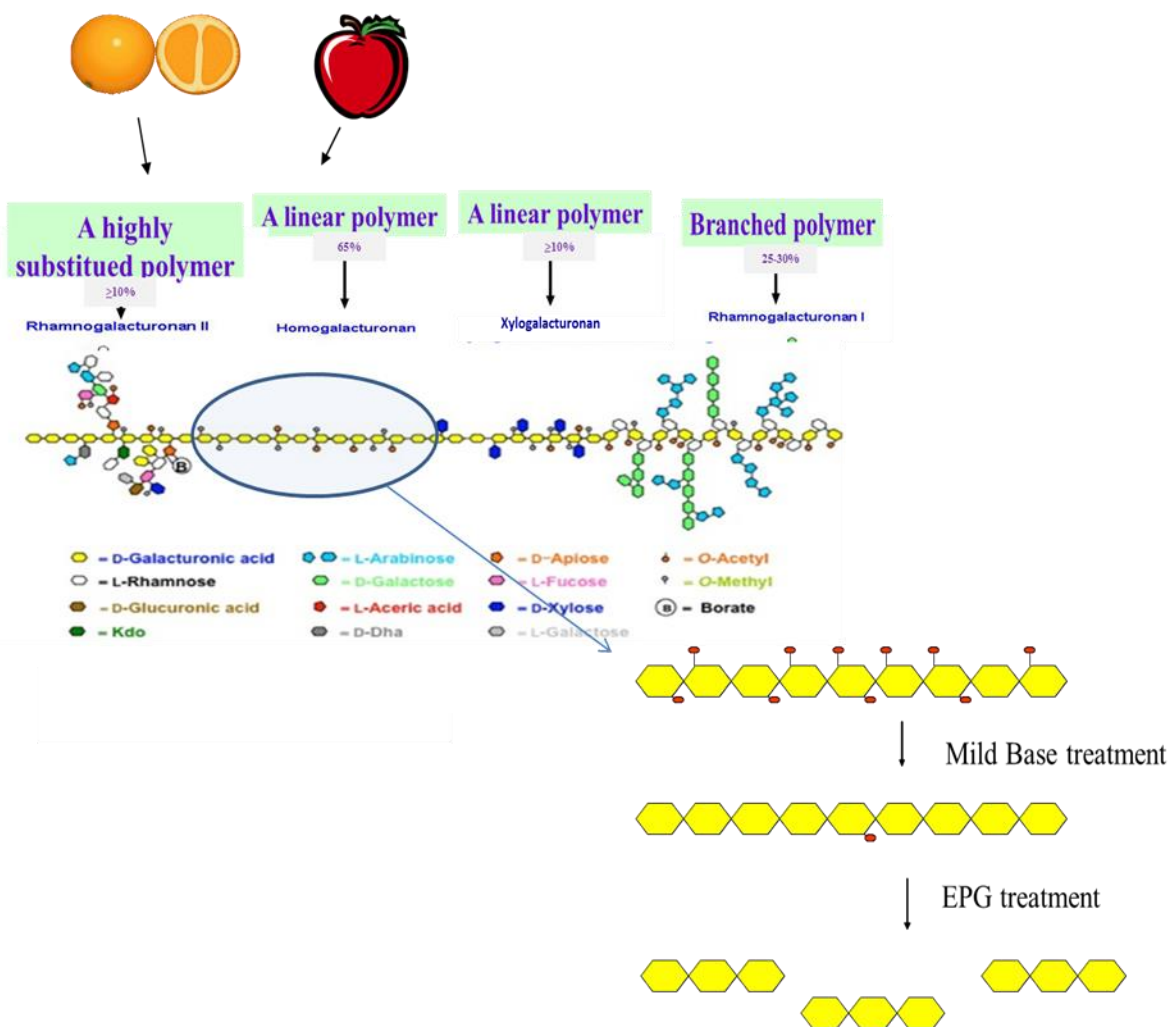
Figure 1.1. Isolation of pectic polysacharrides from the plant cell wall. When pectin is isolated from the primary plant cell wall, a diverse mixture of enzymes is used, often including pectinases like endopolygalacturonases (EPG), along with mild base deesterifiation. Modified from Harholt *et al*. 2010.
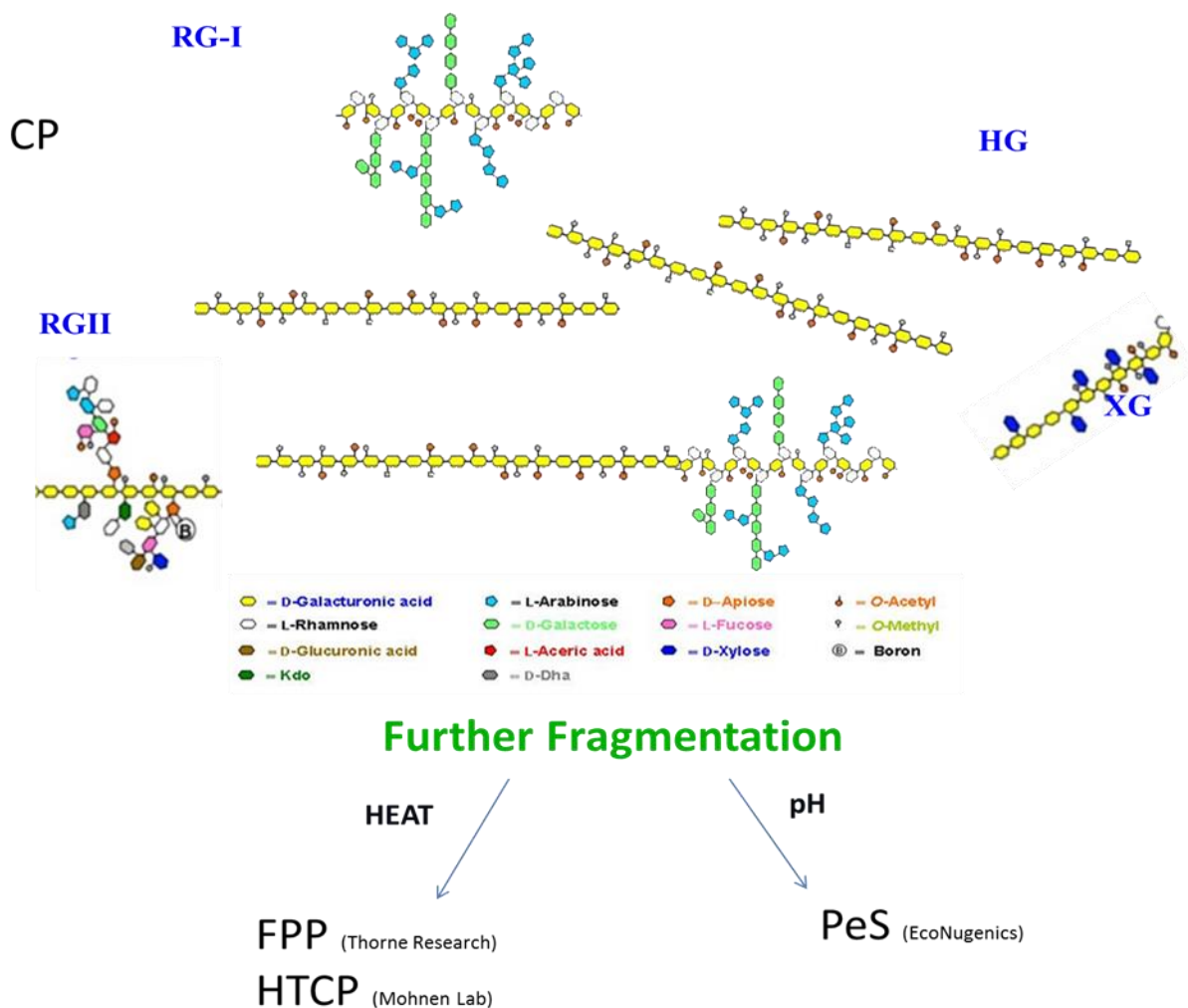
Figure 1.2. Pectic polysaccharides isolated from plant cell walls and their fragmentation to yield modified pectin. RG-II, RG-I, HG, and XG are the polysaccharides released by chemical and enzymatic methods when pectin is isolated from the cell wall. Further fragmentation by heat or pH yield biologically active modified citrus pectin. Modified from Harholt *et al*. 2010. CP, citrus pectin.

categories including type I arabinogalactans (AGI), linear $\beta-(1\text{-}4)$-galactans with single units of $\alpha-$L-Ara$f$ at O3 (Clarke *et al.*, 1979) and linear or branched arabinans consisting of $\alpha-$L-(1,5)- L-Ara$f$ with 1,2 and/or 1,3-linked $\alpha-$L-arabinosyl side chains at O2 or O3. Interestingly, arabinogalactan proteins (AGPs) that have $\beta-(1-3)$ and $\beta-(1-6)$ galactan-rich glycans (arabinogalactan type II, AGII) often co-elute with RG-I (Vincken et al. 2003) and Immerzeel et al. (2006) have suggested that AGPs may be covalently linked to carrot pectin. More recently, Tan *et al* (2013) have identified an AGP with covalently linked pectin and xylan in *Arabidopsis* and have named this proteoglycan arabinoxylan pectin arabinogalactan protein 1 (APAP-1). The author also reported that 50% or more of RG-I isolated from *Arabidopsis* suspension culture cells appears to be covalently linked to the same protein core as in APAP1. AGPs are members of the large family of hydroxyproline rich glycoproteins (HRGPs) (Zhang et al., 2003) in plants, and consist of more than 90% AGII (Majewska-Sawka and Nothnagel, 2000; Nothnagel, 1997). The AGII in AGPs is linked to the protein core as $\beta-(1-3)$ linked galactan with $\beta-(1\text{-}6)$-Gal$p$ side chains, terminated by $\alpha-$L-Ara$f$ at O6 of the galactan backbone, and in some cases with GlcA and Rha (Tan *et al.*2012). After pectin is isolated from the plant cell wall, it is often subjected to further fragmentation by heat and/or acid/base treatment to yield biologically-active pectic structures. The identification and purification of the specific structure, in heat-treated citrus pectin (HTCP), that possesses cancer-inhibiting and apoptosis-inducing ability is the subject of the research presented here.

Heat modification of citrus pectin generates apoptosis inducing activity

As discussed above pectin has been shown to suppress tumor incidence in rats (Heitman *et al*. 1992) and inhibit cancer cell metastasis in mice and rats (Platt and Raz 1992; Pienta *et al*

1995; Nangia-Makker *et al*. 2002). Additionally, in a previous study, large commercial pectin increased homotypic cell–cell aggregation and metastasis to the lung while small, pH-modified pectin inhibited lung metastasis (Platt and Raz 1992). Thus, the cancer-inhibiting effect of pectin differs depending upon the type of pectin used. The pectin used in prior studies was derived from modifications of commercially available material. The structure of commercially available pectin differs depending on the source and method of preparation. Therefore, to examine whether pectins prepared using different extraction protocols have similar biological effects, in our initial studies we treated prostate cancer cells with several commercially available pectin preparations: an unmodified citrus pectin (CP), a pH-modified pectin named Pectasol (PeS), and a heat modified pectin named fractionated pectin powder (FPP). CP represents the starting pectin (i.e. "mother pectin") used to make the modified pectins. PeS is a pH-modified pectin generated by base treatment of CP. PeS is similar to the pH-modified pectins used in some of the previously published studies reporting anti-cancer effects of pectin (Platt and Raz 1992; Pienta *et al* 1995; Chauhan *et al*. 2005). FPP represents another type of commercial pectin generated by heat treatment of CP at 100–132°C for 20 min to 5.5 h. When each of these pectic preparations were incubated with androgen-sensitive and androgen-insensitive lymph node-derived human prostate cancer cells (LNCaP and LNCaP C4-2, respectively) we found that only the heat modified pectin, FPP, had significant apoptosis-inducing activity. Cells incubated in media devoid of pectin served as a negative control and cells treated with thapsigargin, a compound that induces apoptosis by creating endoplasmic reticulum stress and raising cytosolic calcium concentration, served as a positive control. Figure 1.3 shows that, among the pectins tested, FPP induced significant apoptosis in both LNCaP (Figure 1.3A) and LNCaP C4-2 (Figure 1.3B) cells, whereas PeS and CP induced little or no apoptosis.

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As expected the positive control thapsigargin also induced significant apoptosis. In these experiments, apoptosis was quantified using the M30 Apoptosense assay (Peviva), an enzyme-linked immunosorbent assay (ELISA) that measures the presence of an apoptosis-specific neoepitope of cytokeratin-18, that is produced during apoptosis by the activation of caspase. Western blots identifying the presence of activated caspases in the cell lysates of FPP and thapsigargin treatments and the absence of these apoptosis products in the other treatments (Figure 1.4) supported the Apoptosense ELISA data. In an effort to prove that the activity was pectin-related and not due to an artifact that was introduced during commercial preparation, we attempted to create a heat-fractionated pectin by heat treating citrus pectin. Apoptosis analysis of our heat-treated citrus pectin (HTCP) showed that it was as effective as the commercially prepared FPP in inducing apoptosis (Figure 1.3C). HTCP was produced as in Jackson *et al.* (2007) by heating a 0.1% aqueous citrus pectin dispersion at 123.3°C at 17.2-21.7 psi for 60 min.

Linkages other than carboxy-methyl esters play a dominant role in the apoptosis-inducing activity

When pectic polysaccharides are isolated and purified from the plant cell wall, they are often treated with mild base to remove ester linkages and with the enzyme endopolygalacturonase (EPG) to release the individual pectic polysaccharides and oligosaccharides. The initial deesterification removes linkages such as methyl esters on the GalA residues in HG making the pectin more accessible to enzymes like EPG. To determine the effects of these treatments on the apoptotic pectin, we subjected heat modified pectin to both mild base deesterification and EPG cleavage. EPG cleavage alone, before ester removal resulted in only a slight reduction of apoptotic activity, however mild base deesterification completely abolished

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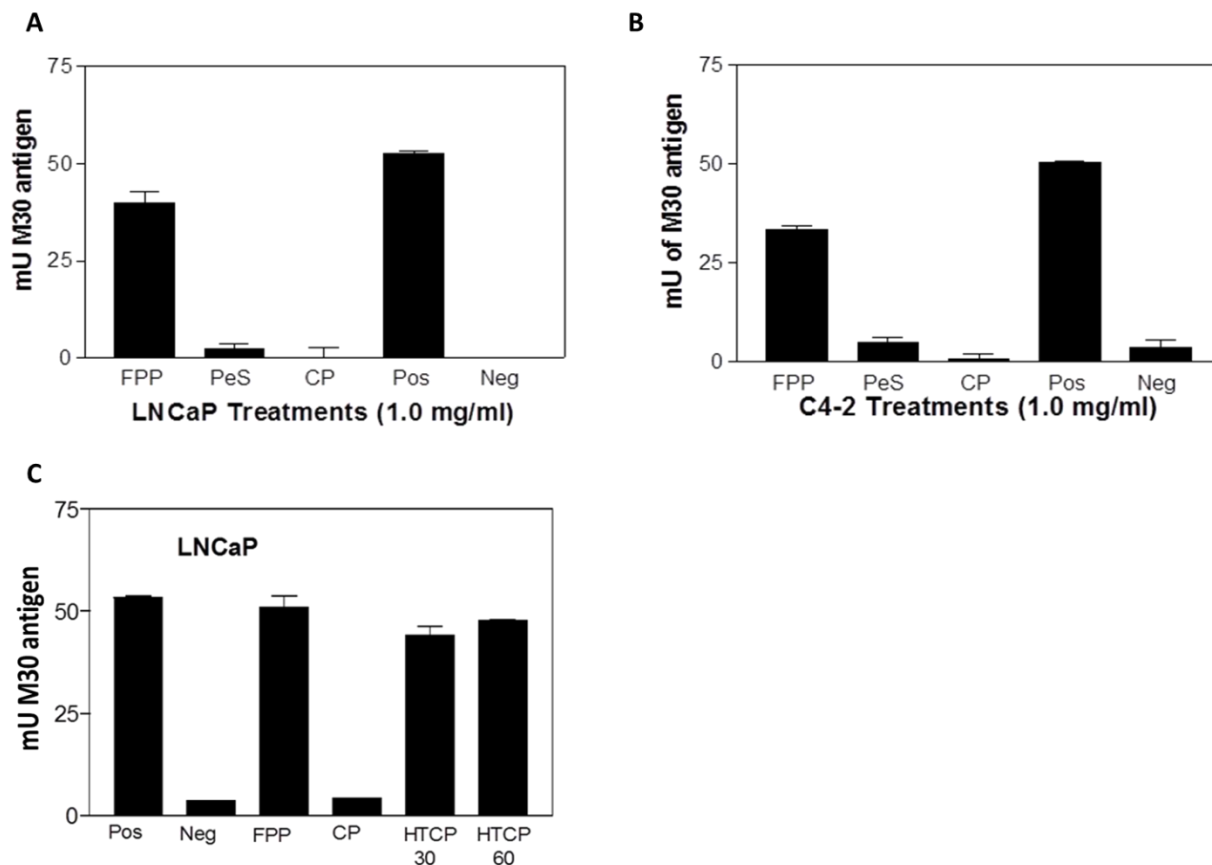
Figure 1.3. Induction of apoptosis by FPP and HTCP. **(A)** LNCaP cells were treated with 1 mg/mL FPP, PeS, CP, or with 0.01 mM thapsigargin (Pos) for 48 h. Incubation with media alone served as the negative control (Neg). Equal amounts of cell extracts (12 ug protein) were used to measure apoptosis. Apoptosis was assayed using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts+SEM. Comparable results were obtained in at least two experiments. **(B)** Induction of apoptosis in LNCaP C4-2 cells. See (A) above for details. **(C)** Comparison of apoptotsis-inducing activity of FPP with HTCP. LNCaP cells were treated for 48 h with 0.01 mM thapsigargin (Pos) or with 1 mg/mL FPP, CP, or HTCP (generated by either 30 (HTCP30) or 60 (HTCP 60) minute heat treatment of CP). Incubation with media alone served as the negative control (Neg). Equal amounts of cell extracts (12 ug protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay as described above. Data are the average of duplicate apoptosis assays of duplicate cell extracts+SEM. Comparable results were obtained in at least two experiments. Modified from Jackson *et al* (2007).
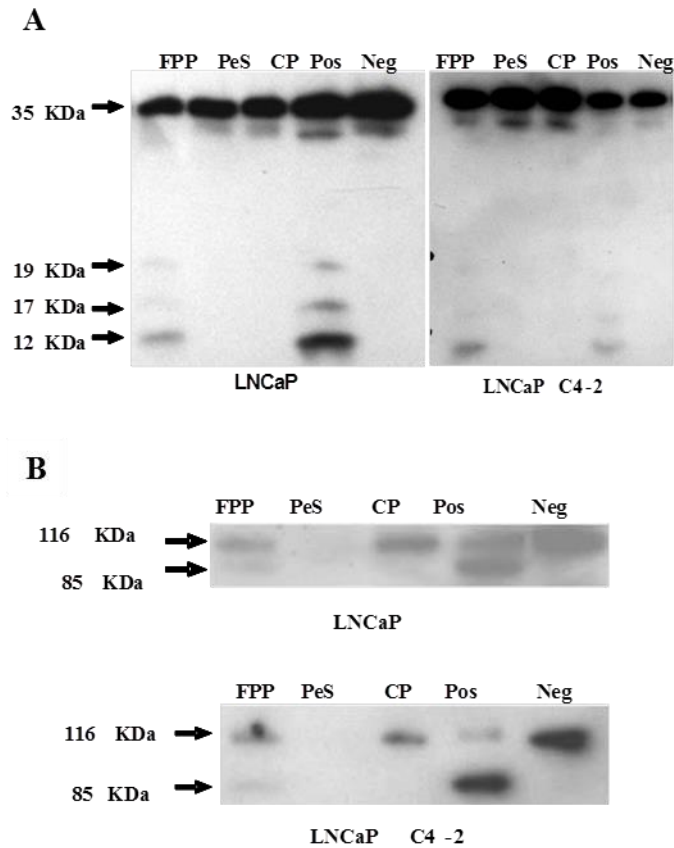
Figure 1.4. Activated Caspase-3 in LNCaP and LNCaP C4-2 cells treated with FPP. Western blot analysis of 30 µg protein from LNCaP and LNCaP C4-2 cells treated as described in Figure 1.3 using anti-caspase-3 antibody (see experimental procedures). (**A**) Procaspase-3 is shown at 35 KDa. The 19 KDa, 17 KDa and 12 KDa cleaved products are indicated. (**B**) Activated poly ADPribose-polymerase (PARP) in LNCaP and LNCaP C4-2 cells treated with FPP. Western analysis was done using an anti-PARP antibody. P A R P is shown at 116 KDa and its cleavage product at 85 KDa. Data are from Jackson *et al* (2007).

apoptotic activity (Figure 1.5A). In order to determine whether removal of only the methyl esters present resulted in loss of activity, heat modified pectin was treated with the enzyme pectinmethylesterase (PME). Both PME-treated and PME+EPG treated heat-modified pectinretained high levels of apoptotic activity suggesting that one or more base-sensitive linkages other than carboxy-methyl esters in HG play a dominant role in the apoptosis-inducing activity (Figure 1.5B).

Taken together the results revealed that the apoptotic pectic structure contained HG that was partially esterified, but that other structural moieties were present in the apoptotic pectin. To further define and purify the structures of the apoptotic pectin and to attempt to identify the smallest active apoptotic pectin within the diverse pectic structures present in the pectin fraction used, we used a series of biochemical separation and glycan fragmentation techniques and characterized the active fraction for carbohydrate composition and linkage.

2. Results

We first compared the apoptotic heat-treated pectin we generated (HTCP) to the commercially available heat-treated apoptotic pectin (FPP) by separation of the intact pectins and the enzyme and mild-base-treated (deesterified) pectins by gel electrophoresis. We visually assessed the effect of these enzymatic treatments by separating the pectin using high percentage polyacrylamide gel electrophoresis (PAGE). When pectin is separated using a high polyacrylamide resolving gel (30%), high molecular weight material remains at the top of the gel while low molecular weight material migrates towards the bottom of the gel due to the presence of the negatively-charged galacturonic acid in the material that electrophoreses towards the positive pole. At this polyacrylamide concentration, discretely-sized oligogalacturonides (as

A

B
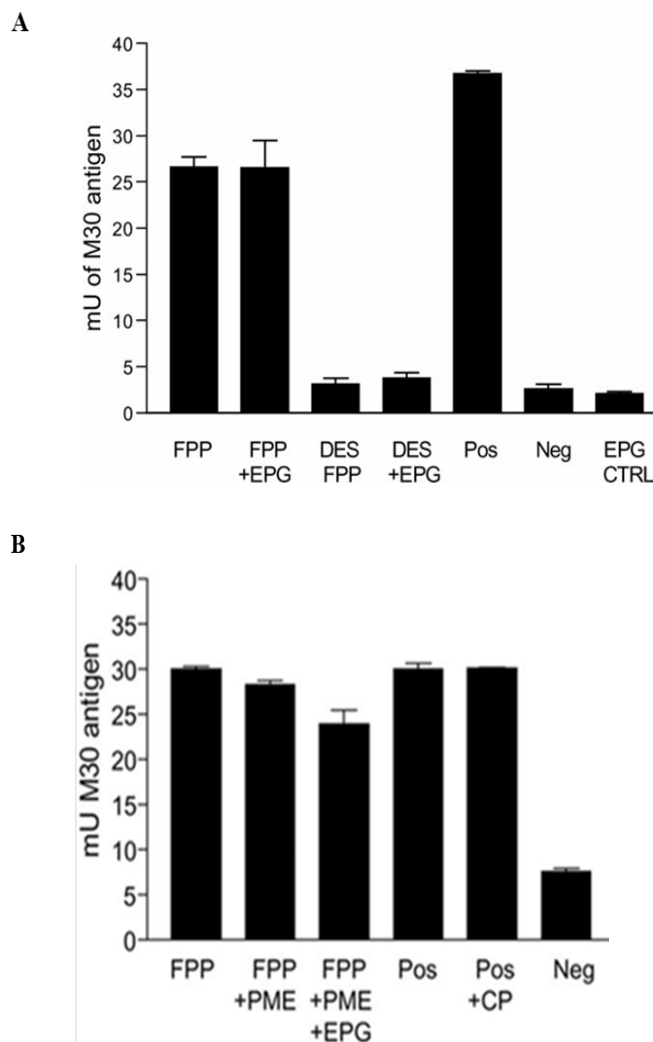
Figure 1.5. (A) Effect of mild base deesterification and/or endopolygalacturonase treatment of FPP on its ability to induce apoptosis. LNCaP cells were treated with 1 mg/ml FPP, FPP treated with endopolygalacturonase (EPG+FPP), FPP deesterified by mild base treatment (DES+FPP), deesterified FPP that was subsequently treated with endopolygalacturonase (DES+EPG), 0.01 mM thapsigargin (Pos), or media only (Neg). Data are the average of duplicate apoptosis assays of duplicate cell extracts (30 µg protein) ± SEM. Similar results were obtained in two independent experiments. **(B)** The effect of pectinmethylesterase (PME) and endopolygalacturonase (EPG) treatment of FPP (a commercially available HTCP) on its ability to induce apoptosis in LNCaP cells. Equal amounts of protein (30 mg) from cells treated with 1 mg/mL FPP, PME-treated FPP (FPP+PME), pectinmethylesterase and endopolygalacturonase-treated FPP (FPP+PME +EPG), 0.001 mM thapsigargin (Pos), 0.001 mM thapsigargin + 1 mg/mL CP (Pos+ CP), or media only (Neg) were tested for their ability to induce apoptosis in LNCaP cells as measured by M30 antibody binding. Data are the average of duplicate apoptosis assays of duplicate cell extracts+SEM. Similar results were obtained in at least two separate experiments. Data are from Jackson *et al* (2007).

small as a degree of polymerization of four, i.e. tetramer) can be visualized if present in the sample. In Figure 1.6, we show that the heat modified pectin created in-house, HTCP, appears identical to the commercially prepared FPP when visualized by PAGE (Figure 1.6A). When the effects of the above chemical and enzymatic manipulations on HTCP and FPP were analyzed by PAGE, the data show that PME treatment alone shifts the bulk of the dark staining material from the top of the gel to the bottom half of the gel (compare Figure 1.6B and C, lane 3 and lane 5). We also demonstrated that non-specific deesterification with mild base treatment, which removes several types of esters including carboxymethyl, acetyl and other esters and could also lead to β-elimination in some linkages under these conditions, leads to the generation of fast moving fragments (Figure 1.6B lane 4 and D lanes 3 and 5) suggesting that this treatment not only cleaves methyl esters, but also cleaves additional linkages leading to extensive fragmentation of the heat modified pectin and loss of apoptotic activity. Interestingly, both PME treatment and deesterification generate material that moves faster into the gel, however, a large portion of methyl ester-cleaved heat modified pectin migrates from the top to the middle of the gel in contrast to the oligomeric ladder that is created by deesterification (compare Figure 1.6B lanes 4 and 5 and Figure 1.6C lane 5 with D lane 5) in the lower part of the gel. Since the PME-treatment of heat-modified citrus pectin (Figure 1.6B and C lane 5) retains apoptotic activity, it is likely that the moderately-sized material near the center of the gel contains fragmented material that is responsible for apoptotic activity. As expected, the OGAs present in intact HTCP and FPP were lost upon treatment with EPG (FPP: compare Figure 1.6B lanes 5 and 6; HTCP: compare Figure 1.6C, lanes 3 and 4). Treatment of the PME-treated FPP or HTCP with EPG leads to the loss of OGAs and the appearance of a broad band of stained material suggesting that a significant amount of the pectin remains cross-linked as diversely-sized oligosaccharides and
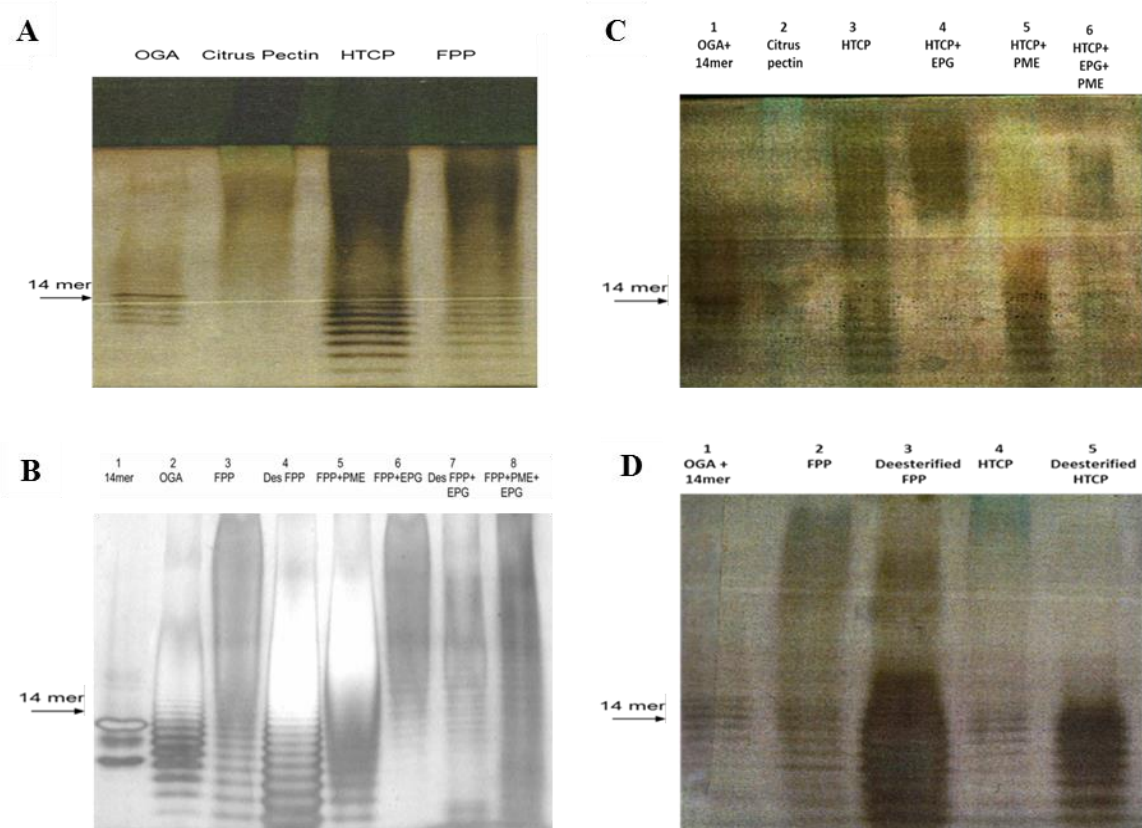
Figure 1.6. Polyacrylamide gel electrophoresis (PAGE) comparing unmodified citrus pectin, inhouse heat-modified citrus pectin (HTCP) and commercially available heat modified citrus pectin (FPP). **(A)** Lane 1, 0.01 µg oligogalacturonides (OGA) of DP 14 (see arrow) with 0.1 µg OGA mix of approximate DP 7–23. Lanes 2-4 (Citrus Pectin, HTCP and FPP respectively) were each loaded with 6 µg material. **(B)** PAGE separation of FPP and base and enzyme treated FPP to study the effects of mild-base treatment, and treatment with pectinmethylesterase (PME) and endopolygalacturonase (EPG) on apoptotic pectin. Lane 1, 0.1 µg OGA of DP 14 (see arrow). Lane 2, mixture of OGAs of DP ~7-23. Lane 3 intact FPP; Lane 4 base-deesterified FPP (Des FPP); Lane 5 pectin methylesterase-treated FPP (FPP+PME); Lane 6 endopolygalacturonase-treated FPP (FPP+EPG); Lane 7 deesterified FPP that was subsequently treated with EPG (Des FPP + EPG) and pectin methylesterase and endopolygalacturonase-treated FPP (FPP+PME+EPG) (Lane 8); (Lanes 3-8 were each loaded with 6 µg of material). **(C)** PAGE separation of HTCP and enzyme-treated HTCP to study the effects pectinmethylesterase (PME) and endopolygalacturonase (EPG) on apoptotic pectin. Lane 1, 0.01 µg oligogalacturonides (OGA) of DP 14 (darkest band) and 0.1 µg OGA mix of approximate DP 7–23; Lane 2, unmodified citrus pectin; Lane 3, intact HTCP; Lane 4, HTCP treated with endopolygalacturonase (EPG); Lane 5, HTCP treated with pectinmethylesterase (PME); Lane 6, HTCP treated with both pectinmethylesterase and endopolygalacturonase (Lanes 2-6 were each loaded with 6 µg material). **(D)** PAGE separation of HTCP/FPP and mild base-treated (deesterified) HTCP/FPP to study effects of mild base deesterification on apoptotically active pectin. Lane 1, 0.01 µg OGA of DP 14 (darkest band) with 0.1 µg OGA mix of approximate DP 7–23; Lane 2, commercially available heat-treated citrus pectin, FPP; Lane 3, mild base deesterified FPP; Lane 4, HTCP (created in-house); Lane 5, mild base-treated HTCP (Lanes 2-5 were each loaded with 6 µg material). Data in A and B taken from Jackson *et al* (2007) to allow for comparison with new data in C and D.

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polysaccharides (Figure 1.6B lane 8 and 1.6C lane 6). An important observation was that less pectic material is cleaved by EPG following PME treatment of FPP compared to treatment with mild base ((Figure 1.6B compare lanes 7 and 8) again suggesting that base treatment causes the loss of linkages in addition to methyl esters.

There is no direct correlation between the size and apoptotic activity of pectin

We also separated the three commercial pectins analyzed in our initial studies (FPP, PeS and CP) by size exclusion chromatography to ascertain whether differences in pectin size correlated with activity. The relative sizes of heat modified FPP, pH modified PeS, and unmodified CP were established by separation over a Superose 12 HR10 (10-300 nm) size exclusion chromatography (SEC) column in 50 mM sodium acetate and 5 mM EDTA at 0.5 ml min^{-1} using a Dionex DX500 system. The eluted pectins were detected using an uronic acid colorimetric assay (Blumenkrantz and Asboe-Hansen 1973). Figure 1.7A shows that FPP is intermediate in size between the polydisperse and large CP (Figure 1.7C), which has an estimated molecular mass range of 23-71 kDa and the relatively uniformly-sized and low molecular weight PeS (Figure 1.7B), which has a molecular mass range of 10-20 kD.

There is no significant difference in the sugar composition of the active and inactive pectins

Our prior sugar composition analysis (Figure 1.8) comparing FPP, PeS and CP to determine whether the apoptotic effects of the heat-treated pectin were due to uniquely enriched specific pectic carbohydrates showed that, as expected, galacturonic acid (GalA) was the major component in all three pectins tested. No consistent correlation was found between the apoptosis-inducing activity and the glycosyl residue composition of heat-modified FPP compared to
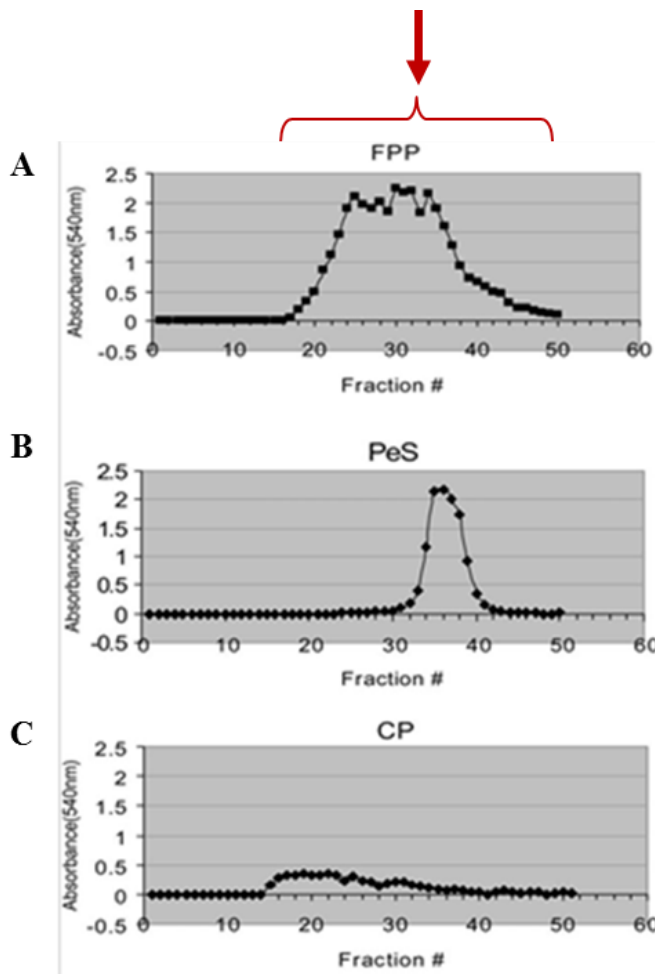
Figure 1.7. Determination of the active structural components in heat modified citrus pectin (here FPP) that contribute to its apoptosis-inducing activity. (A-C) The size distribution of the pectins as determined by SEC over a Superose 12 HR10 column in 50 mM sodium acetate and 5 mM EDTA and detection by an uronic acid colorimetric assay. **(A)** Data for FPP; **(B)** Data for PeS; and **(C)** Data for CP. The arrow and bracket indicate the expected size range of HTCP which was generated in a manner similar to that used to generate the FPP. Adapted from Jackson (2005; Master's thesis).

inactive unmodified CP and pH-modified PeS. For example, FPP was arabinose-rich compared to PeS, but had comparable amounts of Ara to CP. Likewise, both FPP and PeS had less Gal (74% and 77% less, respectively), slightly less Rha (30% and 40% less, respectively), and more GalA (16% and 24% more, respectively) than CP. Glycosyl residue analysis of HTCP (the in-house generated heat modified citrus pectin) and HTCP+PME+EPG, which will be referred to as HPE from here on, revealed that PME-EPG-cleaved heat-modified citrus pectin (HPE) contained measureable amounts of the same relative mole percentages of sugar residues as the undigested HTCP and commercially available heat modified citrus pectin FPP. It is important to note that the entire reaction mixture following enzyme treatment was analyzed for sugar composition. Thus the composition data reflect the total sugars in the polymeric and the cleaved reaction products. This was the same mixture used to test for apoptotic activity. None of the pectins contained ribose, fucose, mannose, N-Acetyl Galactosamine (GalNAc), N-Acetyl Glucosamine (GlcNAc) or N-Acetyl Mannosamine (ManNAc). FPP, however, was arabinose-rich compared to HTCP and HPE (FPP=~5 mole%; HTCP and HPE=0.6 mole%) and the two pectins created in-house contained a small amount of xylose (0.1 mole%) which was absent in FPP.

There are subtle differences in sugar linkages in the apoptotic pectin

Glycosyl residue linkages present in FPP, CP, PeS, HTCP and HPE were also determined. FPP, CP and PeS were analyzed using both a single and double methylation procedure (Tables 1.1 and 1.2) and the HTCP and HPE were analyzed using the double methylation procedures (Materials and Methods) (Table 1.3). The single methylation method has the disadvantage of yielding incomplete methylation with the resulting incomplete linkage results, but has the advantage of avoiding or reducing fragmentation of the pectin because of β-
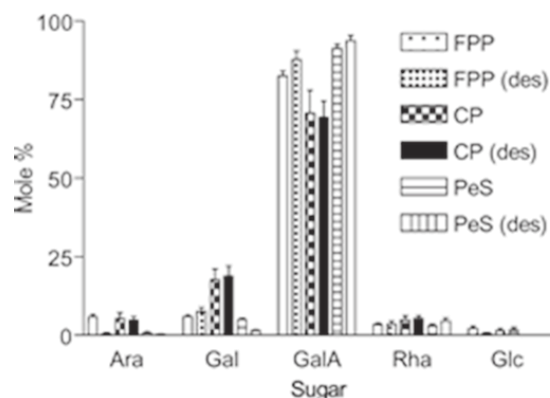
A   **B**
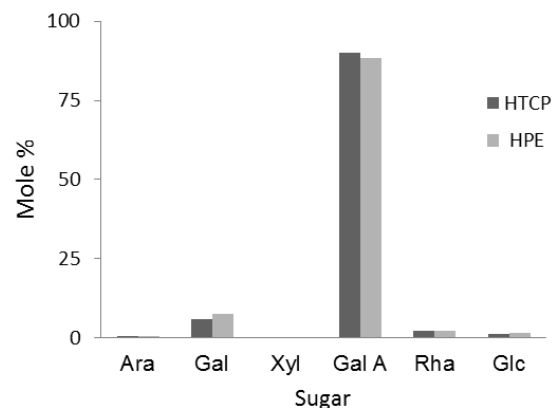
Figure 1.8. **(A)** Glycosyl residue composition analyses of unmodified and base-treated FPP, PeS and CP. (B) Glycosyl residue composition analyses of HTCP and HTCP that was digested with PME and EPG. The entire reaction mixture following enzyme treatment was analyzed for sugar composition. Composition analyses were done by GC/MS of TMS derivatives of methyl glycosides produced by acid methanolysis (York *et al.* 1985). Data are the average ± SEM mole % specific sugar from duplicate analyses from two separate experiments (N = 4) (A). (B) N=1. Data in A taken from from Jackson (2005) and Jackson *et al.* 2007 to allow comparison with new data in B.

elimination of the glycosyluronic acid linkages in the HG. On the other hand, the double methylation method leads to more complete methylation, but can lead to β-elimination of the glycosyluronic acid linkages (York *et al*. 1985), and thus, to an apparent increase in the amount of terminal GalA and a loss in the apparent 4-linked GalA. Comparison of the linkage data showed that FPP, CP and PeS (Tables 1.1 and 1.2) contain primarily HG (the presence of 4-linked GalA) and RG-I (2-linked Rha and 2,4-linked Rha). The only linkage data that correlated with the apoptosis-inducing activity of FPP was the higher amounts of terminal and 5-linked Ara in FPP. These linkages were lost in the base-treated FPP. FPP and CP contained 5-linked arabinan and 4-linked galactan, which are known to occur as side chains of RG-I, whereas these linkages were absent or greatly reduced in PeS. HTCP and HPE predominantly contain 6-linked glucose, HG (4-linked GalA) and terminal rhamnose, often linked to the GalA backbone. There is also evidence of RG-I backbone structure(s) (2-Rha, 2,4-Rha and 2,4-GalA), and arabinan and galactan side chains of RG-I (5-Araf and 4-Gal respectively). T-Araf or t-Gal could be indicative of AG type II, however there is no evidence for the 3-, 6-, 3, 6-Gal*p* linkages present in the AGII galactose backbone and side chain.

Anion-exchange chromatography of heat modified pectin reveals only four uronic acid positive peaks that are apoptotically active

In an effort to further characterize the active component in heat modified pectin, the smallest active pectin generated to this point, a heat fractionated pectin (FPP or HTCP) that was treated with PME and then with EPG, was separated by high performance anion exchange chromatography (HPAEC) and monitored using in-line electrochemical (PAD) and multiple wavelength detection (analytical scale preparations only). The pectic structures of both the

Table 1.1 Glycosyl linkage analysis of untreated and mild base-treated FPP, CP and PeS by the single methylation method[a]

Glycosyl Residue	Relative amount					
	FPP	FPP (des)	CP	CP (des)	PeS	PeS (des)
T-Ara (f)	4 ± 0	0	2.5 ± 0.5	1 ± 0	0	0
5-Ara	3 ± 0	0	2 ± 0	1 ± 0	0	0
T-Gal	4 ± 0	2 ± 0	2.5 ± 0.5	1.5 ± 0.5	5 ± 0	2 ± 0
3-Gal	0.5 ± 0.5	0	1.5 ± 0.5	0.5 ± 0.5	0	0
4-Gal	12 ± 0	7.5 ± 2.5	11.5 ± 1.5	8 ± 1	3 ± 0	0
2,4-Gal	3 ± 0	1.5 ± 1.5	2.5 ± 0.5	1.5 ± 0.5	0	0
3,4-Gal	1.5 ± 0.5	1 ± 1	2 ± 0	1 ± 1	0	0
T-GalA	9 ± 2	4.5 ± 2.5	2.5 ± 0.5	2 ± 0	23.5 ± 0.5	7 ± 0
4-GalA	36.5 ± 5.5	37.5 ± 6.5	32 ± 1	40.5 ± 4.5	28 ± 0	45 ± 4
2,4-GalA	7.5 ± 0.5	10.5 ± 0.5	10 ± 0	10.5 ± 1.5	7 ± 0	10 ± 1
3,4-GalA	8 ± 1	13.5 ± 1.5	10.5 ± 1.5	14 ± 1	7.5 ± 0.5	14.5 ± 1.5
GalA (U)[b]	5 ± 1	8 ± 1	6 ± 1	10 ± 1	7.5 ± 0.5	11.5 ± 1.5
T-Rha	T[c]	0	T	T	0	0
2-Rha	7 ± 1	4 ± 0	5 ± 0	3 ± 0	10± 0	5 ± 0
2,3-Rha	2 ± 0	1.5 ± 0.5	2 ± 0	1.5 ± 0.5	3.5 ± 0.5	2 ± 0
2,4-Rha	3 ± 0	1 ± 1	3 ± 0	2 ± 0	3.5 ± 0.5	2 ± 0
2,3,4-Rha	1.5 ± 1.5	1 ± 0	1.5 ± 0.5	1 ± 0	1.5 ± 0.5	1 ± 0
4-Glc	2 ± 0	2.5 ± 1.5	3 ± 1	1 ± 0	0	0

[a]Linkage analysis was carried out by GC/MS of partially methylated alditol acetates (PMAAs) produced by permethylation, reduction, depolymerization, reduction and acetylation as descrived by York *et al.* (1985). Data are average % of glycosyl residues with specified linkages ± SEM from duplicate analyses (N=2).
[b]U: undemethylated; apparent 2,3,4,-linked GalA
[c]T: trace; <1
Taken from Jackson *et al.* (2007)

Table 1.2 Glycosyl linkage analysis of untreated and mild base-treated FPP, CP and PeS by the double methylation method[a]

Glycosyl Residue	Relative amount					
	FPP	FPP (des)	CP	CP (des)	PeS	PeS (des)
T-Ara (f)	1.5 ± 1.5	0	0	0	0	0
5-Ara	0.5 ± 0.5	0	0	0	0	0
T-Gal (f)	0	0	0	0	5.5 ± 0.5	0
T-Gal	2 ± 0	3.5 ± 1.5	2.5 ± 0.5	1 ± 0	1.5 ± 0.5	1 ± 0
4-Gal	5 ± 0	11.5 ± 6.5	4.5 ± 1.5	3.5 ± 0.5	0	0
2,4-Gal	1 ± 0	T[c]	1 ± 0	0.5 ± 0.5	0	T
T-GalA	20.5 ± 4.5	12 ± 1	20.5 ± 1.5	12.5 ± 1.5	17.5 ± 3.5	17.5 ± 1.5
4-GalA	43 ± 1	48 ± 9	47.5 ± 2.5	58.5 ± 1.5	55 ± 7	58.5 ± 1.5
2,4-GalA	1.5 ± 0.5	1.5 ± 1.5	2.5 ± 0.5	4 ± 1	2.5 ± 0.5	3.5 ± 1.5
3,4-GalA	4.5 ± 1.5	2.5 ± 2.5	3.5 ± 0.5	6 ± 0	3 ± 0	3.5 ± 0.5
GalA (U)[b]	3.5 ± 1.5	1.5 ± 1.5	4.5 ± 0.5	5.5 ± 0.5	2.5 ± 0.5	2.5 ± 1.5
T-Rha	1 ± 1	0	0	0	0	0
2-Rha	2.5 ± 0.5	5.5 ± 3.5	4 ± 2	2 ± 0	3.5 ± 0.5	2 ± 0
2,4-Rha	3 ± 1	3.5 ± 0.5	2.5 ± 0.5	4 ± 0	2 ± 0	2 ± 1
T-Glc	3.5 ± 0.5	0	0	0	2 ± 0	2 ± 2
4-Glc	5 ± 0	9.5 ± 5.5	4.5 ± 0.5	4 ± 4	2.5 ± 0.5	4 ± 0

[a]Linkage analysis was carried out as explained in Table 1.1, except that following the first methylation the permethylated material was reduced by super-deuteride and the reduced sample was re-methylated using the NaOH/MeI method (Materials and Methods). Data are the average percentage of glycosyl residues with the specified linkages. ± SEM from duplicate analyses (N=2).
[b]U: undemethylated; apparent 2,3,4,-linked GalA
[c]T: trace; <1
Taken from Jackson *et al.* (2007)

Table 1.3 Glycosyl linkage analysis of untreated HTCP and PME and EPG treated HTCP (HPE) by the double methylation method[a]

Glycosyl Residue	Relative amount	
	HTCP	HPE
T–Ara (f)	0.7	0.6
5-Ara	0	0
T-Gal (f)	0	0
T-Gal	1.2	0.9
4-Gal	4.9	5.9
2,4-Gal	0	0
4,6-Gal	0.7	
T-GalA	3.2	0.9
4-GalA	38.0	13.9
2,4-GalA	0.7	0
3-4-GalA	0.7	0
T-Rha	17.8	30.0
2-Rha	1.7	1.2
2,4-Rha	0.6	0.4
3-Rha	0.6	1.3
2,3-Rha	1.2	1.8
T-Glc	0.8	3.1
4-Glc	1.9	3.4
4,6-Glc	0.3	0.5
6-Glc	24.0	35.4
T-Xyl	0.2	0.4
4-Xyl	0	0.5

Linkage analysis was carried out as explained in Table 1.1, except that following the first methylation the permethylated material was reduced by lithium deuteride and the reduced sample was re-methylated using the NaOH/MeI method (Materials and Methods). Data are the average percentage of glycosyl residues with the specified linkages.

enzyme-treated FPP and HTCP were cleaved into small HG oligosaccharides (oligogalacturonide (OGA)) fragments or fragments of RG-I backbone or other pectic structures as seen in the uronic acid assay profiles (Figure 1.9A and B) which identified four distinct peaks near the beginning of the fractionation. The results indicated that these fractions contained either GalA-containing oligogalacturonides ranging from monomers to oligosaccharides of DP 7 to 8 or fragments with relatively few GalA residues and with neutral sugars. Contrastingly, deesterified FPP/HTCP had a loss of uronic acid positive material (Figure 1.9C and D). Furthermore, when the individual uronic acid positive peaks seen in Figure 1.9A and B were pooled and incubated with LNCaP cells, there was low but significant apoptosis activity in all but one pool (Figure 1.9E).

Aopototic structure is sensitive to exo-β-galactosidase and RG-I lyase treatment

The smallest active pectin generated for use in these studies, HPE, was subjected to a series of enzymatic digests to remove side chains and to further fragment the rhamnogalacturonan region of the pectic backbone. The enzymes were added individually and in combination to correlate any loss of activity with removal of a particular sugar or linkage and the resulting treated pectin was assayed for apoptosis activity. As seen in Figure 1.10A there was a significant reduction in apoptotic activity when HPE was digested with the enzyme exo-β-galactosidase which hydrolyses terminal non-reducing β-D-galactose residues in β-D-galactosides, indicating that terminal galactose residues in the galactan side branches of RG-I and/or a linkages involving terminal galactose residues in the arbabinogalactan side chains of RG-I are important for apoptotic activity. Furthermore, cleavage of the RG-I backbone by RG-I lyase which results in α-4-5 unsaturated RG oligosaccharides, abolishes the apoptotic activity (Figure 1.10C). Curiously, HPE-treated simultaneously with both RG-I lyase and

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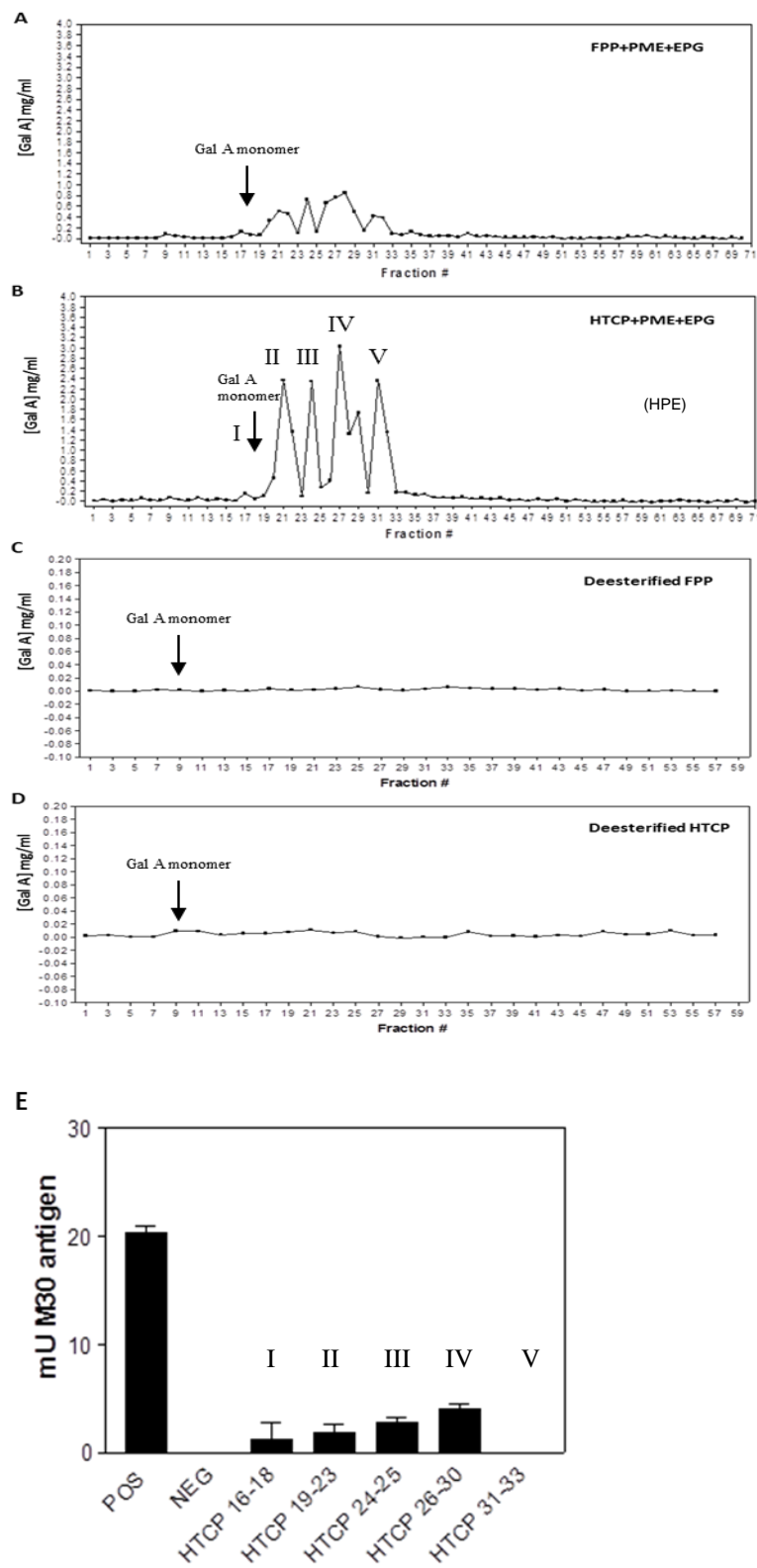
Figure 1.9. (A-E) Uronic acid assay of fractions collected from FPP+PME+EPG **(A)**, HTCP+PME+EPG (HPE) **(B)**, Deesterified FPP **(C)**, and Deesterified HTCP **(D)** separated over an anion exchange column. For A and B, separation was over preparatory scale column. Sample (40 mg) was loaded onto the column and eluted at a rate of 4 ml/min with 1M ammonium formate buffer. For C and D the separation was over an analytical scale onto column. Two mg sample was loaded onto the column and eluted at a rate of 1 ml/min with 1M ammonium formate. **(E)** Apoptotic activity in LNCaP cells treated with (0.3 mg/ml) uronic acid positive pooled fractions from HPE fractionated over an anion exchange column (B) or 0.01 mM thapsigargin (Pos) for 48 h. Incubation with media alone served as the negative control (Neg). Equal amounts of cell extracts (12 ug protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts+SEM.

I=Pooled fractions 16-18
II=Pooled fractions 19-23
III=Pooled fractions 24-25
IV=Pooled fractions 26-30
V=Pooled fractions 31-33

arabinofuranosidases showed a lesser inhibition of apoptotic response than HPE treated with only RG lyase. This was unexpected since removal of arabinan side chains improves RG-I lyase activity (Mutter *et al*. 1998). Incubation of HPE with endo-β−1,4-galactosidase alone (Figure 1.10A), to hydrolyze linkages in type I arabinogalactan did not significantly affect activity while digestion with α-L-arabinofuranosidases alone to hydrolyze terminal non-reducing α-L-arabinose residues, or with endo-1,5-α-L-arabinofuranosidases alone resulted in slightly reduced activity (Figure 1.10A and C). When all enzymes, werecombined there was only a slight reduction in activity indicating that the apoptosis-abolishing activities of exo-β-galactosidase and RG-lyase by themselves are inhibited in some manner when all the enzymes are mixed together (Figure 1.10B and C). Mutter *et al*. (1998) showed that removal of galactose side chains from RG-I inhibited the catalytic efficiency of RG-lyase.

Determining the presence of an APAP-1-like structure in the apoptotic pectin

Tan *et al.* (2013) have recently obtained evidence for a new plant cell wall glycoconjugate, APAP1 (Arabinoxylan-Pectin-Arabinogalactan Protein 1), in which pectin and arabinoxylan glycans are covalently linked to an arabinogalactan protein. The AGPs associated with APAP1 are soluble in Yariv-reagent and show no labeling at the reducing end with the aldehyde-reactive fluor 2-amino-benzoamide (2-AB), suggesting that the pectins are covalently linked to the protein core. In contrast, heat treated citrus pectin does absorb at 254 nm when labeled with 2-AB. In addition, when LNCaPs were treated with the individual pectic polysaccharides HG, RG-I and with an RG-I that contained AGP, apoptosis was not induced (Figure 1.11). However, when the unmodified citrus pectin, pH-modified and inactive Pectasol, inactive deesterified heat-treated citrus pectin, and heat-treated citrus pectins HTCP and FPP
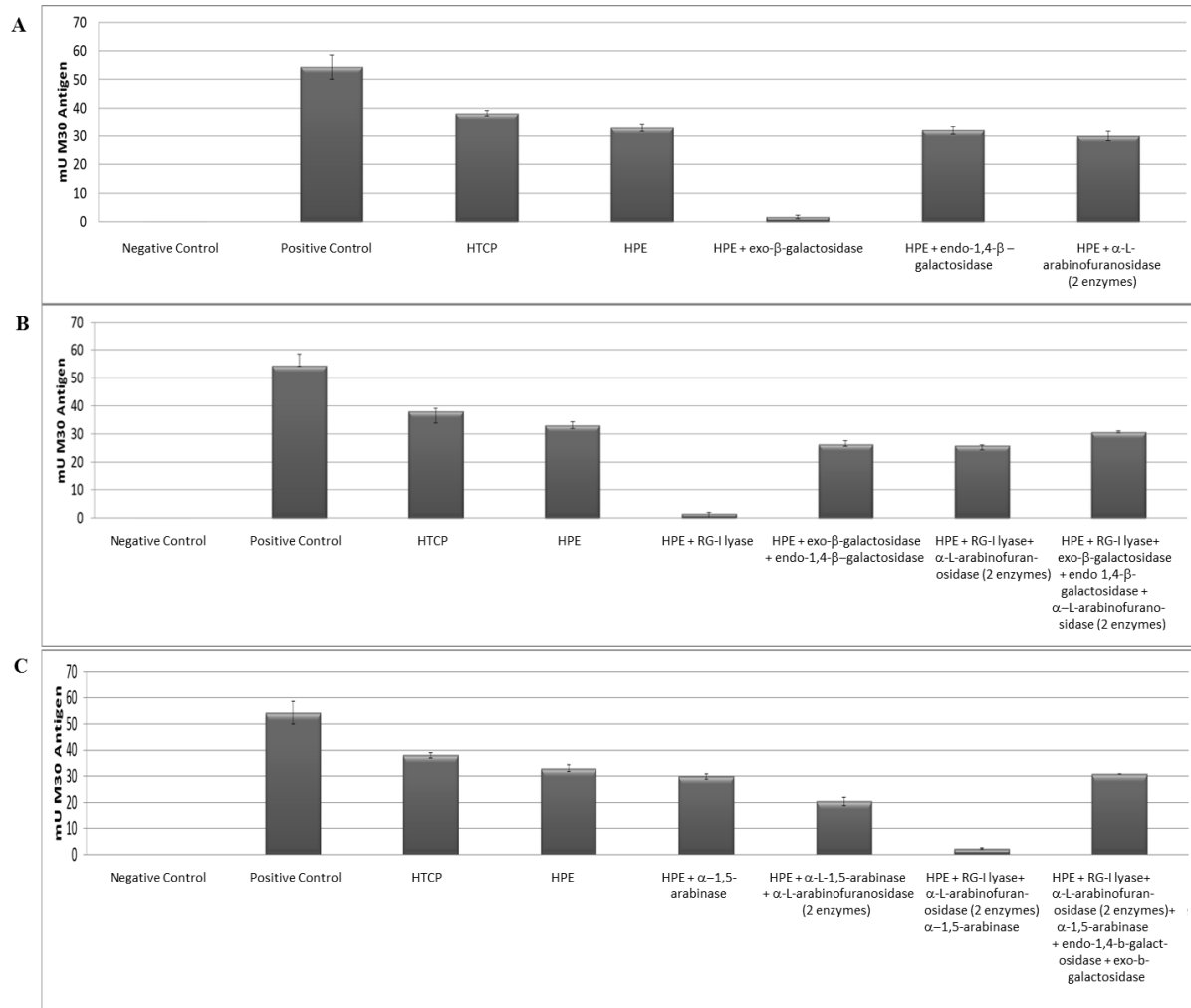
Figure 1.10. (A-C) Analysis of apoptotic activity of HPE digested with RG-I backbone and side chain-degrading enzymes. LNCaP cells were treated with 1 mg/mL of the designated pectins as described or with 0.01 mM thapsigargin (Pos) for 48 h. Incubation with media alone served as the negative control (Neg). Equal amounts of cell extracts (10 ug protein) were used for measuring apoptosis. Apoptosis was assayed using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts+SEM. Comparable results were obtained in two independent experiments. **(A)** Apoptotic activity of digested HPE with enzymes that degrade arabinan and galactan side branches of RG-I. **(B)** Apoptotic activity of digested HPE with RG-I lyase to cleave the RG-I backbone. **(C)** Apoptotic activity of HPE digested with endo-1,5-arabinase and with all the described enzymes.
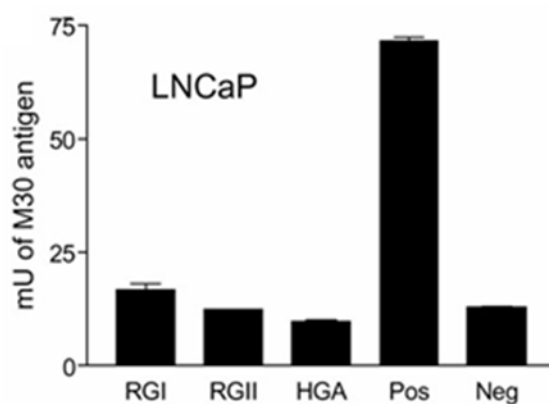
Figure 1.11. Determination of the active structural components in FPP that contribute to its apoptosis-inducing activity. Effects of treatment of LNCaP cells with the purified pectins. LNCaP cells were treated with 1 mg/ml homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) for 48 hr and equal amounts of protein (12 μg) were assayed for apoptosis using the M30 Apoptosense assay. Data are the average of duplicate apoptosis assays of duplicate cell extracts ± SEM. Comparable results were obtained in three independent experiments. This supporting data is from Jackson (2005) and Jackson *et al*. (2007).
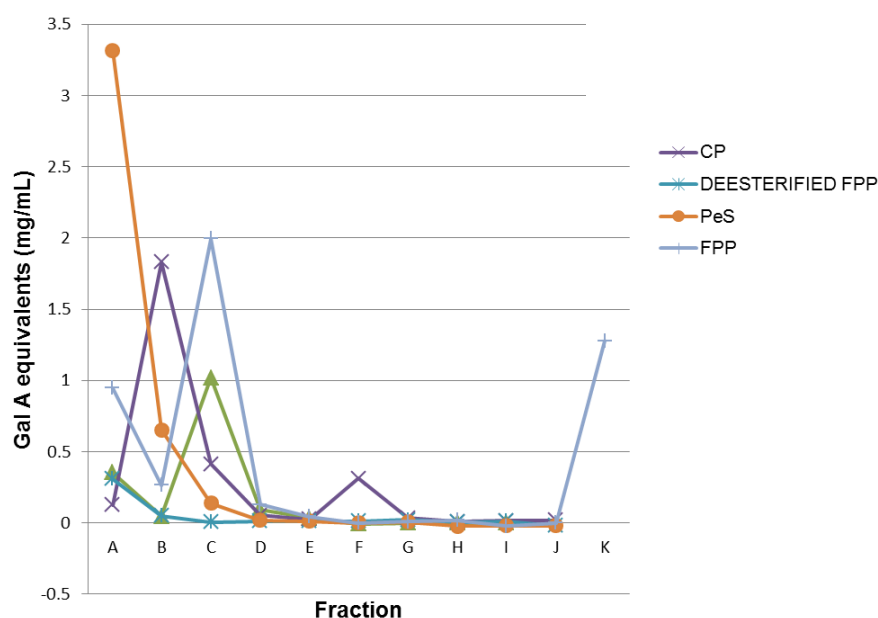
Figure 1.12. Uronic acid assay of fractions collected after separation of the indicated pectin over a reversed-phase HPLC chromatography column. Two milligrams of each pectin was separated with a solvent A (0.1% [v/v] TFA aqueous) to 50% solvent B (80% [v/v] acetonitrile in 0.1% [v/v] TFA aqueous) gradient over 100 min. A-K represents pooled fractions eluted from the column collected at 10 minute intervals.

were analyzed by reversed-phase chromatography (Figure 1.12), only deesterified FPP and Pectasol voided the column, suggesting that the other polysaccharides which bound the column in a manner comparable to APAP1, may contain a protein component. Proteinase K treatment of heat-treated citrus pectin was performed, however, there was no effect on apoptotic activity. This incomplete enzymatic cleavage could be due to heavy glycosylation of a potential AGP glycoconjugate.

Determining the presence of AGII in the active pectin

Based on the results obtained from glycosyl residue composition and linkage analyses and from the enzymatic digestions, we concluded that the apoptosis-inducing pectin may require HG, RG-I, galactan, type I arabinogalactan domains or a combination of these polysaccharides for its activity. There is limited data supporting the presence of a type AGII structure in the apoptotic pectin. Specifically, the heat treated pectin contained RG-I, HG and type AGI sugars and linkages such as 2- and 2,4-Rha*p* (RG-I backbone); 4-GalA*p* (RG-I and HG backbone); 5-Ara*f* (RG-I side chain) , 4-Gal*p* (RG-I side chains/type AGI backbone). There was no evidence for the 3-, 6-, 3, or 6-Gal*p* Type IIAG linkages. There was evidence for the presence of t-Ara*f*. However this linkage can be found on structures other than on type II AG such as in the arabinose side chains of RG-I and in type I arabinogalactans.

3. Discussion

Purification efforts to date to isolate and characterize the apoptotic pectin structure(s) have revealed that key residues and/or linkages are necessary for pectin's apoptotic activity. While the apoptotic pectin may contain a protein component, a proteoglycan such as APAP-

1does not appear to be responsible for on the apoptosis-inducing abilities of pectin. Linkages involving terminal galactose residues in the galactan side branches of RG-I and/or a linkages involving terminal galactose residues in the arbabinogalactan side chains of RG-I appear to be essential. Furthermore, activity was abolished by treatment of HPE with RG-I lyase, suggesting that the RG-I backbone is also necessary for the preservation of apoptotic activity. In conclusion, we provide further evidence that specific structural characteristics of pectin are responsible for inducing apoptosis in cancer cells. Our results demonstrate that different extraction protocols may alter the structure of pectin and can lead to differences in pectin's apoptosis-inducing activity. This is of particular therapeutic significance, as we have demonstrated that manipulating the structure of pectin results in a compound that is capable of inducing apoptosis and that this structure can be extensively fragmented without destroying apoptotic activity. Generation of the smallest pectic fragment that can induce apoptosis in human prostate cancer cells could impact the development of pectin as a therapeutic agent and/or lead to more targeted structure function studies.

4. Materials and Methods

Two prostate cancer cell lines, LNCaP and LNCaP C4-2, were utilized in these experiments. LNCaP obtained from American Type Culture Collection (Rockville, MD) are androgen- responsive prostate cancer cells, whereas LNCaP C4-2 cells were derived from LNCaP cells as androgen-refractory prostate cancer cells (purchased from Grocer Inc., Oklahoma City, OK). HUVECs were from American Type Culture Collection. EBM-2 media and EGM-2 supplements were from Cambrex Bio Science (Walkersville, MD). RPMI-1640 media supplemented with 25 mM HEPES and L-glutamine was purchased from Hyclone

(Logan, UT). Fetal bovine serum (FBS), penicillin/streptomycin, citrus pectin (P-9135), sodium hydroxide, alcian blue, and the caspase-3 colorimetric assay kit were from Sigma-Aldrich (St Louis, MO). Fungizone was obtained from Invitrogen (Carlsbad, CA). Bio-Rad Protein Assay dye reagent concentrate was purchased from Bio-Rad (Hercules, CA). M-30 Apoptosense ELISA was from Peviva AB (Sweden). Sodium carbonate and sodium acetate were purchased from J.T. Baker (Phillipsburg, NJ) and acetic acid from EM Science (Gibbstown, NJ). PeS was purchased from EcoNugenics (Santa Rosa, CA), FPP from Thorne Research (Dover, ID), and CP (P-9135) from Sigma- Aldrich. Purified PME (from Aspergillus niger 2.2 mg/mL,1.0 U/mg, 1 U/1 mmol/min) and EPG (from A. niger, 0.5 mg/mL, 1.2 U/mL, 1 U/1 mmol/min) were a gift of Carl Bergmann (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Exo-β-Galactosidase (*A. niger)*, α-L-arabinofuranosidases (*A. niger* and *Bifidobacterium adolescentis)*, endo-β−1,4-galactosidase (*A. niger)*, and endo-1,5-α-L-arabinase (*A. niger)* were from Megazyme (Wicklow, Ireland). RG-I lyase was a gift of Henk Schols (via Melani Atmodjo). All other chemicals unless otherwise stated were from Fisher Scientific.

Purified pectins

Purifed HG, RG-I, and RG-II were a gift of Stefan Eberhard (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). The HG was a mixture of oligogalacturonides of degrees of polymerization of approximately 7 − 23 that were produced by partial endopolygalacturonase treatment of commercial polygalacturonic acid as described by Spiro et al. (1993). RG-I was isolated from sycamore (*Acer pseudoplatanus*) suspension culture cells as described in Marfà et al. (1991). RG-II was isolated from red wine as

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described by Pellerin et al. (1996).

Fractionation of citrus pectin by heat treatment

HTCP was prepared as described in Jackson *et al*. (2007). Specifically, a 0.1% aqueous pectin dispersion was prepared by dissolving 500 mg of unmodified Citrus Pectin (CP) in 500 ml of de-ionized, filtered water. The solution was autoclaved at 123.2^{o}C at 17.2-21.7 psi for 30 minutes, cooled to room temperature and stored overnight at 4°C, resulting in the formation of a gel-like precipitate (batch 1). The following day, the aqueous phase was collected, frozen at -80°C and lyophilized. Subsequent batches did not yield a visible precipitate after incubation overnight at 4°C, therefore the entire solution was frozen at -80°C and lyophilized. The pH of the resulting heat mofified pectin was 4.0. There was no detectable difference in the structural characteristics or apoptotic activity between HTCP batches. HTCP represented 80% of the starting CP on a % mass basis.

Pectinmethylesterase and endopolygalacturonase treatment of pectins

The first step to produce the smallest-apoptotically active pectin was fragmentation of HTCP by pectinmethylesterase (PME) and endopolygalacturonase II (EPG) isolated from *Aspergillus niger*. We have shown that even when subjected to treatment with EPG, which cleaves HG at contiguous nonesterified GalA residues (Chen and Mort 1996), subsequent to digestion with PME to remove methyl ester groups and thereby make HG more accessible to EPG cleavage, HTCP retains ~80% of its apoptotic activity (Jackson *et al* 2007). To carry out this digestion in conditions favorable to both enzymes, ammonium formate, pH 4.5, was added to 20 mg/mL HTCP to give a final ammonium formate concentration of 10 mM. Purified *A. niger*

endopolygalacturonase (EPG) 1.2 U/mL and pectinmethylesterase (PME) 1 U/mg were added to the solution. PME activity was confirmed by the detection of methanol released using the method of Klavons and Bennett (1986). As a negative control, the effect of HTCP in buffer with no enzyme was compared. The samples were incubated overnight at room temperature (RT) to ensure complete digestion. The enzymes were inactivated by boiling for 10 min at 100°C and the digested pectin was frozen at -80°C and lyophilized. To confirm reproducibility with past lots, the dry samples were analyzed by high-percentage acrylamide PAGE and tested for apoptotic activity in an M-30 Apoptosense ELISA.

Use of PAGE to probe the effects of enzyme treatment of HTCP on its apoptosis-inducing activity

We subjected the PME+EPG-digested HTCP to the same separation by PAGE as described earlier and analyzed the reaction products by alcian blue staining using a modification of the procedures of Corzo *et al.* (1991) and Reuhs *et al*. (1993; 1998) as described by Djelineo (2001) and Jackson *et al.* (Jackson *et al* 2007). Specifically, the pectin sample was mixed in a 5:1 ratio with 6X sample buffer (0.63 M Tris-Cl/ pH 6.8, 0.05% phenol red, 50% glycerol), loaded onto a resolving gel (0.38 M Tris pH 8.8, 30% (wt/vol) acrylamide (37.5:1 acrylamide: bis-acrylamide, wt/wt) overlaid with a stacking gel (5% acrylamide, 0.13 M Tris pH 6.8 M) and separated at 17.5 mA for 60 minutes or until the phenol dye was within 1 cm of the end of the gel. The gel was stained 20 min with 0.2% alcian blue in 40% ethanol, 20 min with 0.2% silver nitrate containing 0.075% formaldehyde, and incubated in 4% sodium carbonate containing 0.05% formaldehyde until bands appeared (with washing between each incubation). Once the bands developed, the carbonate solution was removed immediately and the gel stored overnight in 5% acetic acid followed by final preservation in water or by desiccation.

Apoptosense assay

Apoptotic activity was measured to determine the ability of each pectin-containing sample to induce programmed cell death in LNCaP cells. Each new preparation of PME+EPG-digested HTCP was tested to confirm that it contained at least 80% of its apoptosis-inducing activity as previously observed (Jackson *et al* 2007). LNCaP cells were incubated in medium containing the pectin and the level of apoptosis quantified. LNCaP cells grown in RPMI-1640 medium supplemented with 25 mM HEPES, 2.0 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 U/ml Penicillin, 0.05 mg/ml Streptomycin, and 0.25 μg/ml Fungizone in the presence of 5% CO_2 at $37^{o}C$ were seeded at a density of 1.6 x 10^5 cells per well in 6-well culture plates and allowed to adhere to the plate for 24 hr. The medium was removed and replaced with medium containing 1 mg/ml filter-sterilized (0.20 μm nylon filters) PME+EPG-treated HTCP (HPE), 1 μM thapsigargin (positive control) a compound that induces endoplasmic reticulum stress and thereby raises cytosolic calcium concentration and leads to apoptosis, or with medium only (negative control). The cells were treatmented 48 hrs and thereafter lysed in ice cold lysis buffer (10 mM Tris-HCL, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40), incubated on ice for 5 min, and soluble protein collected by centrifugation at 4°C.   The protein concentration was determined using a Bradford/Bio-Rad Protein Assay (Bio-Rad Protein Assay Dye Reagent Concentrate). Apoptotic activity was measured using the M30-Apoptosense ELISA (Peviva AB, Sweden) using equal amounts of protein per sample. This assay detects the presence of the apoptosis-specific cytokeratin-18 neoepitope (generated by cleavage of cytokeratin-18 by caspases activated in response to treatment). In brief, the protein extract was added to 96-well plates coated with mouse monoclonal M30 antibody. Horseradish peroxidase tracer solution was added to the wells in a dark room illuminated with a green safety light, and the plate was

incubated with agitation for four hr at room temperature. Color development occured with the addition of tetramethyl benzidine solution and incubation in darkness for 20 min. Optical density was determined at 450 nm using Spectra MAX 340 (Molecular Devices, Menlo Park, CA) or Finstruments Model 347 (Vienna, Virginia, USA) microplate readers. The amount of cytokeratin-18 neoepitope was determined based on standard curves generated using standards provided by the manufacturer.

Size exclusion chromatography

The relative size of apoptotically active PME+EPG-treated heat modified citrus pectin was established by separation over a Superose 12 HR10 (10-300 nm) size exclusion chromatography (SEC) column in 50 mM sodium acetate and 5 mM EDTA at 0.5 ml min^{-1} using a Dionex DX500 system. The eluted pectins were detected using a uronic acid colorimetric assay (Blumenkrantz and Asboe-Hansen, 1973) and the profile compared to the previously analyzed pectins. Size-fractionated pectins were used for calibrating the column. The pooled fractions were assayed for apoptotic activity and analyzed by OGA-PAGE as described above. A series of dextran standards of varying molecular weights (Sigma-Aldrich) were included in each run.

Enzymatic digestions as a means to determine the presence of RG-I, RG-I arabinans, RG-I galactans and RG-I arabinogalactans in the apoptotic pectin

The PME+EPG-treated size-fractionated, apoptotically active HTCP (i.e. HPE) was subjected to a series of enzymatic digestions to remove side chains and to further fragment the pectic backbone. The enzymes were added separately (in addition to simultaneously) in order to correlate any loss of activity with removal of a particular sugar or linkage and the pectin was

assayed for apoptosis activity after each treatment. HPE was fragmented by exo-β-galactosidase (*A. niger)*, α-L-arabinofuranosidases (*A. niger* and *Bifidobacterium adolescentis)*, endo-β−1,4-galactosidase (*A. niger)*, endo-1,5-α-L-arabinase (*A. niger)* and RG-I lyase. To carry out this digestion in conditions favorable to the enzymes, ammonium formate, pH 4.5, (except for lyase reactions which were carried out at pH 5.0) was added to 20 mg/mL HPE to give a final ammonium formate concentration of 10 mM. Enzymes as described above were added to the solution. As a negative control, HPE in buffer with no enzyme was compared. The samples were incubated overnight at room temperature (RT) to ensure complete digestion. The enzymes were inactivated by boiling for 10 min at 100°C, and the soluble digested pectin frozen at -80°C and lyophilized.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

HPAEC was used to analyze the apoptotically active enzyme-degraded size-fractionated pectic products using an HPLC system with a Dionex CarboPac P1 column and a Dionex CarboPac Guard Column (3x25 mm). The guard column was used for analytical scale reactions only. The less negatively-charged structures eluted off of the column first followed by the more negatively charged structures. Before fractionating large quantities of each sample, a smaller scale analytical column (4x250 mm) was run and the eluent monitored using in-line electrochemical (PAD) and multiple wavelength detection. The analytical separation provided information on the elution profile and verified that the proposed elution gradient was sufficient. Subsequently, at least 100 mg, ideally, of each apoptotic sample, was loaded onto a large-scale preparatory column (22x250 mm). Oligomeric products were separated with a linear gradient of

50-1000 mM ammonium formate at flow rate of 4 ml min^{-1} with one fraction collected per minute for 70 min. Electronic detection and pulsed amperometric detection (PAD) was not used on the preparatory scale because the NaOH used for PAD could alter the polymeric state of the pectin samples that were subsequently incubated with the cancer cells. Because the pectin samples do not absorb UV light, the fractions were analyzed based on the level of GalA using a uronic acid assay (Blumenkrantz and Absoe-Hansen 1973). Sugar nucleotides (UDP-Glc/ UDP-GlcA or UDP-Gal/ UDP-GalA) which do absorb UV light were included with each sample to ensure stability and consistency of the column. Additionally, a mixture of size specific oligogalacturonides (HG oligosaccharides; OGAs) were also collected and assayed using uronic acid assays to generate a size versus elution time profile.

Glycosyl residue composition analysis

Glycosyl residue composition analysis was performed at the Complex Carbohydrate Service Center (University of Georgia, Athens) by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the samples by acid methanolysis (York *et al*., 1985). Methyl glycosides were prepared by methanolysis in 1M HCl in dry methanol at 80°C for 18-22 hr, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The samples were per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80° C for 0.5 hr. GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5070 MSD using a Supelco DB1 fused silica capillary column (HTCP and HPE) or an HP5890 interfaced to a 5970 NSD using a Supelco DB5 fused silica capillary column.

Glycosyl residue linkage analysis

Glycosyl linkages were determined by GC-MS analysis of partially methylated alditol acetate (PMAA) derivatives. Glycosyl residue linkage analyses was conducted at the Complex Carbohydrate Service Center at the University of Georgia, Athens basically as described by York *et al.* (1985) using both single and double methylation procedures. For the "single methylation" linkage analysis (Table 1.1), the samples were permethylated, reduced, depolymerized, reduced, acetylated and the resultant partially methylated alditol acetate residues analyzed by GC-MS. Specifically, the samples was permethylated by the method of Hakomori (1964) by treatment with dimethylsulfinyl anion (potassium dimsyl anion was used for HTCP and HPE) and methyl iodide in DMSO. The permethylated material reduced by super-deuteride was hydrolyzed in 2M trifluoroacetic acid (TFA) for 2 hr at $121^{\circ}C$, reduced with $NaBD_4$, and acetylated using acetic anhydride/TFA. The resulting partially methylated alditol acetates were separated on a 30 m Supelco 2330 bonded phase fused silica capillary column and analyzed on a Hewlett Packard 5890 GC interfaced to a 5970 mass detector in selective electron impact ionization mode. For the "double methylation" linkage analysis (Tables 1.2 and 1.3), the methods are as described above except that following the first methylation the permethylated material was reduced by super-deuteride (Table 1.2) or lithium deuteride (Table 1.3) and the reduced sample re-methylated using the NaOH/MeI method of Ciucanu and Kerek (1984). The remethylated samples were hydrolyzed using 2M TFA and processed as described above. The single methylation method often yields incomplete methylation resulting in incomplete linkage data. An advantage of the single methylation procedure is that it avoids or greatly reduces fragmentation of the pectin caused by β-elimination, and yielding 4-5 unsaturation of the glycosyluronic acid linkages in HG. In comparison, the double methylation method leads to more complete

methylation, but can lead to β-elimination of the glycosyluronic acid linkages (York *et al*. 1985), and therefore, to an apparent increase in the amount of terminal GalA and a loss in the apparent 4-linked GalA. Thus, the best analysis will come from a close scrutinization of the results of each method on the individual pectic subfractions, keeping in mind the potential limitations of each method.

Reversed phase HPLC column chromatography

Reversed phase chromatography was as described in Tan *et al.* (2013). Two milligrams of each pectin were separated in a gradient over 100 min with solvent A (0.1% [v/v] TFA aqueous) to 50% solvent B (80% [v/v] acetonitrile in 0.1% [v/v] TFA aqueous). Fractions that eluted from the column over 10 minute intervals were pooled and used for analysis.

CHAPTER 2

PECTIN INDUCES APOPTOSIS IN HUMAN PROSTATE CANCER CELLS: A METHOD

FOR CREATING AN APOPTOTICALLY-ACTIVE PECTIN BY DOMESTIC HEAT

TREATMENT OF A PECTIN SOURCE READILY AVAILABLE TO THE U.S.

CONSUMER[2]

[2]Crystal L. Jackson[A,B], Natalia Suarez[B], Debra Mohnen[A,B]
[A]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, GA 30602-4712
[B]Department of Biochemistry and Molecular Biology, UGA Cancer Center, The University of Georgia, Athens, GA 30602
To be submitted

Abstract

   Prostate cancer is the second leading cause of malignancy among men worldwide, with an annual incidence of 240,000 cases and an annual mortality load of 30,000 deaths, making it the second leading cause of cancer deaths among men (American Cancer Society). We previously found that pectin, a major component of the plant primary cell wall, is apoptotically active against both androgen-sensitive (LNCaP) and androgen-insensitive (LNCaP C4-2) lymph node-derived human prostate cancer cells while these same pectins did not induce apoptosis in noncancerous cells. Pectins are composed of three main polysaccharides, homogalaturonan (HG), rhamnmogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) that contain 4-linked α-D-galacturonic acid residues. We have shown that unmodified Citrus Pectin (CP) and pH-modified CP have little or no apoptotic activity against lymph node-derived human prostate cancer cell lines *in vitro*. Heat treatment of CP, however, as used to create the commercially available pectin Fractionated Pectin Powder (FPP), and to produce Heat-Treated CP (HTCP), a heat-modified pectin created in our laboratory, leads to the induction of significant levels of apoptosis in prostate cancer cells (approximately 40-fold above non-treated cells). These results provide a means to generate apoptotic pectic structures. The two apoptosis-inducing pectins, FPP and HTCP, are not easily accessible to the average consumer. However, pectin is a food product and is available domestically in grocery stores. The goal of the present study was to determine if locally-available grocery store-bought pectin, when heated in a manner consistent with methods that may be used by the average consumer using standard kitchen appliances, yielded an apoptotically-active pectin. Four methods of so-called "domestic heat treatment" were developed and used to test the effects of domestic heat treatment on easily accessible commercial pectin. To characterize the resulting products, the store bought pectins were analyzed for uronic acid content, for relative size by polyacrylamide gel electrophoresis, and for apoptotic activity using

an Apoptosense assay. Three of the four domestically heat-treated pectins showed significant levels of apoptotic activity, comparable to FPP and HTCP. The results identify additional sources of apoptotic pectin for structure-function relationship studies and show that domestic heat treatments of grocery store bought pectin can produce pectin with apoptotic activity against human prostate cancer cells *in vitro*.

1. Introduction

Prostate Cancer (PC) has the highest mortality rate among male genital tract neoplasms and is the second leading cause of death from cancer in men in the United States (U.S. Cancer Statistics Working Group. *United States Cancer Statistics: 1999–2009 Incidence and Mortality Web-based Report.* Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute; 2013). Treatment options vary depending on the stage of the cancer and the age of the subject, but common therapies include surgery, external beam radiation, or radioactive seed implants (brachytherapy) to treat early stage disease; and hormonal therapy, chemotherapy, radiation, or a combination to treat more advanced disease. Androgen deprivation therapy is often initiated in an effort to limit or control the growth of prostate cancer for long periods of time by shrinking the size of the cancer. However, this and other treatments can lead to urinary, bowel and/or erectile dysfunction, infertility and other effects attributed to the loss of testosterone. Additionally, androgen deprivation therapies target only those cells that are hormone sensitive, and are ineffective against androgen insensitive cells. These cells are, however, still capable of expiring by apoptosis. Thus, the identification of novel methods to induce apoptosis in prostate cancer cells, irrespective of their androgen sensitivity, is therapeutically significant.

Human prostatic carcinoma has numerous clinical presentations, but most forms are thought to be derived from mutant surface epithelium. Given the number of possible PC histotypes, as well as diverse patient specificities, it is unlikely that any one prostate cancer therapeutic will be curative for all. Increasingly important, however, is the role of dietary components in cancer prevention and progression. It is now evident that there is a correlation between diet and cancer occurrence. According to the American Institute of Cancer Research

(AICR) and the World Cancer Research Fund (WCRF), 30-40% of cancer cases worldwide could be prevented by monitoring diet. It is recognized that diets rich in fruits and vegetables protect against cancers of the mouth, pharynx, esophagus, stomach, colon, rectum, pancreas, lung, larynx, breast and bladder. A component of all fruits and vegetables, pectin, is a complex plant polysaccharide. Pectin accounts for approximately 30% of the primary walls of all higher plants, except the grass family, where it makes up 2-10% of wall biomas (Atmodjo *et al.*, 2013; Mohnen, 2008). While no associations have been found between fruit intake and protate cancer risk, a high consumption of cruciferous vegetables is associated with reduced risk of prostate cancer (Cohen *et al.*, 2000). Pectins have multiple health promoting effects in humans (Yamada *et al.*, 1995; Yamada *et al.*, 2003) including the ability to induce apoptosis in several types of cancer cells. There is also evidence that dietary components and their digestion products have immunostimulatory capabilities (Schley and Field, 2002). Although the usefulness of pectins in cancer therapy is beginning to be appreciated, the mechanism by which pectins induce apoptosis is not known.

Pectin consists mainly of three types of polysaccharides. Homogalacturonan (HG), a linear polymer of 1,4-linked α-D-galactopyranosyluronic acid (GalA) that is partially methylesterified and acetylated, accounts for 57-69% of pectin (Mohnen 2002). Rhamnogalacturonan-I (RG-I) accounts for 7-14% of pectin and consists of a backbone of the repeating disaccharide [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→]. Twenty-eighty percent of the rhamnoses of RG-I are substituted arabinans, galactans, or arabinogalactans (O'Neill *et al.*, 1990; Mohnen, 1999; Ridley *et al.*, 2001). Rhamnogalacturonan II (RG-II) is a substituted galacturonan that accounts for 10-11% of pectin and has a structure that is highly conserved across plant species. RG-II consists of a homogalacturonan backbone with four structurally-

complex side branches (O'Neill *et al.*, 2004). Recently, RG-I and HG domains have also been shown to be present in the cell wall proteoglycan arabinoxylan pectin arabinogalactan I (APAP) (Tan *et al.*, 2013).

Commercially available pectin is usually produced by an acid extraction of pectin from dried citrus peels or apple pomace (Thakur *et al.*, 1997), a process that results in the partial loss of RG-I and RG-II. Commercial citrus pectin may be further treated with base or heat to yield partially fragmented and structurally modified pectin. Therefore, in order to evaluate and understand the biological efficacy of pectin, the structure of the biologically active pectin must be addressed.

We previously showed that the apoptotic activity and structure of commercially available pectin differs depending on the method of its preparation (Jackson *et al.*, 2007). For example, several commercially available pectin preparations, Citrus Pectin (CP), Pectasol (PeS) and Fractionated Pectin Powder (FPP), differ in their ability to induce apoptosis in human prostate cancer cells (Jackson *et al.*, 2007). CP, which represents the starting pectin used to make the modified pectins, has little or no apoptotic action on LNCaP cells. Likewise, the pH-modified pectin, PeS, generated by base treatment of CP had very little ability to induce apoptosis in human prostate cancer cells (Jackson *et al.*, 2007). FPP, however, a type of commercial pectin generated by heat treatment of CP, induced significant apoptosis in LNCaP cells compared to cells incubated in media devoid of pectin. Of particular relevance to this paper, we found that a heat-modified citrus pectin created in our laboratory, HTCP, induces apoptosis in androgen sensitive LNCaP and androgen unresponsive LNCaP C4-2 lymph node-derived human prostate cancer cell lines at levels comparable to that of FPP and comparable to the positive control thapsigargin, a compound that induces high levels of apoptosis in prostate cancer cells (Jackson

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et al., 2007). We also previously showed that chemical and enzymatic manipulation of the structure of the heat modified pectin reduces its apoptotic activity and thus conclude that a base-sensitive linkage is necessary for the apoptotic activity of pectin. Apoptosis in the afore mentioned work was quantified using an ELISA to measure the generation of an apoptosis-specific neoepitope of cytokeratin-18, a substrate of activated caspase (Materials and Methods).

The two apoptosis-inducing pectins, FPP and HTCP are not easily accessible or economical to the average consumer. However, pectin is sold as a gelling agent in grocery stores and is available in such a form to the consumer. The goal of the present study was to determine if apoptotic heat-modified pectin could be generated domestically using a readily-available pectin source from the grocery store and standard kitchen appliances and cooking methods. The rationale is that Fractionated Pectin Powder (FPP), a commercially available pectin generated by heat modification of citrus pectin, as well as heat-treated citrus pectin created in our laboratory, both induce apoptosis in prostate cancer cells. Thus it was likely that a simple heat treatment of domestically available pectin used in normal kitchen food preparation methods, could yield apoptotic pectin. Such an approach is analogous to the actions by indigenous people world-wide who utilize local flora and simple distillation and maceration techniques to yield pectic polysaccharide-enriched fractions possessing mitogenic activity (Nergard *et al.*, 2004). Here we report that three of the four methods employed to produce domestically accessible and heat-modified pectin yield a pectin that induces apoptosis in human prostate cancer cells *in vitro*.

2. Results

Strategy

FPP and HTCP are not easily available or economical to the average consumer. In an effort to determine if apoptotically-active pectin is generated using publically available pectin, we applied a series of heat treatments, using standard kitchen appliances, to a grocery store bought pectin, 100% Natural **Sure Jell®** Premium Fruit Pectin.

We previously showed that heat treatment of the non- or low apoptotic Commercially available citrus pectin (CP), by autoclaving at $123.2^{o}C$ and 17.2-21.7 psi for 30 or 60 minutes, significantly increased the apoptotic response, making heat-treated CP (HTCP) as apoptotically active as FPP (Jackson *et al.*, 2007). The application of a heating cycle longer than 60 minutes only slightly increased the apoptotic response of the treated CP compared to the 30 minute treatment. In this report, four methods (Table 2.1) were developed to create domestically heat-treated pectins (DOM HTP). A 0.1% solution of store bought Sure Jell® pectin was heated using standard kitchen appliances including a microwave oven, a stove top range, and/or a pressure cooker. For three of the methods, pectin was first dissolved in hot water that had been heated in a microwave oven at 100% power for three minutes. The exception was DOM HTP II, in which pectin powder was first heated alone before being dissolved in the microwaved water. All solutions were then further heated either in a glass dish in a microwave or in a sauce pan or pressure cooker on a stove top range as indicated (see Table 1 and Materials and Methods). The effects of the domestic heat treatments on the store bought pectin were analyzed by uronic acid assays, polyacrylamide gel electrophoresis (PAGE) and an Apoptosense assay to test for apoptotic activity.

Table 2.1. Summary of methods used to produce domestically heat-treated pectins (DOM HTP).

Pectin Sample	DOM HTP I (Domestic Heat-Treated Pectin I)	DOM HTP II [b] (Domestic Heat-Treated Pectin II)	DOM HTP II (Domestic Heat-Treated Pectin III)	DOM HTP IV (Domestic Heat-Treated Pectin IV)	AUTO HTP [c] (Autoclaved pectin)
Method[a]	Dissolve in pre-heated water 5 min; Microwave 10 min	Heat pectin powder 3 min; dissolve in pre-heated water 5 min; Microwave 10 min	Dissolve in pre-heated water 3 min; Cook on stove for 10 min at medium heat	Dissolve in pre-heated water 3 min; Cook in pressure cooker for 3 min	Dissolve in de-ionized water; Autoclave at 123.2ºC, 17.2-21.7 psi, 30 min

[a]All DOM HTP pectins were made at a concentration of ~0.1% by dissolving 1/8 teaspoon (~500 mg) of Sure Jell Pectin in 500 mL water. For all pectins except DOM HTP II, the pectin was dissolved in water that had been pre-heated in a microwave at 100% power for three minutes. Standard kitchen supplies and appliances were used to generated all the DOM HTP pectins.

[b]For DOM HTP II, as indicated, the pectin powder itself was first heated in a microwave before being dissolved in the microwaved water (Experimental).

[c]Sure Jell pectin was autoclaved in the laboratory for a 30 min cycle.

Uronic acid analysis of pectins modified by domestic heat treatment

The effects of the heat treatments on the pectins were analyzed by uronic acid assays of the domestically heat-treated Sure Jell® pectins DOM HTP 1-IV and the autoclaved Sure Jell® pectin and the results were compared those from previously characterized heat-treated citrus pectin, HTCP (Jackson *et al.*, 2007). Uronic acid assays were used to measure the total uronic acid content in the domestically heat-treated pectins. The uronic acid content of the DOM HTP I-IV is shown in Figure 2.1 as ug uronic acid/ ug of total mass of material. DOM HTP I was created by dissolving pectin in microwave-oven heated water and then heating the 0.1% pectin solution in a microwave. DOM HTP II was produced by utilizing a microwave to first heat the pectin before dissolving it in hot water. DOM HTP III and DOM HTP IV were produced by the same method as DOM HTP I with the exception that instead of heating the pectin solution in a microwave, the solutions were heated on a stove in a sauce pan or pressure cooker, respectively. Pectins from dicotyledonous plants, such as citrus, contain approximately 57-69 mole% galacturonic acid (Mohnen *et al.*, 1996). The uronic acid content of 0.51 ug uronic acid/total mass of HTCP (heat-treated citrus pectin) is consistent with the expected uronic acid content of pectin. The uronic acid content of the DOM HTP ranged from 11-26%, which was 22-51% of the value expected for pectin. The low uronic acid content in the DOM HTP was due, at least in part, to the presence of other ingredients in the Sure Jell® pectin (i.e. dextrose and citric acid). The possibility that the method of heat treatment used to produce HTCP in the laboratory, autoclaving, more fully dissociated or dissolved the pectin and thus allowed for more effective detection of the uronic acid present in the HTCP was ruled out since autoclaving Sure Jell® in the same manner as HTCP (see Autoclaved DOM in Figure 2.1), did not yield an increased uronic acid content compared to the other DOM HTP treatments.
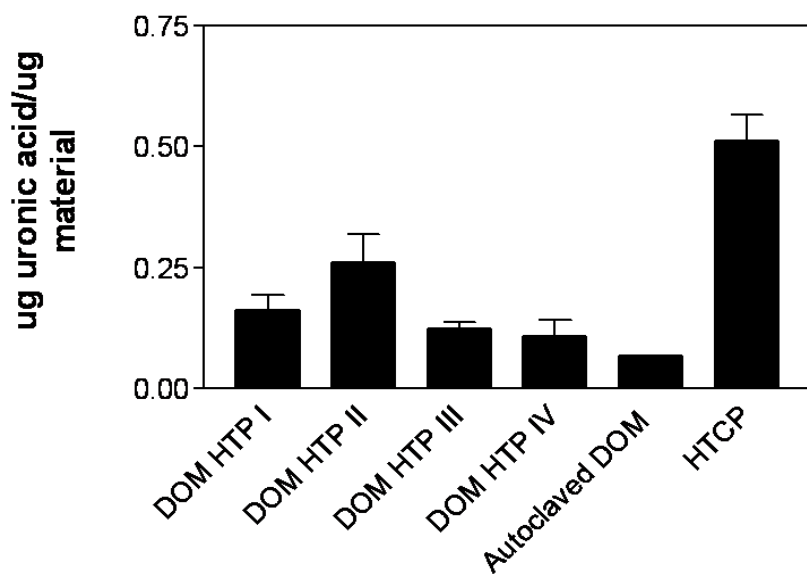
Figure 2.1. Uronic acid analysis of pectins produced by domestic heat treatment (DOM HTP I-IV) compared to HTCP and Autoclaved DOM. One mg of Sure Jell® (Kraft Foods, Tarrytown, NY) was mixed in one mL water and heated as described. The Autoclaved DOM was the same pectin source as the DOM HTPs, but was autoclaved in the laboratory as opposed to domestic heat treatment. Data are the average of duplicate assays of 3 replicates ± SEM. Similar results were obtained in at least three experiments. DOM HTP I-IV as in Table I.

Polyacrylamide gel electrophoresis of domestically heat-treated pectins

The domestically heat-treated samples DOM HTP I-IV were separated by high percentage polyacrylamide gel electrophoresis (PAGE) to visually compare the polymeric structural characteristics of the domestically heat-treated pectin to FPP and HTCP, the previously analyzed apoptotic heat-treated pectins (Jackson *et al.*, 2007). PAGE separates pectin based on charge and molecular weight and such gels are particularly useful to separate HG oligosaccharides (oligogalacturonides, OGAs) (Djelineo 2001). The PAGE gels were first stained with alcian blue and then silver nitrate to detect the pectins. Alcian blue is a positively-charged dye that binds the negatively charged GalA in the pectin. Figure 2.2 shows that the control pectins, HTCP and FPP (Figure 2.2A lanes 2 and 7; Figure 2.2B lanes 3 and 8), had the expected structural characteristics as previously reported (Jackson *et al.*, 2007). The previous study showed that whereas unmodified citrus pectin, due to its large polymeric structure, barely enters these PAGE gels, the heat-treated apoptotic pectins, HTCP or FPP, separate in PAGE as a smear of dark staining polymeric pectin near the top of the gel and HG oligosaccharides (oligogalacturonides, OGAs) lower in the gel.

Equal masses of the DOM HTP samples and HTCP or FPP were loaded into the PAGE gels shown in Figure 2.2. The HTCP and FPP separated as smears of dark staining polymeric pectin near the top of the gel with discrete bands representing OGAs in the lower portion of the gel (Figure 2.2A, lanes 2 and 7; Figure 2.2B, lanes 3 and 8), however, the commercial pectins prepared by heating with standard kitchen appliances, DOM HTP I-IV, separated as smear of dark staining in the upper portion of the gel, with no discernible discrete OGA bands at the bottom of the gel (Figure 2.2A , lanes 3-6; Figure 2.2B, lanes 4-7). As expected from the uronic

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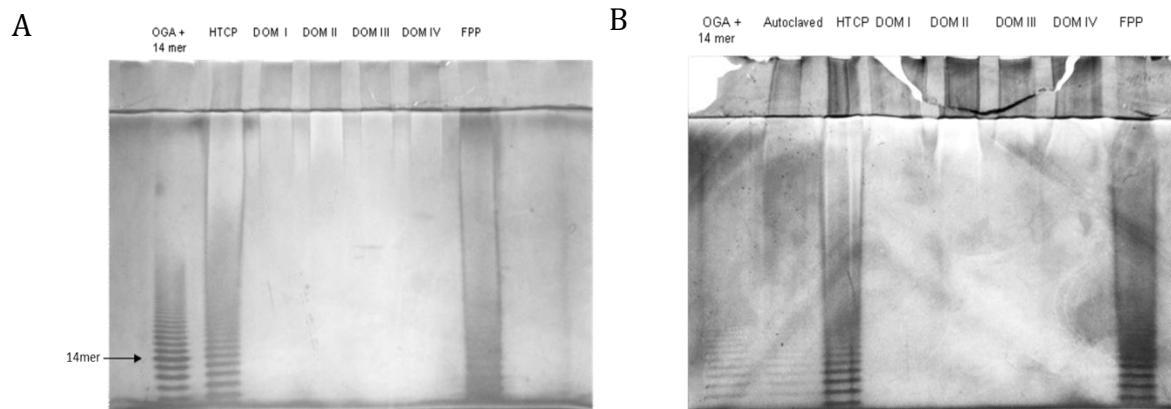
Figure. 2.2 Separation of pectins by polyacrylamide gel electrophoresis. (A) DOM HTPs, FPP, HTCP separated by PAGE (see Materials and Methods). Lane 1, 0.01 µg oligogalacturonide (OGA) of DP 14 (see arrow) plus 0.1 µg of OGAs of DP ~7-23; Lanes 2-7, 6 µg of HTCP, DOM I-IV and FPP, respectively. **(B)** Same as in 2A with the addition of Autoclaved DOM in lane 2. Lane 1, 0.01 µg OGA of DP 14 (faint) plus 0.1 µg mixture of OGAs of DP ~7-23; Lanes 2-8, 6 µg of Autoclaved DOM, HTCP, DOM I-IV and FPP, respectively.

acid results, DOM HTP II gave the most intense staining. Figure 2.2B also shows that the Autoclaved DOM (lane 2), more closely resembles FPP and HTCP when visualized by PAGE in that the smear of alcian blue staining material in the autoclaved DOM more broadly separates lower into the gel, suggesting that the longer and higher temperature heat treatment either more fully dissociated, or partially cleaved the pectin.

Apoptotic activity of domestically heat-treated pectins on lymph node-derived human prostate cancer cell line (LNCaP)

An Apoptosense Assay was utilized to determine whether one or more of the domestic heat treatments yielded a pectin with significant apoptosis activity on human prostate cancer cells in culture (Figure 2.3). LNCaP human prostate cancer cells were treated for 48 hr with 1 mg/mL of each pectin, DOM HTP 1-IV, autoclaved DOM and the control apoptotic pectin HTCP. Untreated LNCaP cells served as the negative control and LNCaP cells treated with the apoptosis-inducing compound, thapsigargin, served as the positive control. The non-treated Sure Jell® was not tested as it was not miscible in water without heating. As expected, HTCP induced considerable levels of apoptotic activity, approximately 25-fold above the negative control. Autoclaved DOM induced comparable levels of apoptosis, indicating that the same heat treatment used to convert the non- or low apoptotic citrus pectin to apoptotic pectin (Jackson et al., 2007) yielded apoptotic Sure Jell®. Of the domestically heat treated pectins, DOM HTP I showed the highest levels of apoptosis, though all domestically heat-treated pectins gave some induction of apoptotic activity. DOM HTP III and IV provided significant levels comparable to DOM I, approaching the levels induced by treatment of LNCaPs with HTCP.
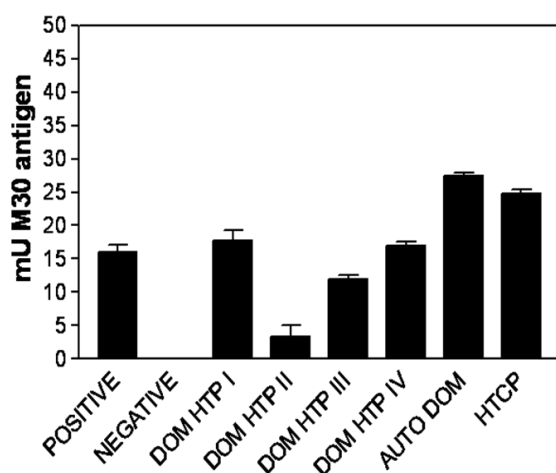
Figure .2.3. Induction of apoptosis by DOM HTPs. LNCaP cells were treated with 1 mg/mL of DOM HTP 1-IV, HTCP or with 0.01 mM thapsigargin (Positive) for 48 hr. Incubation with media alone served as the negative control (Negative). Equal amounts of cell extracts (10 µg protein) was used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts ± SEM. Comparable results were obtained in at least two experiments.

3. Discussion

Citrus pectin has been shown to induce apoptosis in cultured human prostate cancer cells (Jackson *et al.*, 2007).The amount of this apoptotic activity varied, depending on the source of the pectin and the manner in which the pectin was produced and/or treated. Heat treating pectin by autoclaving to produce the so-called 'heat-treated citrus pectin (HTCP)' was particularly effective in producing pectin that induced apoptosis in human prostate cancer cells *in vitro* (Jackson *et al.*, 2007). The goal of the present study was to determine whether one or more methods of heating pectin in the home, using an easily available source of pectin from the grocery store, also yielded a pectin that induces apoptosis in human prostate cancer cells *in vitro*. Four treatment protocols were developed wherein a 0.1% pectin solution was heated utilizing the standard kitchen appliances of a microwave oven, a stove top range with a pan and a pressure cooker.

Three of the domestic heat treatment methods (DOM HTP I, III, IV) generated pectins with appreciable apoptotic activity on LNCaP cells. Each of these three methods included an initial step of dissolving pectin in hot water prior to the subsequent heat treatments of microwaving for ten minutes, cooking on a stove top in a pan for ten minutes, cooking in a pressure cooker for three minutes. Of these three methods, DOM HTP I (microwave treatment) and DOM HTP IV (heating with a pressure cooker) generated pectin with the most apoptotic activity. The apoptotic activity of DOM HTP II was significantly less than the other domestically prepared pectins. The method used to produce DOM HTP II included heating the dry pectin powder before dissolving in hot water. The results suggest that this step was detrimental to recovery of apoptotic activity.

Figure 2.2 shows that, when visualized by PAGE, DOM HTPs I-IV, contain polymeric material similar, in part, similar in size to the apoptosis inducing pectins FPP and HTCP. This supports the hypothesis previously presented in Jackson *et al.* (2007), that linkage into a polymeric or oligomeric structure is responsible for the apoptosis-inducing activity of pectin. Furthermore, Figure 2.2.B shows that when the pectin used to create DOM HTP I-IV is autoclaved, it separates into a smear of dark staining polymeric pectin near the top of the gel and oligogalacturonides (OGAs) lower in the gel, a pattern that is very similar to the previously described apoptotic FPP and HTCP (Jackson *et al.*, 2007). Detailed structural differences among the DOM HTPs cannot be visualized by PAGE.

Three of the four domestic heat treatments generated a pectin that had significant apoptotic activity, comparable to the FPP, HTCP and thapsigargin controls, signifying that multiple means of heating pectin may be employed to produce pectin with apoptotic activity on human prostate cancer cells *in vitro*. DOM HTP I had the highest relative level of apoptosis of the domestically heated pectins (Figure 2.3), indicating that microwave heating of pectin is sufficient to obtain a pectin structure that is capable of inducing apoptosis, comparable to if not more effective than, heating pectin under pressure as was done for DOM HTP IV.

Whether or not longer or alternative heat treatments would yield a pectin with enhanced apoptotic activity was not investigated. When compared to FPP and HTCP, a considerable amount of material remained in the stacking portion of the PAGE gel with the DOM HTP samples. Perhaps a longer heating period would yield a more apoptotically active pectin. However, a longer heat treatment could also alter the DOM structures in a deleterious manner. Further experimentation would be required to discern between these two possibilities.

In Jackson *et al.* (2007), we presented the first analysis that correlates structural features of pectin, a plant polysaccharide fiber, with apoptosis-inducing activity on human prostate cancer cells *in vitro*. In the work reported here we show that several methods consistent with cooking practices used in the home generate a pectin that has apoptotic activity on human prostate cancer cells in the laboratory. However, further experimentation on a clinical scale will be required to determine whether oral update of pectins cooked in the manner described in this paper significantly affects the occurrence of prostate cancer in humans.

4. Materials and Methods

Materials

The androgen-responsive LNCaP human prostate cancer cell line (obtained from American Type Culture Collection, Rockville, MD) was utilized in these experiments. RPMI-1640 medium supplemented with 25 mM HEPES and L-glutamine was purchased from Hyclone (Logan, Utah). Fetal Bovine Serum (FBS), penicillin/streptomycin, citrus pectin (P-9135), sodium hydroxide, alcian blue and caspase-3 colorimetric assay kit were from Sigma-Aldrich (St. Louis, MO). Fungizone was obtained from Invitrogen (Carlsbad, CA). Bio-Rad Protein Assay Dye Reagent concentrate was purchased from Bio-Rad (Hercules, CA). The M-30 Apoptosense ELISA kit was from Peviva AB (Sweden). Sodium carbonate and sodium acetate were purchased from J. T. Baker (Phillipsburg, NJ) and acetic acid from EM Science (Gibbstown, NJ). Fractionated Pectin Powder was purchased from Thorne Research catalog #SF778 (Dover, ID). HTCP was made as in Jackson *et al*. (2007). 100% natural *Sure Jell®Premium* Fruit Pectin (Kraft Foods, Tarrytown, NY) for homemade jams and jellies was purchased at a Walmart Super Store. All other chemicals, unless otherwise stated, were from

Fisher Scientific. *Other materials:* The stove top range, microwave oven, pressure cooker, Pyrex glass dish (7" x 5" x 1.5"), and Pyrex measuring cup were from standard domestic vendors.

Preparation of Domestic Heat-Treated Pectin I (DOM HTP I)

Tap water (500 ml) in a covered Pyrex glass container was heated in a microwave at the highest power setting for three minutes and a 0.1% aqueous pectin dispersion was prepared by dissolving 500 mg (1/8 tsp) of Sure Jell® Premium Fruit Pectin. The pectin was slowly dissolved in the warm water over a five minute time span. The pectin solution was microwaved for ten minutes in a covered Pyrex glass container. At the end of the heat treatment, the solution was allowed to cool to room temperature and stored overnight in a -20 °C freezer. The next day the gelled solution was lyophilized.

Preparation of Domestic Heat-Treated Pectin II (DOM HTP II)

Five hundred mg (1/8 tsp) of Sure Jell® powder was heated in a microwave for 3 min and mixed into 500 ml of water that had been previously heated for 3 minutes in a microwave in a covered Pyrex glass container. The pectin was dissolved in the warm water over a five minute time span. The 0.1% pectin solution was microwaved at the highest power setting for ten minutes in a covered Pyrex glass container. Following the heat treatment, the solution was allowed to cool to room temperature and stored overnight in a -20°C freezer. The next day the gelled solution was lyophilized.

Preparation of Domestic Heat-Treated Pectin III (DOM HTP III)

Tap water (500 ml) in a covered Pyrex glass container was heated in a microwave at the highest power setting for three minutes and a 0.1% aqueous pectin dispersion was prepared by

dissolving 500 mg (1/8 tsp) of Sure Jell® Premium Fruit Pectin in the warm water over a three minute time span. The aqueous solution was heated (while covered) for ten minutes on a stove top range over medium heat. At the end of the heat treatment, the solution was allowed to cool to room temperature and stored overnight in a -20°C freezer. The next day the gelled solution was lyophilized.

Preparation of Domestic Heat-Treated Pectin IV (DOM HTP IV)

Tap water (500 ml) in a covered Pyrex glass container was heated in a microwave at the highest power setting for three minutes and a 0.1% aqueous pectin dispersion was prepared by dissolving 500 mg (1/8 tsp) of Sure Jell® Premium Fruit Pectin in the warm water over a three min time span. The aqueous solution was heated for three min on a stove top range under pressure using a pressure cooker. The heat treatment began as soon as the aqueous solution was poured into the pressure cooker. At the end of the heat treatment, the solution was allowed to cool to room temperature and stored overnight in a -20 °C freezer. The next day the gelled solution was lyophilized.

Preparation of Autoclaved Domestic Pectin (Autoclaved DOM)

A 0.1% aqueous pectin dispersion was prepared by dissolving 500 mg of unmodified Sure Jell® Pectin in 500 ml of de-ionized, filtered water. The solution was autoclaved at 123.2^{o}C, 17.2-21.7 psi for 30 minutes. Following autoclaving, the solution was allowed to cool to room temperature and stored overnight in -20°C freezer. The next day the gelled solution was lyophilized.

Polyacrylamide Gel Electrophoresis of Pectins

Pectin samples were dissolved (there was no visual precipitate) in deionized water (1 mg/mL) and separated by high percentage acrylamide PAGE and analyzed by alcian blue staining as previously described (Jackson *et al*. 2007). Pectin samples were mixed in a 5:1 ratio with 6X sample buffer (0.63 M Tris-Cl/ pH 6.8, 0.05% phenol red, 50% glycerol), loaded onto a resolving gel (0.38 M Tris, pH 8.8, 30% (wt/vol) acrylamide (37.5:1 acrylamide: bis-acrylamide, wt/wt, Bio-Rad)) overlaid with a stacking gel (5% acrylamide, 0.64 M Tris, pH 6.8 M) and separated at 17.5 mAmps for 60 minutes or until the phenol dye was within 1 cm of the end of the gel. The gel was stained with 0.2% alcian blue (Sigma-Aldrich A5268) in 40% ethanol for twenty minutes while rocking, washed three times for twenty seconds and then incubated for twenty minutes in water on the rocker. The gel was incubated while rocking in 0.2% silver nitrate containing 0.075% formaldehyde, rinsed three times for twenty seconds with water, and incubated in 4% sodium carbonate containing 0.05% formaldehyde until bands appeared. The carbonate solution was immediately removed and the reaction stopped with 5% acetic acid. The gel was stored overnight in water.

Uronic Acid Assay

Triplicate galacturonic acid standards were made by mixing the appropriate volume of galacturonic acid (1 mg/mL stocks) with water to give a final volume of 50 ul in Sarstedt #73.1055 RIA vials arranged in Sarstedt #95.1010 RIA metal vial racks. A mass of 0, 1, 2, 4, 6, 8, and 10 ug per well was made for each uronic acid assay standard. Triplicate five µl samples of OGA, HTCP, FPP, DOM HTP I, DOM HTP II, DOM HTP III, and DOM HTP IV were assayed. Three hundred µl of ice-cold sodium tetraborate solution (0.0125 M sodium tetraborate in

concentrated sulfuric acid) was added to each vial. The rack was covered and mixed for 5 minutes and the samples heated for fifteen minutes on a heating block at 100°C. The plate was placed in ice for five minutes or until cool to the touch. Five μl meta-hydroxybiphenyl reagent (0.15% m-hydroxybiphenyl in 0.5% NaOH) was added to each vial and the vials mixed (500 rpm) for five minutes. Two hundred microliters (200 ul) of the solution was carefully placed into a microtiter plate. Each well was mixed again by pipeting 3 to 4 times before being transferred to the microtiter plate to ensure complete mixing in the wells. The absorbance was measured at 540 nm on a Microplate Manager Bio-Rad Laboratories, Inc.

Cell Culture, Apoptosense Assay, and Preparation of Cell Lysates

LNCaP cells were grown in RPMI-1640 medium supplemented with 25 mM HEPES, 2.0 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 U/ml Penicillin, 0.05 mg/mL Streptomycin, and 0.25 g/ml Fungizone in the presence of 5% CO_2 at 37°C. Cells were maintained in logarithmic growth phase by routine passage every 10-12 days (LNCaP). Cells were plated at a density of 1.6 x 10^5 cells per well in 6-well culture plates and allowed to adhere for 24 hr. The medium was removed and replaced with medium containing filter-sterilized pectin (0.20 μm nylon filters; Fisher Scientific). Treated cells were incubated in media containing the following compounds (1 mg/mL final concentration) as indicated: Heat-Treated Citrus Pectin (HTCP), Fractionated Pectin Powder (FPP), Domestic Heat-Treated Pectin I (DOM HTP I), Domestic Heat-Treated Pectin II (DOM HTP II), Domestic Heat-Treated Pectin III (DOM HTP III, Domestic Heat-Treated Pectin IV (DOM HTP IV), Autoclaved DOM Pectin (AUTO DOM) and Thapsigargin positive control (Sigma, St. Louis, MO). The negative control was untreated cells cultured in media alone. Cells were harvested after 48 hr and lysed in ice cold Lysis Buffer (10 mM Tris-HCL, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40),

incubated on ice for five minutes, and soluble protein was collected by centrifugation at 4°C. Protein concentration was determined in triplicate using a Bradford/Bio-Rad Protein Assay (Bio-Rad Protein Assay Dye Reagent Concentrate).

Apoptotic activity was assayed using the M-30 Apoptosense ELISA. Cells grown in culture flasks and treated as described above were harvested and total protein extracted as described above. Protein was assayed for the presence of the apoptosis-specific cytokeratin-18 neoepitope (generated by cleavage of cytokeratin-18 by caspases activated in response to treatment) using the M30-Apoptosense ELISA (Peviva AB, Sweden). In brief, protein extract (10 μg) was added to 96-well plates coated with mouse monoclonal M30 antibody. Horseradish peroxidase tracer solution was added to the wells in a dark room illuminated with a green safety light, and the plate was incubated with agitation for 4 hr at room temperature. Color was developed by adding tetramethyl benzidine solution and incubating in darkness for 20 min. Optical density was determined at 450 nm using Spectra MAX 340 (Molecular Devices, Menlo Park, CA) or Finstruments Model 347 (Vienna, Virginia, USA) microplate readers. The amount of cytokeratin-18 neoepitope was determined based on standard curves generated using standards provided by the manufacturer.

Abbreviations

FPP, Fractionated pectin powder; HG, Homogalacturonan; HTCP, heat-treated citrus pectin; RG-I, Rhamnogalacturonan-I; RG-II, Rhamnogalacturonan II; PeS, Pectasol; CP, Citrus Pectin; DOM HTP, domestically heat-treated pectin; PAGE, polyacrylamide gel electrophoresis

CHAPTER 3

PROGRESS TOWARDS DETERMINING THE MECHANISM OF ACTION OF APOPTOTIC

PECTIN[3]

[3]Crystal L. Jackson[A,B], Debra Mohnen[A,B], Richard Steet[A,B]
[A]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, GA 30602-4712
[B]Department of Biochemistry and Molecular Biology, UGA Cancer Center, The University of Georgia, Athens, GA 30602
To be submitted

Abstract

Our prior published data (Jackson *et al.* 2007) showed that heat-treatment of commercial pectin generates a structure that induces apoptosis in a lymph node-derived human prostate cancer cell line, LNCaP, and that digestion of the apoptotic pectin with pectinases and pectinmethylesterases (mimicking the treatments utilized to isolate pectin from the cell wall) does not destroy the apoptotic activity. Since the cell line was developed, LNCaPs have been the most extensively utilized model of androgen-responsive prostate cancer *in vitro* (Horoszewicz *et al.*, 1980). The LNCaP C4-2 cell line, also used as an *in vitro* model in addition to the LNCaPs used in the prior study (Jackson *et al.* 2007), was derived from LNCaP cells by passing twice through nude mice. LNCaP C4-2 cells, unlike LNCaP cells, are androgen independent. The goal of this research was to study the mechanism of pectin-induced apoptotic activity in these *in vitro* cancer models. Our results suggest that pectin may be sensitizing LNCaP cells to TRAIL (Tumor necrosis factor (TNF)-related apoptosis inducing ligand) by inhibiting phosphatidylinositol PIP3 kinase (PI3K) activity. The results also suggest that pectin may increase the sensitivity of androgen-resistant LNCaP cells to death by androgen withdrawal. Furthermore, the inability of TGF beta1 alone or in the presence of varying concentrations of the apoptotic pectin to induce apoptosis in LNCaPs suggests that pectin does not sensitize LNCaPs to TGF beta1 signaling but instead TGF beta1 inhibits the apoptotic response of pectin. Taken together, these data confirm that cell proliferation is indeed inhibited in FPP-treated LNCaP cells through some mechanism that affects the $NF_\kappa B$ signaling pathway, perhaps one involving sensitization to TRAIL.

Keywords: **Apoptosis, Prostate Cancer, LNCaP, TRAIL, Mechanism, Pectin, Fractionated Pectin Powder**

1. Background and Introduction

Apoptosis vs. necrosis

In all adult systems, except for the nervous system and muscle, there is regular cellular replication throughout life (Cooper 2000). Occasionally cells proliferate at an atypical rate, forming a tumor/neoplasm, a localized mass of growing cells (Ewing 1921). As long as this neoplasm is kept clustered together in a single mass, the tumor is said to be benign. It is not until rapidly-proliferating, abnormal cells invade surrounding tissues that they become malignant or cancerous (Ptot1986). Therefore, the pathogenicity of many diseases is a result of too little cell death, particularly of too little apoptosis. Apoptosis was first recognized as a form of cell death in mature cells in 1972 (Kerr *et al.* 1972). The mechanism of how apoptosis is induced and executed has only become clear in the last 15-20 years. Unlike necrosis, which is characterized as the premature and accidental death of cells and living tissue resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents (Fink and Cookson 2005), apoptosis is a programmed cell suicide. In cancer, cells evade normal growth regulatory mechanisms including apoptosis and begin to divide and invade surrounding tissues without constraint. Therefore, programmed cell death is critical not only for early development, but also for homeostasis throughout life.

Apoptotic pathways

Apoptosis is characterized by membrane inversion (leading to exposure of phosphatidyl serine residues on the cell surface), membrane blebbing, DNA fragmentation, and chromatin condensation (as a result of cell shrinkage) and is the result of two distinct cellular pathways (Kerr *et al.* 1972; Fink and Cookson, 2005). In the extrinsic pathway, transmembrane death receptors are activated by their respective ligands such as Fas-L and TRAIL (Tumor necrosis

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factor (TNF)-related apoptosis inducing ligand) which bind to the cell surface causing the aggregation of death receptors (Table 3.1; Table 3.2; Figure 3.1). This triggers initiator caspases (cysteine-dependent aspartate-directed proteases) 8, 2, 9, and 10 to cleave the pro-forms of effector caspases (caspase-3,-6 and -7) resulting in their activation in the initiation of apoptosis. Apoptosis is ultimately displayed by the destruction of multiple proteins including PARP (poly-ADP ribose polymerase) cleaved by caspase-7, proteins involved in RNA synthesis and splicing, cytoplasmic and nuclear proteins including DFF (DNA fragmentation factor), α-Fodrin (a noerythroid secretin that is universally associated with membrane-bound cytoskeletal proteins), and Lamin A/C (nuclear proteins involved in DNA and chromatin organization) (Figure 3.1). Lamins are cleaved by caspase-6 resulting in nuclear disorder and chromatin condensation. Caspases-3, -6, and -7 can be inhibited by IAP family proteins which include XIAP, Survivin (a G2/M phase checkpoint protein), livin, cIAP1, and cIAP2. IAPs (inhibitor of apoptosis proteins) bind to caspases through their BIR (baculovirus inhibitor of apoptosis protein repeats) inhibiting them directly. In response to stress, the mitochondrial associated proteins SMAC and DIABLO are released to compete with the binding of IAPs to caspases (Figure 3.1). When SMAC and DIABLO bind IAPs, caspases are uninhibited and apoptosis progresses.

Table 3.1. Summary of apoptotic death receptors and their respective ligands.

Receptor	Ligand
TNFR1 (DR1)	TNF-α, LT-α
FAS (CD95, APO-1, DR2)	FasL
TRAIL-R1 (DR4)	TRAIL
TRAIL-R2 (DR5)	TRAIL
DR3 (APO2)	TL1, VEGI
DR6	?
NGFR	NGF
EDAR	Eda

Source: Methods and compositions for detecting receptor ligand mimetics; http://www.google.com/patents/EP2089711A2?cl=en

Table 3.2. Pro- and anti-apoptotic factors.

Anti-apoptotic	Pro-apoptotic
Bcl-2	BID
Bcl-XL	BIM
Bcl-w	BAD
Mcl-1	T-IKBα
Bfl/A1	
p-IKBα	

In the second pathway, the intrinsic pathway, an apoptotic signal is amplified through the involvement of Cytochrome C in mitochondria. The pro-apoptotic protein Bid is cleaved by an active caspase-8 in response to a death stimulus, resulting in tBid or truncated Bid. tBid translocates to the mitochondria where it disrupts the mitochondrial inner membrane potential causing the release of apoptogenic factor Cytochrome C. Cytochorome C is important in mitochondrial respiration and energy production. Upon its release from the mitochondria, Cytochrome C binds to Apaf-1 (apoptosis activating factor-1) triggering a conformational change and the formation of the apoptosome complex (7 molecules of cytochrome C, Apaf-1 and ATP). A caspase activating and recruiting death domain (CARD) binds to the apoptosome and sequesters caspase-9. Caspase-9 sequestration activates caspase-3 leading to downstream apoptosis (Figure 3.1) (Fulda and Debatin 2006).

Apoptosis in lymph node derived human prostate cancer cells (LNCaPs)

LNCaPs, like some other cancer cells, are resistant to TRAIL-induced apoptosis because of a mutation in the tumor suppressor gene PTEN (phosphatase and tensin homolog) (Chow and Clement 2006; Chen *et al.* 2001). This mutation causes these cells to lack an active lipid phosphatase PTEN, a negative regulator of the phosphatidylinositol PIP3 kinase (PI3K) ➔ Akt
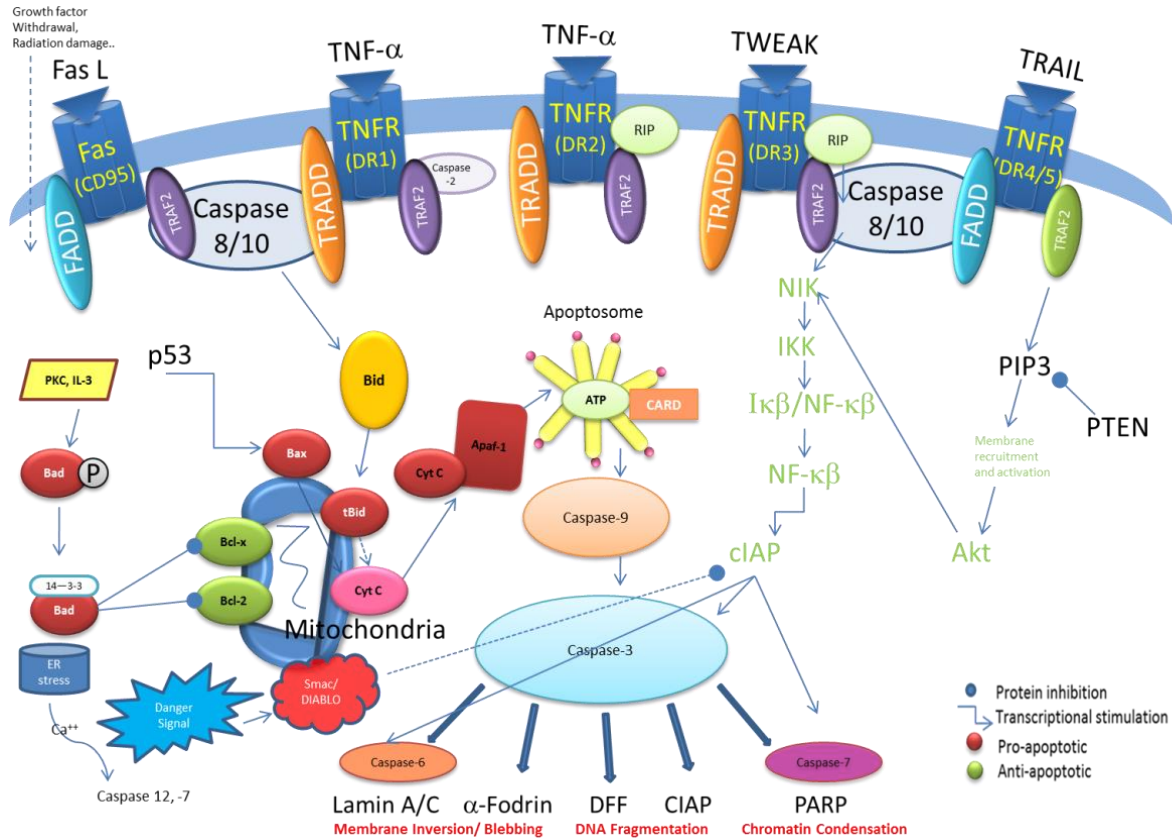
Figure 3.1. Apoptotic signaling pathways. In the extrinsic pathway, transmembrane death receptors are activated by their respective ligands for example Fas-L, TNF-α (Tumor necrosis factor-alpha), TWEAK (TNF-like weak inducer of apoptosis), and TRAIL (Tumor necrosis factor (TNF)-related apoptosis inducing ligand) which bind to the cell surface causing the aggregation of death receptors. This activates protease caspase-8 which in turn leads to the activation of caspase-3 resulting in apoptosis and ultimately in the destruction of nuclear proteins including PARP (poly-ADP ribose polymerase), DFF (DNA fragmentation factor), α-Fodrin, and Lamin A/C. In the intrinsic pathway, the pro-apoptotic protein Bid is cleaved by an active caspase-8 in response to a death stimulus such as growth factor withdrawal or radiation damage, resulting in tBid or truncated Bid. tBid translocates to the mitochondria where it disrupts mitochondrial inner membrane potential causing the release of apoptogenic factor Cytochrome C. Upon its release from the mitochondria, Cytochrome C binds to Apaf-1 (apoptosis activating factor-1) triggering a conformational change and the formation of the apoptosome complex (7 molecules of cytochrome C, Apaf-1 and ATP). A caspase activating and recruiting death domain (CARD) binds to the apoptosome and sequesters caspase-9. Caspase-9 sequestration activates caspase-3 leading to downstream apoptosis.
Modified from http://www.cellsignal.com/reference/pathway/Death_Receptor.html

(protein kinase B) cell survival pathway. Cell survival promoter Akt is constitutively activated by phosphorylated PIP3, which anchors Akt in the plasma membrane. Among prostate cancer cell lines, LNCaP cells express the highest level of constitutively activated Akt (Chen et al 2001), conferring the TRAIL resistant phenotype. The protein encoded by PTEN, which LNCaPs do not make, dephosphorylates PIP3 (phosphatidylinositol-3,4,5-triphosphate) and leads to an induction of apoptosis. It has been shown that inhibition of PIP3 kinase by kinase inhibitors wortmannin and LY-294002 (Lin *et al.*1999) suppresses constitutive Akt activity in LNCaPs, sensitizing them to TRAIL-induced apoptosis.

Furthermore, in order for androgen-responsive prostate cells to overcome the growth arrest instituted by the androgen withdrawal and to survive in the absence of androgens, a small population of malignant cells must overcome the acute onset of androgen deprivation and develop the ability to proliferate in an androgen-independent manner. After removal of androgen support, LNCaPs initially arrest in G1 state and eventually resume androgen-independent proliferation. In fact, androgen removal promotes the increased expression of growth factors, thereby contributing to the formation of the abnormal autocrine loops seen in advanced PCa. Thus, androgen removal may increase growth factor signaling, which could contribute to acquisition of the androgen-independent phenotype. Specifically androgen ablation alone can increase PI3K⟶Akt activation.

To determine if pectin acts through the TRAIL-mediated pathway or by inhibiting growth factor receptor binding or by enhancing the apoptotic effect of androgen deprivation or possibly by a combination of the three mechanisms, we incubated LNCaPs with pectin in the presence and absence of TRAIL. We also incubated the cells with TGF beta1 (transforming growth factor beta) and with pectin in the absence of androgens through serum withdrawal. Our results suggest that pectin may be sensitizing LNCaPs to TRAIL by inhibiting PI3K activity

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and that pectin may increase sensitivity of apoptosis-resistant LNCaPs to death by androgen withdrawal. Furthermore, the inability of TGF beta1 alone or in the presence of varying concentrations of the apoptotic pectin to induce apoptosis in LNCaPs, suggests that pectin does not sensitize LNCaPs to TGF beta1 signaling but instead TGF beta1 inhibits the apoptotic response of pectin.

2. Results

Pectin sensitizes LNCaPs to TRAIL (TNF-related apoptosis inducing ligand)-induced apoptosis

LNCaP cells have a mutation in the tumor suppressor gene PTEN which lead to elevated levels of phosphorylated PIP3. When PIP3 is phosphorylated, the cell survival promoter Akt is constitutively activated. This constitutive activiation makes LNCaP cells resistant to TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis (Chow and Clement 2006; Chen *et al.* 2001). To test whether pectin acts through the PIP3 → Akt pathway by inhibiting PI3K from phosphorylating PIP3, and thereby inhibiting activation of Akt-induced cell survival (Figure 3.2 and 3.3), we incubated LNCaPs with 0.1, 1.0 or 3.0 mg/mL heat modified pectin (HTCP) in the presence and absence of TRAIL. We previously showed in Jackson *et al* 2007 that heat modified pectin does not induce apoptosis in LNCaP cells at low concentrations 0.01 and 0.10 mg/mL but does induce apoptosis at 0.5, 1.0 and 3.0 mg/mL (with 1.0 and 3.0 inducing the highest levels comparable to positive control thapsigargin) before the activity drops off at 5.0 mg/mL and higher concentrations (our unpublished results). The heat modified pectin initially used in our studies was one that was commercially available, fractionated pectin powder (FPP). We subsequently generated a heat modified pectin in house, HTCP, (Jackson *et al* 2007) and found that it induced apoptosis at levels comparable to FPP. When LNCaPs were incubated with 0.1, 1.0 and 3.0 mg/mL

HTCP in the presence of TRAIL, there was an increased apoptotic effect compared to HTCP by itself (Figure 3.3). The results suggested that pectin, in some manner, may have sensitized LNCaPs to TRAIL by inhibiting PI3K activity.

Pectin increases sensitivity of LNCaPs to death by androgen withdrawal

When LNCaP cells were incubated with pectin in the absence of androgens the apoptotic activity of pectin was slightly enhanced compared to pectin-induced apoptosis in LNCaP cells grown in the presence of androgens. These results suggest that the effects of androgen ablation and pectin are additive. However, because LNCaP cells treated with serum-deprived medium alone exhibited no apoptosis and cells under the same conditions in the presence of pectin show slightly higher apoptotic activity than that observed in normal testing conditions (with media containing serum) (Figure 3.4), one possible mechanism for pectic-apoptotic activity is increasing the sensitivity of LNCaP cells to androgen withdrawal, thereby lessening the number of cells that are able to progress to, and proliferate in, the androgen-independent state. As previously mentioned, androgen removal may increase growth factor signaling. These results support the hypothesis that when androgens are removed, the increased expression of growth factors contributes a slight protective effect to the cells, and that this effect is lessened in the presence of pectin. Pectin appeared to act synergistically with androgens and as well as through a separate mechanism, independent of androgen ablation, perhaps involving growth factor inhibition.
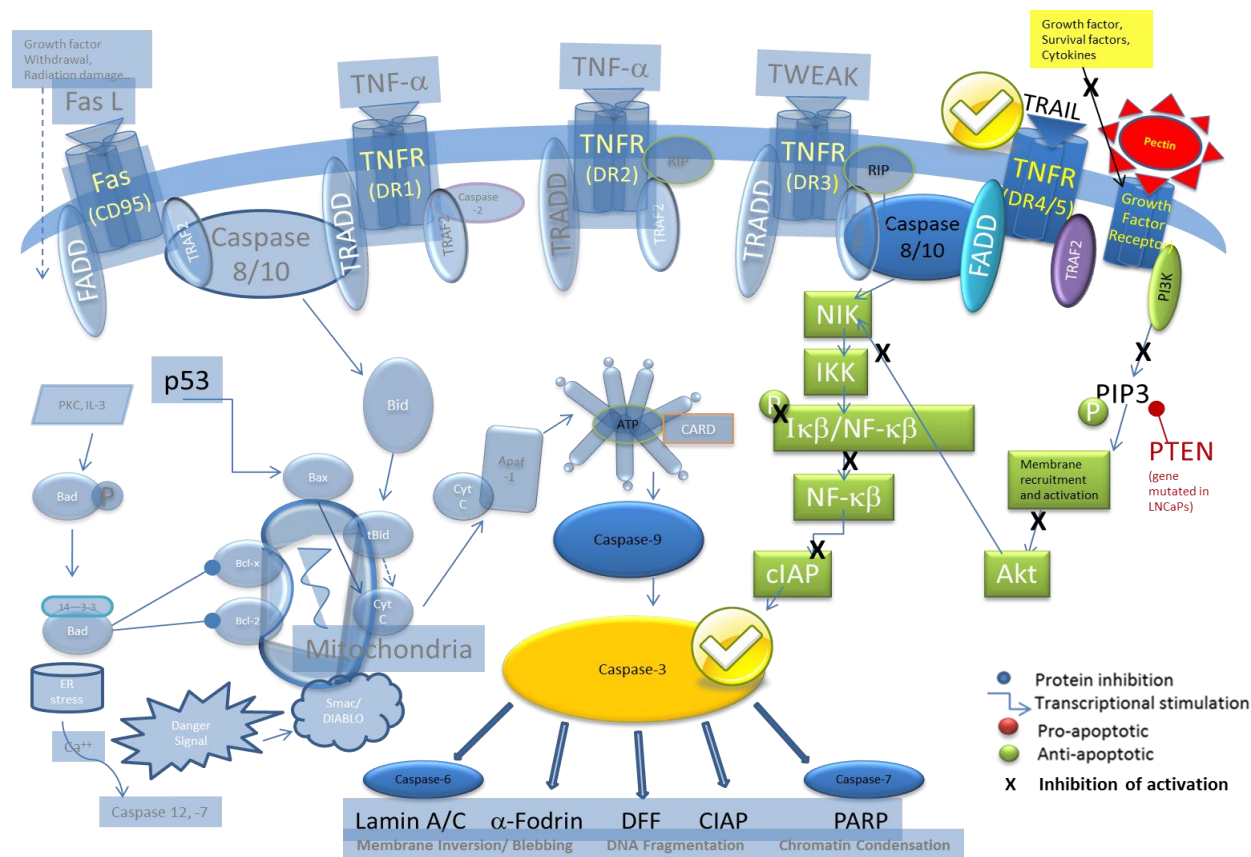
Figure 3.2. Proposed signaling pathway in pectin-mediated apoptosis. A mutation in the PTEN gene confers LNCaP cells resistant to TRAIL-induced apoptosis. This mutation leads to the constitutive activation of cell survival promoter Akt. Pectin may sensitize LNCaPs to TRAIL by inhibiting the phosphorylation of PIP3, in turn inhibiting the constitutive activation of Akt. "Xs" indicate a downstream inhibition of activity of specific anti-apoptotic factors as a result of pectin binding to the LNCaP cell surface.
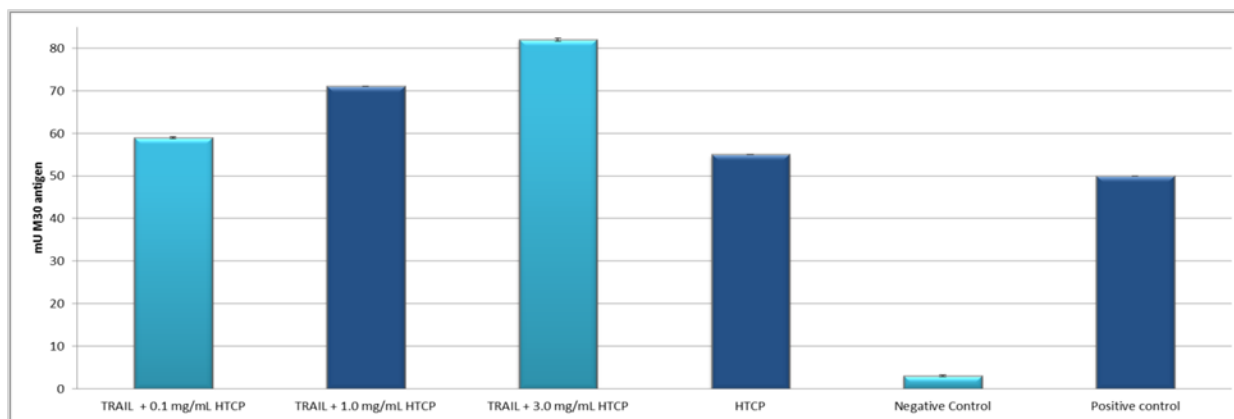
Figure 3.3. **Induction of apoptosis by TNF-related apoptosis inducing ligand (TRAIL) in the presence of pectin.** LNCaP cells were treated for 48 hr with 1 mg/mL heat modified citrus pectin (HTCP), with 0.01 mM thapsigargin (Pos) or with TRAIL in the presence or absence of varying concentrations of HTCP. Incubation with medium alone served as the negative control (Neg). Equal amounts of cell extracts (12 μg protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts + SEM. Comparable results were obtained in at least two experiments.
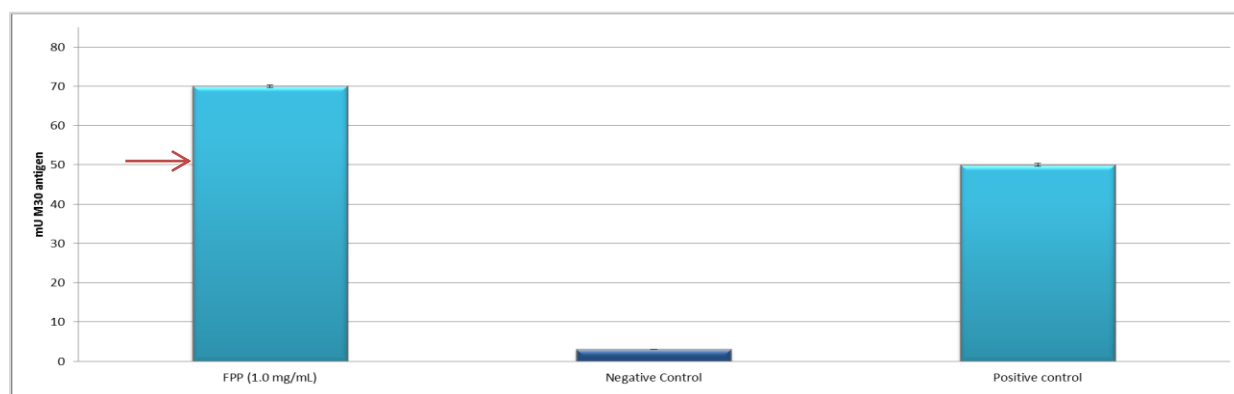
Figure 3.4. Induction of androgen-mediated apoptosis by pectin in LNCaP cell grown in serum free medium. (A) LNCaP cells were treated with 1 mg/mL FPP or with 0.01 mM thapsigargin (Pos) in serum free medium for 48 h. Incubation with serum free medium alone served as the negative control (Neg). Arrow indicates the level of FPP apoptotic activity when cells are grown under the standard culture conditions, i.e. in the presence of serum/androgens. Equal amounts of cell extracts (12 μg protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts + SEM. Comparable results were obtained in at least two experiments.

TGF beta1 inhibits the apoptotic response of pectin

To address the involvement of the growth factor TGF beta1 in pectin-mediated apoptosis, we treated LNCaPs in the presence and absence of TGF beta1 with and without increasing concentrations of pectin in normal culture medium containing serum. The sensitivity of LNCaP cells to TGF beta1 has been controversial (Kim *et* al. 1996). TGF beta1 signals through a heteromeric complex consisting of TGF beta receptor type I and TGF beta receptor type II. Southern blot analyses have revealed that LNCaP cells contain defective TGF beta receptor type I and thus, are insensitive to the growth inhibitory effects of TGF beta1 (Kim *et al*. 1996). Experiments to test the effects of apoptotic pectin on LNCaP cells in the presence and absence of TGF beta1 reveal that TGF beta1 alone does not induce apoptosis in LNCaPs (Figure 3.5). This result is consistent with what is known in the literature. In addition, TGF beta1 in the presence of varying pectin concentrations (0.1, 1.0 and 3.0 mg/mL) (Figure 3.5) does not induce apoptosis. This suggests that pectin does not sensitize LNCaPs to TGF beta1 signaling, but instead TFG beta1 inhibits the apoptotic response of pectin.

Western blot analyses reveal the increased expression of proapoptotic signaling proteins and the reduced expression of anti-apoptotic signaling proteins in the cell lysates of LNCaP cells treated with heat modified citrus pectin

Caspase activity assays use monoclonal antibodies to detect the presence of inactive proenzyme, the intermediate cleavage product or the active form of the cysteine aspartic acid proteases. The level of active caspases, and therefore the level of apoptosis, can be determined by 1) taking lysed cell extracts (containing soluble protein), 2) separating the protein (equal amounts loaded in each well) by SDS-gel electrophoresis, 3) transferring to a PVDF membrane,
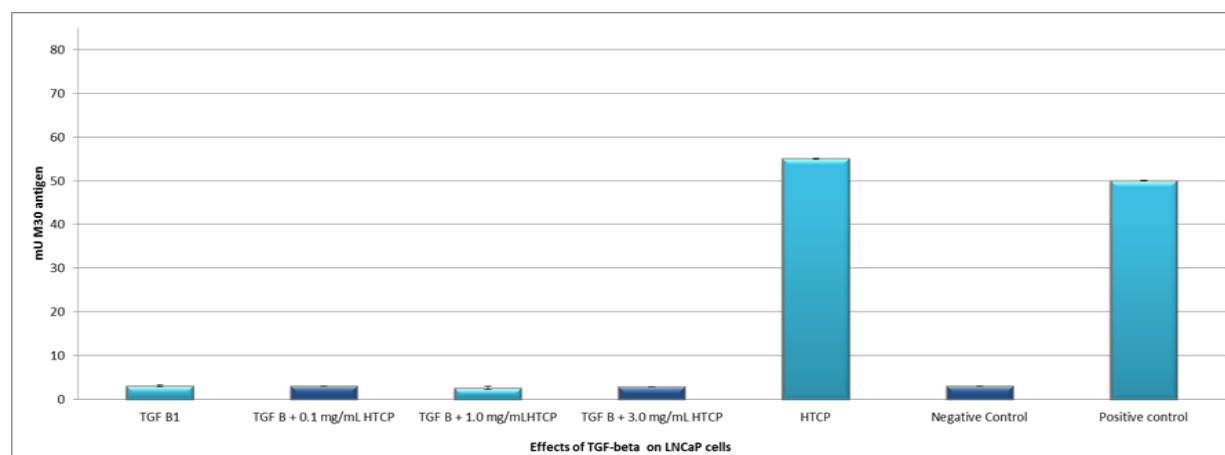
Figure 3.5. Effect of transforming growth factor beta1 (TGF beta1) on LNCaP cells. LNCaP cells were treated with 1 mg/mL apoptosis-inducing heat modified citrus pectin (HTCP), 0.01 mM thapsigargin (Pos), or with TGF beta1 (TGF B1) in the presence or absence of varying concetrations of pectin for 48 h. Incubation with medium alone served as the negative control (Neg). Equal amounts of cell extracts (12 μg protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell extracts + SEM. Comparable results were obtained in at least two experiments.

4) blocking against endogenous phosphatase/peroxidase activity, 5) labeling with an antibody that recognizes either the intermediately processed proenzyme or the active, cleaved form of the caspase, and 6) developing the antibody with a secondary antibody counjugated to an enzymatic substrate (eg. alkaline phosphatase). By comparing the fragment size (KDa) seen on a Western blot one can determine if a caspase is present in the sample of interest in its active (smaller /cleaved fragments) or inactive form, directly indicating whether or not apoptosis has occurred. We previously showed (Jackson *et* al. 2007) that the 19, 17, and 12 kDa activated caspase-3 cleavage products are present in LNCaP cell lysates after treatment of LNCaP cells with heat modified pectin FPP. The presence of specific apoptotic signaling proteins can also be probed to help uncover what pathway an apoptosis-inducing agent such as heat modified pectin targets intracellularly. Therefore, we assayed for the presence of a number of these apoptotic signaling proteins in LNCaP cell lysates collected after pectin-induced apoptosis.

Pectin-induced apoptosis in LNCaP cells is mediated through the NF$_K$B signaling pathway

Nuclear factor kappa light chain enhancer of B cells (NF$_K$B) is an important protein for cytokine signaling and cell survival. In its inactive state NF$_K$B is kept in the cytosol by inhibitor of kappa light chain enhancer of B cells (I$_K$B). Thus we would expect for NF$_K$B protein levels to be low in LNCaPs treated with apoptotic pectin and for I$_K$B expression levels to be high. The western blot analyses of cell lysates from LNCaP cells treated with varying concentrations of the apoptosis-inducing heat modified pectin, fractionated pectin powder (FPP) reveal that there is low expression of the 65 kDa subunit, the nuclear localized subunit of the cell survival promoter NF$_K$B, at the higher (0.5, 1.0 and 3.0 mg/mL) and more apoptotic pectin concentrations (Figure 3.6). Furthermore as expected, there is increased expression of T-I$_K$B the protein that

dephosphorylates $I_\kappa B$ signaling $I_\kappa B$ to sequester $NF_\kappa B$ in the cytosol, keeping it in its inactive state. Specifically, $T-I_\kappa B_\alpha$ is present in high levels for the 0.01, 0.1, 0.5, and 1.0 mg/mL pectin-treated cells, and is present at low levels in the 3.0 mg/mL pectin treatment and in the negative control cells. $T-I_\kappa B_\alpha$ is not present at all in the positive control (Figure 3.7). It should be noted that the positive control used for these studies is thapsigargin (TG). TG induces apoptosis by disrupting the Ca^{++} channels in the ER (endoplasmic reticulum). While TG has been shown to be an apoptosis-inducing agent in LNCaPs, it may not be following the same pathway(s) that is triggered by heat modified pectin. This explains some of the differences in the proteins activated by TG versus pectin treatment. Likewise, there is reduced expression of the phosphorylated form of $I_\kappa B$ (p-$I_\kappa B$), which cannot bind $NF_\kappa B$, allowing it to travel to the nucleus to induce transcription of genes involved in cell proliferation, in LNCaP cells treated with FPP and in the positive and negative controls (Figure 3.7). IKK (inhibitor of kappa kinase) which phosphorylates $I_\kappa B$ is expressed in low amounts in LNCaP cells treated with the positive control and with 0.01, 0.1, 0.5, 1.0 and 3.0 mg/mL FPP (Figure 3.8).

TRADD (TNF-Related Apoptosis Inducing Death Domain) mediates $NF_\kappa B$ activation by binding to TRAF2. TRAF2 (TNF-Receptor associated factor 2) is required for activation of $NF_\kappa B$ and binding of TRAF2 by death domains like TRADD ensures recruitment of inhibitors of apoptosis proteins (IAPs), thereby suppressing apoptosis. TRADD is expressed in low levels in the cell lysates of LNCaP cells treated with apoptotsis-inducing FPP (Figure 3.9).

The anti-apoptotic factor TRAF2 (TNF-receptor associated factor 2) forms a heterodimeric complex with TRAF1 to interact with IAPs (inhibitor of apoptosis proteins) and mediate anti-apoptotic signals from TNF receptors. In some proapoptotic phenotypes, TRAF2 is degraded (Rumble and Duckett 2008). When analyzed by Western blot, the cell lysates of LNCaP

cells treated with apoptotsis-inducing FPP have TRAF2 expressed at low levels in all treatments including positive and negative controls (Figure 3.10).

XIAPs (X-linked inhibitor of apoptosis proteins) are the most potent members of the IAP (inhibitor of apoptosis proteins) family. XIAP binds to and inhibits caspases-3, -7, and -9 (Deveraux and Reed 1999). In response to stress, the mitochondrial associated proteins SMAC (second mitochondrial activator of caspases) and DIABLO are released to compete with IAPs' binding to caspases. When SMAC and DIABLO bind IAPs, caspases are uninhibited and apoptosis progresses. Interestingly, XIAP is present in all FPP treatments, at levels higher than the negative control (Figure 3.11).

Taken together, the data show that the antiapoptotic proteins NF_KB, p- I_KB, IKKγ, TRAF2, and TRADD—which in these studies is likely mediating NF_KB activation by binding to TRAF2, are decreased upon treatment of LNCaP cells with pectin while the pro-apoptotic protein p- I_KB is increased. Interestingly, XIAP which directly binds to and inhibits caspases was expressed at levels higher that the negative control in pectin-treated LNCaPs. These results confirm that survival is indeed inhibited in FPP-treated LNCaP cells through some mechanism that affects the NF_KB signaling pathway, perhaps involving sensitization to TRAIL and/or inhibition of XIAP-mediated activation of NF_KB.

LNCAP cells do not express galectin-3

Lastly, it has been suggested that the inhibitory effects of pectin on metastatic lesions in the lung are mediated through their binding to galectin-3 (a galactoside-binding lectin) (Nangia-Makker *et al.* 2002). Galectins are specific galactoside-binding proteins present on the surface of cancer cells. Galectins facilitate cell-cell interactions by binding to galactose-containing
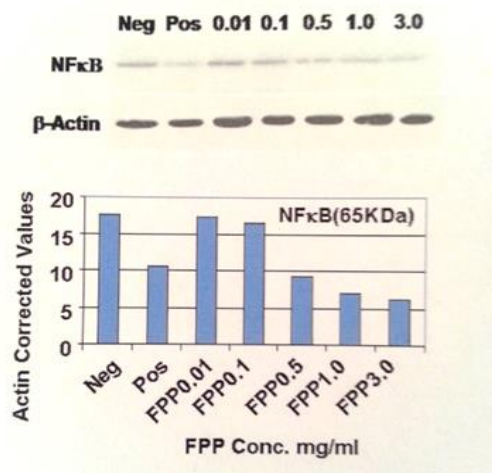
Figure 3.6. Anti-apoptotic NFκB expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis LNCaP cell lysates (30 μg protein) using the respective antibody to analyze levels of the anti-apoptotic protein.

Figure 3.7. Anti-apoptotic p-I$_K$B and pro-apoptotic T-I$_K$B expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis LNCaP cell lysates (30 μg protein) using respective antibody to analyze levels of the proteins.
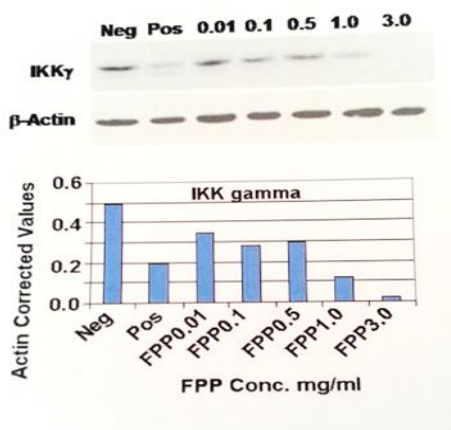
Figure 3.8. Anti-apoptotic IKKγ expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis LNCaP cell lysates (30 μg protein) using respective antibody to analyze levels of the anti-apoptotic protein.
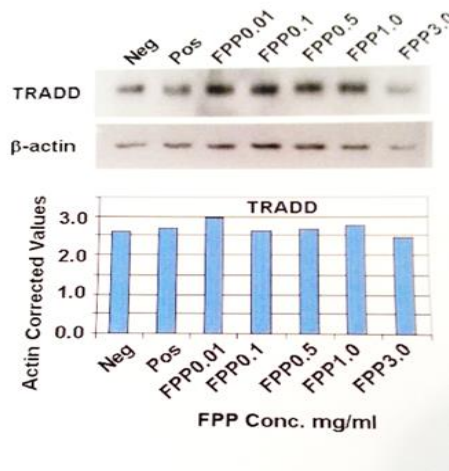
Figure 3.9. Anti-apoptotic (in these studies) TRADD expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis LNCaP cell lysates (30 μg protein) using respective antibody to analyze levels of the anti-apoptotic protein.

Figure 3.10. Anti-apoptotic TRAF2 expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis of LNCaP cell lysates (30 μg protein) using anti-TRAF2 antibody to analyze levels of the anti-apoptotic protein.

Figure 3.11. Anti-apoptotic XIAP expression levels in LNCaP cells treated with increasing concentrations (0.01 to 3 mg/mL) of FPP. Western blot analysis LNCaP cell lysates (30 μg protein) using respective antibody to analyze levels of this inhibitor of apotosis.
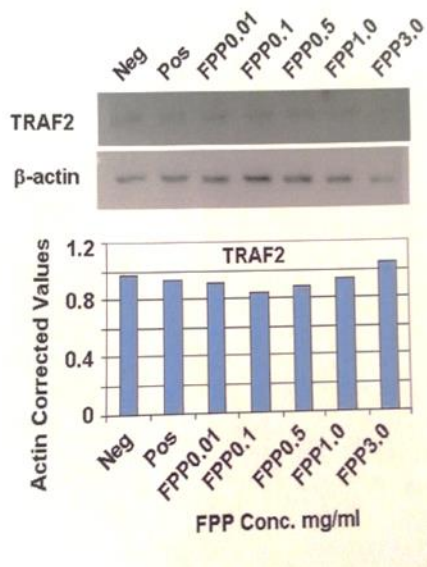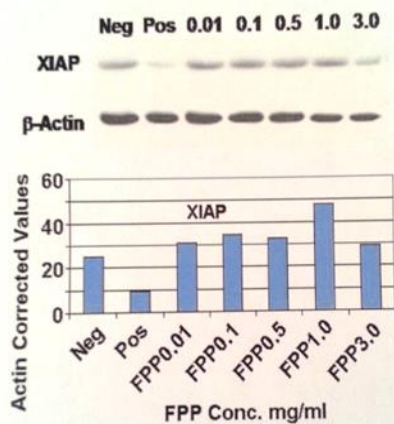
molecules on neighboring cancer cells (Nangia-Makker *et al.* 2002). In human colon, stomach and thyroid cancers, the amount of galectin increased with the progression of cancer (Nangia-Makker *et al.* 2002). Blocking galectin-3 expression in highly malignant human breast, papillary and tongue carcinoma cells led to reversion of the transformed phenotype and suppression of tumor growth in nude mice (Honjo *et al.* 2000), (Honjo *et al.* 2001). It has been proposed that pH-modified citrus pectin blocks binding of galectins, and thus, inhibits tumor cell-cell interactions (Nangia-Makker *et al.* 2002). The potential impact of blocking galectin action includes inhibition of the aggregation of cancer cells to each other and to normal cells, thus inhibiting metastatic lesions. However, LNCaP cells do not express galectin-3 (Ellerhorst *et al.* 1999; Ellerhorst *et al.* 2002; Califice *et al.* 2004; our unpublished observations) suggesting that the apoptotic effects of pectins reported here are due to mechanisms not mediated through galectin-3.

3. Discussion

Until now, no mechanism for the apoptotic effect of heat-modified pectin on human prostate cancer cells has been proposed. Our results suggest that pectin is acting in multiple pathways to induce apoptosis in the lymph node-derived human prostate cancer line LNCaP. Specifically, our results show that *in vitro* LNCaP cells express low levels of the anti-apoptotic proteins TRAF2, IKKγ, pI_κBα, NF$_\kappa$B, andTRADD (TRADD in these studies is likely mediating NF$_\kappa$B activation by binding to TRAF2) and high levels of the pro-apoptotic proteinT-I_κBα when treated with heat modified pectin. These results suggest, increased sensitivity to death-inducing ligands via the NF$_\kappa$B signaling pathway, perhaps involving sensitization to TRAIL, inhibition of

XIAP-mediated activation of NF$_{\kappa}$B, and increased sensitivity to androgen withdrawal. Future studies assessing the mechanism of pectin's apoptotic activity in the androgen independent C4-2 cell line and the presence or absence of pectin-induced apoptotic activity in DU145 and PC3 prostate cancer *in vivo* models (both TRAIL sensitive) may reveal additional mechanisms for pectin-induced apoptosis.

4. Methods

Two prostate cancer cell lines, LNCaP and LNCaP C4-2, were utilized in these experiments. LNCaP cells were obtained from American Type Culture Collection (Rockville, MD) and are androgen- responsive prostate cancer cells, whereas the LNCaP C4-2 cells were derived from LNCaP cells as androgen-refractory prostate cancer cells (purchased from Grocer Inc., Oklahoma City, OK). RPMI-1640 medium supplemented with 25 mM HEPES and L-glutamine was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS), penicillin/streptomycin, citrus pectin (CP) (P-9135), and alcian blue were from Sigma-Aldrich (St Louis, MO). Fungizone was obtained from Invitrogen (Carlsbad, CA). Bio-Rad Protein Assay dye reagent concentrate was purchased from Bio-Rad (Hercules, CA). M-30 Apoptosense ELISA was from Peviva AB (Sweden). FPP was purchased from Thorne Research (Dover, ID) and CP (P-9135) from Sigma- Aldrich. TRAIL and TGF beta1 were gifts of Richard Steet (CCRC, University of Georgia). Anti-actin antibody (Sigma). Other primary antibodies supplied by Dr. Vijay Kumar (Medical College of Georgia). All other chemicals, unless otherwise stated, were from Fisher Scientific.

Cell culture, pectin treatments, and quantification of apoptosis

LNCaP and LNCaP C4-2 cells were grown in RPMI-1640 medium supplemented

with 25 mM HEPES, 2.0 mM L-glutamine, 10% heat-inactivated FBS, 50 U/mL penicillin, 0.05 mg/mL streptomycin, and 0.25 mg/mL fungizone in the presence of 5% CO_2 at $37^{\circ}C$. Cells were maintained in logarithmic growth phase by routine passage every $10-12$ days (LNCaP) or $6-7$ days (LNCaP C4-2). Cells were plated at a density of 1.6 x 10^5 cells per well in 6-well culture plates and allowed to adhere for 24 h. The medium was removed and replaced with medium containing (1 mg/mL) filter-sterilized pectin (0.20 mm nylon filters; Fisher Scientific). Treated cells were incubated in medium containing the following compounds as indicated: TRAIL (25 ng/mL), TGF beta1 (25 ng/mL), pectin (1 mg/mL-HTCP, FPP, PeS, CP), and positive control Thapsigargin (0.01 mM) (Sigma). The negative controls were untreated cells cultured in medium alone. Cells were harvested after 48 h and lysed in ice-cold Lysis Buffer (10 mM Tris $-$ HCL, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40), incubated on ice for 5 min, and soluble protein was collected by centrifugation at $4\text{-}8^{\circ}C$. The protein concentration was determined in triplicate using a Bradford/Bio-Rad Protein Assay (Bio-Rad Protein Assay Dye Reagent Concentrate). Apoptotic activity was assayed using the M-30 Apoptosense ELISA (see the Apoptosense assay section).

Apoptosense assay

Cells grown in culture flasks and treated as described above were harvested and total protein extracted as described above. Protein was assayed for the presence of the apoptosis-specific cytokeratin-18 neoepitope (generated by cleavage of cytokeratin-18 by caspases activated in response to treatment) using the M30-Apoptosense ELISA (Peviva AB, Sweden). In brief, protein extract $(12\text{-}29 \text{ } \mu g)$ was added to 96-well plates coated with mouse monoclonal anti-cytokeratin-18 M30 antibody. Horseradish peroxidase tracer

solution was added to the wells in a dark room illuminated with a green safety light, and the plate was incubated with agitation for 4 h at RT. Color was developed by adding tetramethyl benzidine solution and incubating in darkness for 20 min. Optical density (OD) was determined at 450 nm using Spectra MAX 340 (Molecular Devices, Menlo Park, CA) or Finstruments Model 347 (Vienna, VA) microplate readers. The amount of cytokeratin-18 neoepitope was determined based on standard curves generated using standards provided by the manufacturer.

Preparation of cell lysates and western blotting

Cells were harvested by trypsinization and the washed cell pellets were resuspended in lysis buffer (1X PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL phenylmethyl sulfonyl fluoride, and 1 mg/mL aprotinin) and incubated on ice for 30 min. The lysed cells were centrifuged at 10,000 g for 10 min at 4-8°C and the supernatant was collected. Protein concentration was determined as described above. Proteins (50 g, unless stated otherwise) were separated on NuPAGE 10% Bis-Tris gels (Novex pre-cast mini gels, Invitrogen, Carlsbad, CA) at 100 V for 1 h in the presence of 1X 2-(N-morpholino) ethanesulfonic acid (MES)-SDS running buffer (Invitrogen, Carlsbad, CA). Separated proteins were transferred to (Polyvinylidenedifluoride; PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) at 42 V for 2.5 h using a Novex XCell II blotting apparatus in MES transfer buffer in the presence of NuPAGE antioxidant. Transfer of the proteins to the PVDF membrane was confirmed by staining with Ponceau S (Sigma). The blots were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS), washed twice for 10 min each with TBS containing 0.01%

Tween-20 and incubated for 2 h at RT with primary antibody diluted in TBS containing 0.5% milk. Immunoreactive bands were visualized using the ECL detection system (Amersham, Pharmacia Biotech, Arlington Heights, IL) and signals were developed after exposure to X-ray film (X-Omat films, Eastman Kodak Company, Rochester, NY).

CHAPTER 4

INVESTIGATING THE *IN VIVO* ACTIVITY OF AN APOPTOTIC HEAT MODIFIED

PECTIN BY ORAL TREATMENT OF NUDE MICE WITH HEAT-TREATED PECTIN[4]

[4] Crystal L. Jackson[A,B], Siyuan Zhang[B], Lianchun Wang[A,B], Debra Mohnen[A,B]
[A]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, GA 30602-4712
[B]Department of Biochemistry and Molecular Biology, UGA Cancer Center, The University of Georgia, Athens, GA 30602
To be submitted

Abstract

In Jackson *et al*. 2007 we reported that fractionated pectin powder (FPP), a commercially available pectin generated by heat modification of citrus pectin (CP), as well as heat-treated CP created in our laboratory (HTCP), induce significant levels of apoptosis *in vitro* in the human prostate cancer cell line LNCaP. In contrast, relatively large unmodified citrus pectin (molecular mass range 23-71 kDa) and the small pH-modified pectin (Pectasol,- molecular mass range 10-20 kDa) that was produced by base-treatment of CP, induced little or no apoptosis. By manipulating the structure of pectin, we further demonstrated that a base-sensitive linkage is necessary for the apoptotic activity of pectin and that digestion with pectinases and pectinmethylesterases does not destroy the apoptotic activity. To test the efficacy of the apoptotic pectin in an animal model, we subcutaneously injected 10 million LNCaP cells into athymic nude Foxn1 mice and fed them 1% pectin (either HTCP or CP) in their drinking water. Mice given water alone served as negative controls. After 30 days, mice were euthanized and tumors extracted for measurement. Though there was no evidence for a change in metastasis, compared to water controls, surprisingly both HTCP and CP-treated mice had significantly enhanced primary tumor growth. These initial treatments were done only with the heat-fragmented pectin HTCP. It is possible that the large size of the HTCP utilized in this pilot study led to the increased tumor size. In a previously published study, (Platt and Raz, 1992) showed that B16-F1 melanoma cells pre-incubated with 0.5% CP (70-100 kDa) and then injected intravenously into C57BL/6 mice led to an increase in the number and volume of lung melanoma colonies whereas pre-incubation with a small pH-modified citrus pectin (MCP-10 kDa) led to a reduction in melanoma colony formation. Whether or not apoptosis of prostate cancer cells could be induced by administration of the smallest active

pectin that we have generated from HTCP by treatment with the pectin degrading enzymes, endopolygalacturonase and pectinmethylestersase HPE, remains to be determined.

1. Background

The cancer inhibitory effects of modified pectin

Bioactive dietary components are increasingly prevalent for the treatment of cancer occurence and progression in both the scientific and clinical communities. According to the American Institute of Cancer Research and the World Cancer Research Fund, 30–45% of worldwide cancer cases are estimated to be preventable by dietary means. Specifically, diets rich in fruits and vegetables have protective effects against cancer. Pectin is a natural complex plant polysaccharide present in all higher plant primary cell walls (30% in dicots; $\leq$10% in nongraminaceous monocots) and a dietary component of all fruits and vegetables.

Pectin has been shown to significantly reduce colonic tumor incidence in rats (Heitman *et al*. 1992), inhibit cancer cell metastasis in mice and rats (Platt and Raz 1992; Pienta *et al*. 1995; Nangia-Makker *et al*. 2002), and increase the apoptotic response of chemoresistant tumor cells (Wang et al 2010; Chauhan *et al* 2005). Previous research has attributed pectin's anti-metastatic properties to its propensity to inhibit extravasation by preventing tumor cell adhesion to the endothelium and homotypic cell aggregation in MDA-MB-231 human metastatic breast carcinoma cells and in mouse B16-F1 melanoma cells *in vitro* respectively (Glinsky and Raz 2009). The pectin possessing the noted anti-adhesive properties was a relatively small, pH-modified pectin (10 kDa). *In vivo*, oral administration of a pH-modified citrus pectin (CP) significantly reduced metastasis of rat prostate adenocarcinoma MATLyLu to the lung (Pienta *et al*. 1995). Contrastingly, when injected intravenously in mice, relatively large commercial pectin (70-100 kDa) increased homotypic cell aggregation and metastasis to the lung (Platt and Raz 1992), demonstrating a differential response depending upon the type of pectin used.

Several studies have indicated that pectins not only inhibit metastatic lesions, but also

induce apoptosis in cancer cells either directly or indirectly by sensitizing cancer cells to the effects of chemotherapeutics. In one study, when compared with rats fed corn oil and/or cellulose, azoxymethane-injected rats fed a citrus pectin or fish oil/pectin diet had a greater number of apoptotic cells per colon crypt column and a lower incidence of adenocarcinoma (51.5%) than animals fed cellulose/corn oil (75.6%) (Chang *et al.* 1997; Chang *et al*. 1997-*Carcinogenesis*). Furthermore, the apoptotic index in the distal colon of rats fed a pectin-rich diet was higher than that in rats fed a standard diet. These results were accompanied by activation of caspase-1 and poly(ADP-ribose) polymerase (PARP) and reduced expression of the anti-apoptotic protein Bcl-2 (Avivi-Green *et al*. 2000; Avivi-Green *et al*. 2000b; Kossoy *et al*. 2001). Similarly, dimethylhydrazine-injected Sprague-Dawley rats administered a pectin-rich 15% orange-pulp diet showed a decreased number of endophytic tumors, an activation of caspase-3, and an increased activity of T-cell killers in the tumors; all characteristic anti-tumor effects (Kossoy *et al*. 2001). In human colon adenocarcinoma HT29 cells, caspase-3 activity significantly increased when cells were treated with 10 mg/mL low-methylated apple pectin (Olano-Martin *et al*. 2003).

While animal and cell studies are very encouraging, there is little information available about the efficacy of modified citrus pectins in humans. In one published clinical trial, 10 men with prostate cancer were treated with a pH-modified citrus pectin after standard treatment failed. While the study lacked a control group that did not receive the modified pectin, 7 of 10 men in the pectin-treated group had blood tests that were positive for prostate-specific antigen (PSA), a controversial but commonly utilized marker of prostate cancer growth that is present at low levels in healthy men. Despite considerable PSA levels, the PSA doubling time (a measure of how fast PSA goes up) improved in comparison to measurements done before taking MCP,

indicating that MCP may slow prostate cancer growth (Strum *et al*. 1999). In preclinical studies using the modified a citrus pectin, GCS-100, showed induction of apoptosis in human multiple myeloma cell lines that are resistant to conventional and bortezomib therapies without altering normal lymphocyte cell viability (Chauhan *et al*. 2005). In the myeloma lines, GCS-100 induced DNA fragmentation and the activation of caspase-8, caspase-3, and PARP indicating that GCS-100 triggered apoptosis primarily through the extrinsic pathway. In addition, GCS-100 also inhibited the growth of multiple myeloma cells directly purified from patients who had relapsed following treatment with multiple chemotherapeutics (Chauhan *et al*. 2005), providing evidence that GCS-100 can induce apoptosis in chemoresistant myeloma cells. In a similar study, GSC-100/MCP increased cisplatin-induced apoptosis of PC3 cells (Wang *et al*. 2010). In a broader 2007 pilot study, patients with advanced solid tumors from various carcinomas received a 5 g MCP solution 3 times a day for at least 8 weeks. The MCP utilized was heat-modified with a degree of esterification of less than 20%. Following treatment, improvements were reported in some measures of quality of life, including physical functioning, global health status, fatigue, pain, and insomnia. In addition, 22.5% of participants had stable disease after 8 weeks of MCP treatment, and 12.3% of participants had disease stabilization lasting more than 24 weeks (Azemar *et al*. 2007).

Though encouraging, each of these oral pectic treatments were administered without addressing the rate of absorption in the human gastrointestinal tract. Purifying a specific active structure that is minimally processed and easily absorbed in the gastrointestinal tract could deliver a greater cancer payload than a heterogenous mixture wherein the active component represents a small fraction of the total pectin. To do this effectively, one must have an understanding of how pectin is processed in the human gastrointestinal tract.

Metabolism of pectin

There are no enzymes secreted by the human body that are able to degrade the pectin molecule. Certain bacteria of the gut flora like *Bacteroides, E. coli, Lactobacillus* and *Bifidobacterium*, however, are able to use pectin as a substrate for fermentation in the cecum, colon acendens, and colon, degrading it by 90-95% (Inohara and Raz 1994; Strum *et al*. 1999). Without this process, pectin would pass almost unchanged through the digestive system. Pectin is quite resistant to the acidic conditions of the stomach, undergoing only a slight deesterification. In the intestines, pectin promotes delayed gastric emptying (Holt *et al*. 1979; Schwartz *et al*. 1983; Schwartz *et al*. 1988; Sandu *et al.* 1987) and delayed resorption of nutrients (Fluourie *et al*. 1984). Pectins reduce amylase activity by 10-40%, lipase activity by 40%-80% (Tsujuta *et al*. 2003; Tsujuta *et al*. 2003) and trypsin activity by 15-80% (Isaksson *et al*. 1982b; Isaksson *et al*. 1982a; Dutta and Hlasko 1985; Hansen 1987; Hansen 1986). This reduction in activity of pancreatic enzymes is caused by an increase of viscosity of the digestive fluids due to the presence of pectin and in some cases due to pectin interacting with substrates, thereby diminishing the contacts between enzymes and substrates. In fact, high molecular weight pectin, i.e that >300,000 Da, inhibits gastrointestinal enzymatic activity to a greater extent than more fragmented commercial citrus pectin (Edashige *et al*. 2008).

Pectin degradation increases with decreasing deesterification and fermentation of low esterified pectin is faster than with higher degrees of esterification. Products from the fermentation process in humans are short-chain fatty acids (SCFA)-acetate, propionate, and butyrate (molar proportions 84:14:2) (Dongowski *et al*. 2000) and gases including methane, CO_2 and hydrogen. In addition to the degree of esterification, pectin concentration has an influence on the molar proportion of SCFA. At low pectin concentrations, for example 0.25%, the molar

proportion of 81:10:9 (SCFA-acetate:proprionate:butyrate) was formed and at high concentrations such as 3% pectin solution, 74:7:20 (SCFA-acetate:proprionate:butyrate) (Cho and Almeida 2009). Pectin might be partially fermented to oligogalacturonic acid with different degrees of polymerization. Nonreducing ends of these oligomers can be unsaturated between carbons 4 and 5 due to β-elimination or by the action of pectin and pectate lyases. Products obtained upon further degradation of galacturonic acid (GalA) are furan-2,5 dicarbonic acid and galactaric acid which are further transformed into acetoacetic acid (Cho and Almeida 2009). In addition, pectin promotes delayed/reduced resorption of nutrients (Fluouri *et al*. 1984) as well as delayed gastric emptying (Holt *et al*. 1979; Schwartz *et al*. 1983; Schwartz *et al*. 1988; Sandu *et al.* 1987). Furthermore, excretion of toxic metals including lead is increased by pectins (Zhao *et al*. 2008).

Rationale for the proposed study

We previously reported (Jackson *et al*. 2007) that fractionated pectin powder (FPP), a commercially available pectin generated by heat modification of CP, as well as heat-treated CP created in our laboratory (HTCP), induce significant levels of apoptosis in human prostate cancer cells *in vitro*. In contrast, large unmodified citrus pectin and small pH-modified pectin (Pectasol, PeS), made by base-treating CP, induced little or no apoptosis activity. Apoptosis was quantified by measurement of a specific epitope of cytokeratin-18 cleaved by caspases late in the apoptotic cascade.

We confirmed the induction of apoptosis by analyzing cell extracts using immunoblots, demonstrating activation in both LNCaP and LNCaP C4-2 cells treated with FPP of caspase-3, an effector caspase in the apoptotic pathway. We showed that the 35 kDa

procaspase-3 was cleaved into 19, 17, and 12 kDa, its active products, when treated with FPP, confirming a significant apoptotic response. The analysis of the cell extracts for the presence of poly ADP ribose polymerase (PARP), a substrate of activated caspases, showed the presence of an 85 kDa cleaved PARP product in the positive control (thapsigargin-treated cells) and in the cells treated with FPP, but not with pH-modified PeS or unmodified CP. By manipulating the structure of pectin, we also demonstrated that a base-sensitive linkage in the pectin is necessary for the apoptotic activity of pectin and that digestion with pectinases and pectinmethylesterases (mimicking the treatments utilized to isolate pectin from the cell wall) does not destroy the apoptotic activity.

Taken together, the results of the *in vitro* studies suggested that a specific pectic structure(s) induced apoptosis in human prostate cancer cells. We therefore determined whether this same pectic structure was effective in an animal system. To test the efficacy of this modified pectin in an animal model, we tested the effect of oral treatment of athymic nude Foxn1 mice subcutaneously injected with 10 million LNCaP cells by providing them 1% pectin (either HTCP or CP) in their drinking water.

2. Results

Athymic nude Foxn1 mice were given either 1% HTCP or CP in their drinking water ad libitum for 30 days. Administration of the pectin to the mice began 7 days prior to injection of LNCaP cell subcutaneously into the Foxn1 athymic nude mice. Mice given water alone served as negative controls. All groups displayed a bimodal distribution of tumor volume (mm3) and tumor weight (g) in the tumors extracted from the 15 mice/group. There was no evidence of metastasis in any of the treatments or controls, defined by the presence of tumors away from the

original injection site. Compared to the water control, both HTCP and CP significantly enhanced primary tumor growth after the course of 30 days (Figure 4.1A and 4.1B). The average tumor volume of mice fed CP in their drinking water was higher than that of the water control group beginning 20 days after tumor cell injection. The HTCP-fed group, however, had a smaller average tumor volume than the water control mice up until 30 days of tumor growth after which it increased by >60%. As expected from the trend seen with tumor volume, the final average mass of tumors were highest in the HTCP-treated group followed by the CP-treated mice and then mice receiving water alone.

Figure 4.2 shows a representative sampling of tumors intermediate in size from each treatment group. A total of 15 tumors were excised (1 from each mouse) for each group with masses ranging from 0.02-0.31, 0.17-0.62, and 0.21-0.71 g for the water controls and the CP- and HTCP-treated groups respectively.
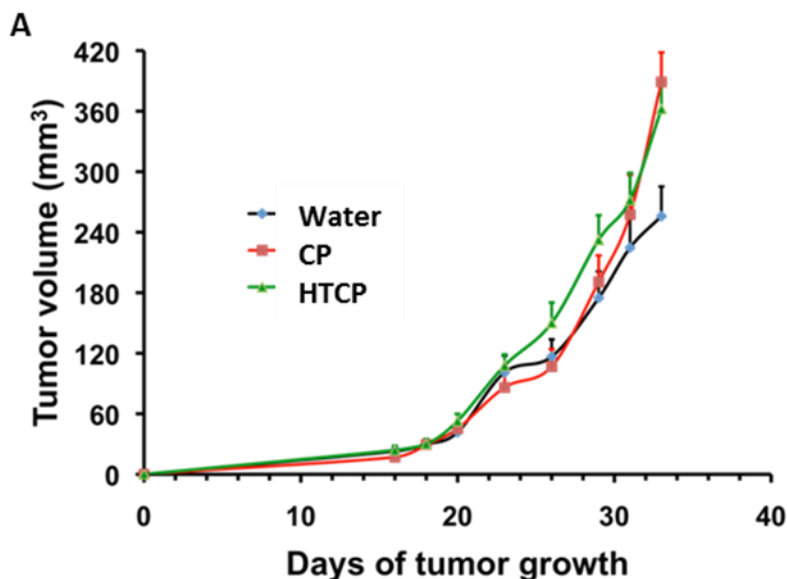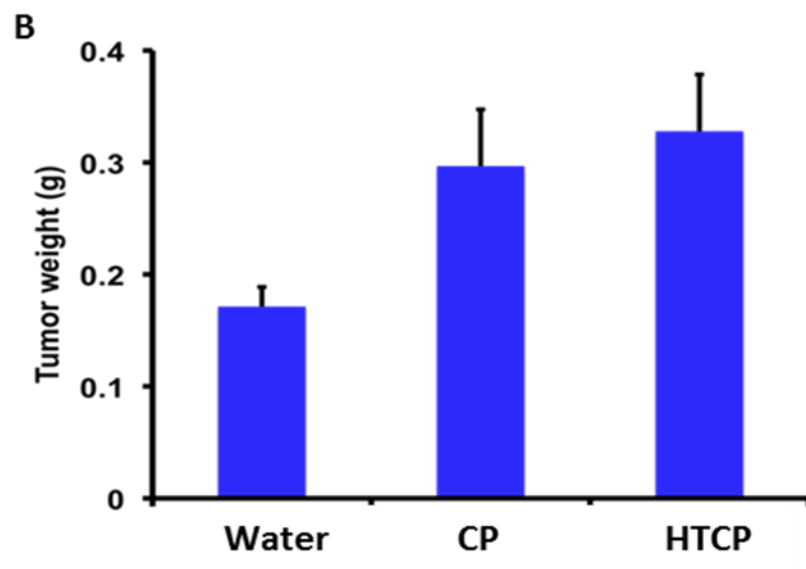
Figure 4.1 (A and B). Analysis of the effect of the heat modified pectin (HTCP) in athymic nude Foxn1 mice subcutaneously injected with 10 million LNCaP cells. The CP and HTCP group received 1% pectin in their drinking water, the water group received no pectin. Treatments began 7 days prior to injection of cancer cells. **(A)** Comparison of tumor volume over 30 days. Some mice died both in the water and CP groups at different timepoints before the completion of the experiment, therefore data are the average of 13-15 tumors/group $\pm$SEM. **(B)** Comparison of final tumor weight in the three groups after the 30 day treatment (n=13, 14 and 13 in the water, CP and HTCP groups, respectively). Data are the average weight of the number of tumors/group +SEM.
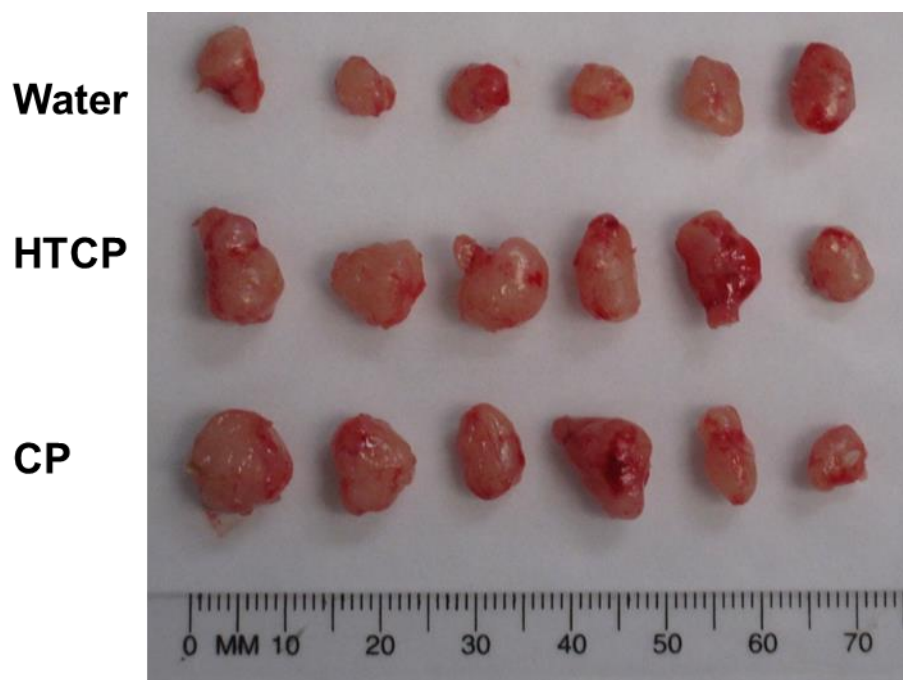
Figure 4.2. Tumors removed from euthanized control mice and mice after 30 days of treatment with the noted pectins. The CP and HTCP group received 1% pectin in their drinking water. The water group received no pectin. Tumors depicted are a sample of the tumors excised from each treatment group and are representative of those intermediate in size.

3. Discussion

In this preliminary study, we orally administered unmodified citrus pectin (CP), heat fragmented citrus pectin (HTCP) and water alone to athymic nude mice over the course of 30 days. Though there was no evidence for changes in the amount of metastasis, compared to water controls, surprisingly the presence of both HTCP and CP in the drinking water significantly enhanced primary tumor growth. The drinking water containing the respective treatments was available *ad libitum* and that pectin-fed mice drank more frequently than water controls. Prior studies have shown that large pectin can induce homotypic cell aggregation *in vivo* (Platt and Raz 1992) and led to an increase in tumor volume. Taking this into account and based on our current results, the size of the pectin utilized in this pilot study may have been too large and have lead to the increase in tumor size. These initial treatments were done with the heat-fragmented CP only. We did not test the smallest active pectin that we have generated to date, HPE, which is further processed with the pectin degrading enzymes, endopolygalacturonase and pectinmethylestersase. We also did not include treatment with a non-pectic polyanion as a negative control. These results stress the importance of considering the size of pectin used for animal studies and reinforce the need to determine the structure of the smallest active apoptotic pectin molecule.

It has been shown that pectin concentration can affect the molar proportion of SCFAs (short chain fatty acids) that are produced (Dongowski 2000) and subsequently absorbed through the intestinal wall. Altering the concentration of the administered treatments may generate a larger amount of fermentation products. Such products have previously been shown to be associated with increased apoptosis in the colon crypt. A subsequent study utilizing the more processed heat-modified pectin reduced in size by treatment with pectinases/pectinesterase-

degraded citrus pectin as a treatment may promote greater absorption of the apoptotic pectic structure.

4. Materials and Methods

The androgen-responsive prostate cancer line LNCaP was obtained from the American Type Culture Collection (Rockville, MD). RPMI-1640 medium supplemented with 25 mM HEPES and L-glutamine was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS), penicillin/streptomycin, and citrus pectin (P-9135) were from Sigma-Aldrich (St Louis, MO). Fungizone was obtained from Invitrogen (Carlsbad, CA). Athymic nude Foxn1 mice were from Jackson Laboratory. All other chemicals, unless otherwise stated, were from Fisher Scientific.

Fractionation of citrus pectin by heat treatment

HTCP was prepared similarly as described in Jackson *et al*. (2007). Specifically, multiple batches of 0.1% aqueous pectin dispersion were prepared by dissolving 500 mg of unmodified Citrus Pectin (CP) in 500 ml of de-ionized, filtered water. The solutions were autoclaved at 123.2^{o}C at 17.2-21.7 psi for 30 minutes, cooled to room temperature and stored overnight at 4°C. The following day, the solutions were combined, frozen at -80°C and lyophilized to dryness.

Preparation of treatments for oral administration

Forty-five athymic nude Foxn1 mice were divided into three groups: water only, HTCP, and CP. The water group received 10 mL of deionized autoclaved filtered (0.2 mm) water/day for 30 days. The CP and HTCP groups received 10 mL of a 10 mg/mL solution of each respective pectin dissolved in deionized autoclaved filtered (0.2 mm) water/day for 30 days.

Tumor cell injection, measurement and processing of tumors

LNCaPs were harvested with trypsin/EDTA (Invitrogen) and were inoculated subcutaneously at 1×10^7 cells per mouse in 50 μl DME into the left hindquarters of 4-8 wk-old mice. Tumor growth was measured using calipers over a 30 day period, and tumor volume was estimated using the following formula: vol = length $\times$ (width)^2/2. Mice were killed after 30 days. Two hours prior to euthanasia, tumors were injected with BrdU-50 mg/kg. Tumors were then removed, measured directly with calipers, and fixed in 4% formalin for histologic analyses.

Acknowledgements

CHAPTER 5

TEST OF THE HYPOTHESIS THAT ANTICANCER PECTIN IS GENERATED DURING

THE FERMENTATION OF ETHANOL FROM PLANT BIOMASS AND MAY SERVE AS A

VALUE-ADDED BY-PRODUCT FROM PECTIN-RICH BIOMASS[5]

[5] Crystal L. Jackson[A,B], Claire Edwards[C], Joy Peterson[C], Debra Mohnen[A,B]
[A]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, GA 30602-4712
[B]Department of Biochemistry and Molecular Biology, UGA Cancer Center, The University of Georgia, Athens, GA 30602
[C]Department of Microbiology, The University of Georgia, Athens, GA 30602

1. Introduction

Lignocellulosic biomass represents the largest source for renewable biomass and is typically composed of 25-60% glucose, 5-30% hemicellulose (rich in xylose and arabinose residues), 10-20% lignin, and 5-35% other sugars. Lignin, causes rigidity in secondary plant cell walls and is associated with cellulose and hemicellulose (Mohnen et al, 2008). Lignin is not fermentable to ethanol and it interferes with the enzymatic degradation of fermentable polysaccharides into their component monosaccharides (Chang and Holtzapple 2000; Berlin et al. 2005; Guo et al. 2009). Thus, the heavy lignification of lignocellulosic biomass and the recalcitrance of this biofuel feedstock to degradation presents a challenging hurdle to process development. Biomass types with low lignin and high carbohydrate content are therefore desirable as fermentation feedstocks for biofuel production.

One high-carbohydrate, low-lignin source for biofuel production is pectin rich-biomass; an under-utilized waste product of the juice and sugar industry that is currently being sold at low value as animal feed or dietary fiber supplement. In the USA, approximately 2.8 million tons (dw) of pectin-rich material is produced each year (Edwards and Doran-Peterson, 2012). Pectin-rich biomass is largely composed of easily digestible polymers that could potentially be exploited to increase biofuel yields (Somerville et al., 2010). As by-products of the juice and sugar industries, commercial pectins are often partially processed making them more susceptible to enzymatic digestion and, subsequently, fermentation by ethanologens. Furthermore, pectin-rich residue is already collected during industrial processing of agricultural products and therefore its use would not increase harvesting or transportation costs.

In the commercial food and drug industries, pectin is most commonly extracted from primary cell walls, the first wall laid down by growing and dividing cells (Mohnen *et al*. 2008)).

Such plant material includes citrus peels, apple pomace (Thakur *et al.* 1997) and sugarbeet pulp. The same pectin-rich materials, sugar beet pulp (Ror- ick et al., 2011), citrus waste (Lopez et al., 2010; Pourbafrani et al., 2010), and apple pomace (Canteri-Schemin et al., 2005) have been analyzed as bioenergy feedstocks (Xiao and Anderson 2013). The cell walls of pectin-rich biomass in dicots and non-graminaceous monocots contain 12–35% pectin (Mohnen 2008) on a dry weight (dw) basis (Edwards and Doran-Peterson, 2012). Specifically, apple pomace, citrus peel, and sugar beet contain 10-15%, 25-35% and 10-20% pectin, respectively (Cho and Almeida, 2009). Only approximately 2% dw of citrus waste and sugar beet pulp is lignin (Edwards and Doran-Peterson, 2012), making residues in pectin-rich biomass more susceptible to enzymatic cleavage. In comparison, cell walls of residual agricultural lignocellulosic biomass that are not characterized as pectin-rich, such as corn cobs, wheat straw and other grasses, and woody biomass (forestry waste), contain only 2-10% dw pectin (Mohnen 2008).

Commercial preparation of pectin

After juice or sugar production, the remaining pectin-rich residue is immediately dried and citrus peels are additionally washed to prevent browning and to separate citrus oil. A pectin-containing extract is produced by treating the raw material with inorganic acid at elevated temperature. These conditions break the bonds of the neutral sugar sidechains that attach to the pectin backbone. The pectin-containing extract is then separated from the insoluble raw material which mainly contains cellulose, and is isolated by alcoholic precipitation. The alcohol-water mixture is separated from the precipitated pectin which is dried, ground, and sieved to defined particle sizes (Cho and Almeida, 2009). Pectin from citrus peel and apple pomace are typically high molecular weight and pectin from sugar beet pectin is low molecular weight. Subsequently

treating the pectic material with diverse mixtures of enzymes including pectin esterase and endopolygalacturonase (Pattathil *et al*. 2010), along with mild base deesterification results in pectic polymers with reduced degrees of esterification and reduced molecular weight. The resulting carbohydrates consist of the linear polymers homogalacturonan (HG) and xylogalacturonan (XGA), the branched polymer rhamnogalacturonan I (RGI) and the highly substituted rhamnogalacturonan II (RGII) (Ridley *et al.* 2001; Albersheim *et al* 1996).

Pectin Structure

Homogalacturonan (HG) accounts for 57-69% of pectin (Mohnen 2002) and is a linear polymer of 1,4-linked α-D-galactopyranosyluronic acid (GalA) (O'Neill *et al.,* 1990) in which some 8-74% (Voragen *et al.* 1986) of the carboxyl groups may be methyl esterified. HG may also be partially O-acetylated at C-3 or C-2. The length of HG remains unclear, but degrees of polymerization of 30 to 200 have been reported (reviewed in Mohnen 1999). Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that accounts for 7-14% of pectin and consists of a backbone of the repeating disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. The average MW of sycamore RG-I is estimated to be 10^5-10^6 Da (O'Neill *et al.* 1990). Roughly 20-80% of the rhamnoses of RG-I are substituted by L-arabinose, D-galactose, L-arabinans, galactans, or arabinogalactans (Ridley *et al.* 2001; O'Neill *et al.* 1990; Mohnen *et al.* 1999). The side branches include α-1,5- and α-1,3-linked arabinans, β-1,4-linked or β-1,3 and β-1,6 linked galactans, and arabinogalactans of diverse linkages (reviewed in Mohnen 1999). Interestingly, arabinoglactan type II (AGII) proteins (AGPs) are often co-eluted with RG-I (Vincken *et al*. 2003) and Immerzeel et al. (2006) have suggested that AGPs may be covalently linked to carrot pectin. Recently, proof of the covalent attachment of pectin to AGP was shown

by Tan *et al*. (2013) upon the identification of the proteoglycan arabinoxylan pectin arabinogalactan 1 (APAP1). APAP 1 contains RG-I attached to the arabinogalactan (AG) domain of the core AGP and HG covalently linked to the RG-I domain. There are also two xylose domains with xylan domain 1 attached to the AG domain and xylan 2 attached to the RG-I domain. AGPs are members of the family of hydroxyproline rich glycoproteins (HRGPs) (Zhang *et al*. 2003) and consist of more than 90% AG (Majewska-Sawka and Nothnagel 2000; Nothnagel 1997). AG in AGPs is $\beta-(1-3)$ linked galactan with $\beta-(1\text{-}6)$-Gal*p* side chains and terminated by $\alpha-$L-Ara*f* at O6 of the galactan backbone or with GlcA or Rha residues (Tan *et al*. 2012). Rhamnogalacturonan II (RG-II) is a substituted galacturonan that accounts for 10-11% of pectin. RG-II consists of a homogalacturonan backbone with four side branches of complex structure. RG-II is arguably the most complicated polysaccharide in nature consisting of 12 different types of sugars joined in over 20 different linkages and contains unusual sugars such as 2-O-methyl xylose, 2-O-methyl fucose, 2-keto-3-deoxy-D-*manno*-octulopyranosylonic acid (KDO), 3-deoxy-D-*lyxo*-2-heptulopyranosylaric acid (DHA) and apiose (reviewed in Atmodjo 2013). Whereas the structure of RG-II is highly conserved across plant species, the structures of HG and RG-I are more variable in different plants and cell types due to differences in polymer size, in the patterns of acetylation, methylation and other modifications of GalA in the backbone of HG, and due to variations in the length and type of side branches on the RG-I backbone. The RG-I side branches include arabinans, β-1,4-galactans that may contain arabinose in their side branches (so called Type I arabinogalactans) and, to a lesser extent the β-1,3 and 1,6-galactans similar to those found in AGPs (i.e. Type II AGs or AGII). The acid extraction process during the production of commercial pectin results in the destruction and loss of RG-II and of some RG-I. In some cases, commercial citrus pectin may be further treated with base or heat to yield

partially fragmented and structurally modified pectin.

In our prior work (Jackson *et al.* 2007) we showed that heat-treatment of commercial pectin generates a structure that induces apoptosis in human prostate cancer cells and that digestion of this pectin with pectinases and pectinmethylesterases (mimicking the treatments utilized to isolate pectin from the cell wall) does not destroy the apoptotic activity. Because heat treatment is one of the processes used in the preparation of biofuel from pectin-rich biomass, we aimed in collaboration with Joy Peterson (Department of Microbiology, University of Georiga, Athens GA) to determine whether biomedically active pectic polysaccharides, i.e. pectins with the ability to induce programmed cell death in human prostate cancer cells (apoptotic pectins), are present in, or generated during, the process of producing ethanol from pectin-rich biomass. Specifically, we asked whether pectic fragments are released during pre-treatment, enzymatic digestion, and/or fermentation of pectin-rich biomass..

After biomass is harvested and mechanically degraded, it is pretreated by chemical or physical means to destroy the lignin shell surrounding cellulose and matrix polysaccharides in the secondary cell wall and to reduce the crystallinity of cellulose making it more accessible to enzymatic digestion. This pre-treatment process includes but is not limited to hot water treatment (170-230°C) and steam-explosion (2,5-7 MPa; 180-280°C). To determine whether biomedically-active pectic polysaccharides are present in, or generated by, the process of producing ethanol from pectin-rich biomass, we evaluated the apoptotic activity on lymph node derived human prostate cancer cells (LNCaPs) cells of pectic fragments released during pre-treatment (e.g. heat), enzymatic digestion (e.g. with pectinases), and fermentation of pectin-rich biomass. Specifically, we asked whether pectins with the ability to induce programmed cell death in human prostate cancer cells (apoptosis-inducing pectins) are generated during the fermentation

of pectin-rich biomass.

2. Results

Effect of bioethanol production processes on the measurement of uronic acid in apple biomass

Similar to high lignocellulosic biomass, structurally complex pectins are not efficiently converted to oligosaccharides that can be effectively fermented without additional processing. However, as by-products of the juice and sugar industry, apple and citrus biomass are often partially processed making them more susceptible to enzymatic digestion and, subsequently, fermentation by ethanologens. In addition, pretreatments like hot water treatment (170-230°C) and steam-explosion (2,5-7 MPa; 180-280°C) are often applied to citrus biomass after juice extraction to isolate limonene, an aromatic monoterpene that comprises about 86–95 % of the essential oils in citrus waste (Shaw 1979, Edwards and Doran-Peterson 2012) and can have an inhibitory effect on ethanol production by yeast (von Loesecke 1934). For these reasons, in our initial studies to determine whether apoptotic pectic structures are present in or generated by the process of producing ethanol from pectin-rich biomass, apple (Figure 5.1B, Figure 5.2B) and steam treated orange peels (Figure 5.1A, Figure 5.2A) were used as bioethanol feedstock. We first evaluated the amount of uronic acid present, using the method of Blumenkrantz and Asboe-Hansen (1973), in the pectic fragments released during pre-treatment (e.g. heat), enzymatic digestion (e.g. with pectinases), and fermentation of pectin-rich biomass.
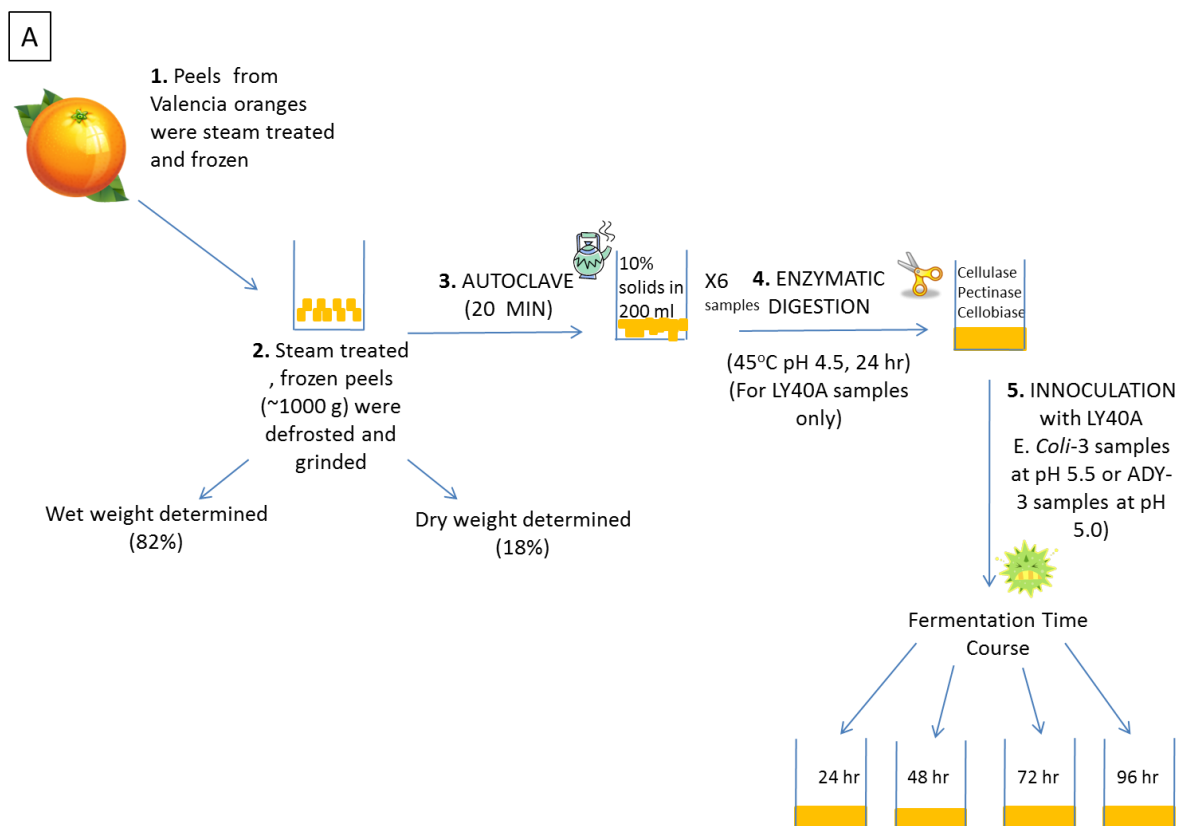
Figure 5.1. Schematic for bioethanol production from fermentation of citrus biomass **(A)** or apple biomass **(B).** Wet and dry weights were determined from pectin-rich biomass and the blended fruit residue was heated to 121°C at high pressure (autoclaved) for 20 min. The material was divided into 6 test samples per group based on the microbe used for fermentation (citrus) or pH of fermentation (apple pomace). Samples in the *E. coli* groups underwent partial saccharification by pectin degrading enzymes. All samples were then inoculated with respective ethanologen (*E. coli* or ADY) for 96 h (citrus) or 72 (apple pomace). Aliquots were taken at each step and after every 24 hr during the fermentation process. Collected samples were centrifuged and supernatants preserved for isolation of pectin.

Figure 5.1. Schematic for bioethanol production from fermentation of citrus biomass **(A)** or apple biomass **(B).** Wet and dry weights were determined from pectin-rich biomass and the blended fruit residue was heated to 121°C at high pressure (autoclaved) for 20 min. The material was divided into 6 test samples per group based on the microbe used for fermentation (citrus) or pH of fermentation (apple pomace). Samples in the *E. coli* groups underwent partial saccharification by pectin degrading enzymes. All samples were then inoculated with respective ethanologen (*E. coli* or ADY) for 96 h (citrus) or 72 (apple pomace). Aliquots were taken at each step and after every 24 hr during the fermentation process. Collected samples were centrifuged and supernatants preserved for isolation of pectin.
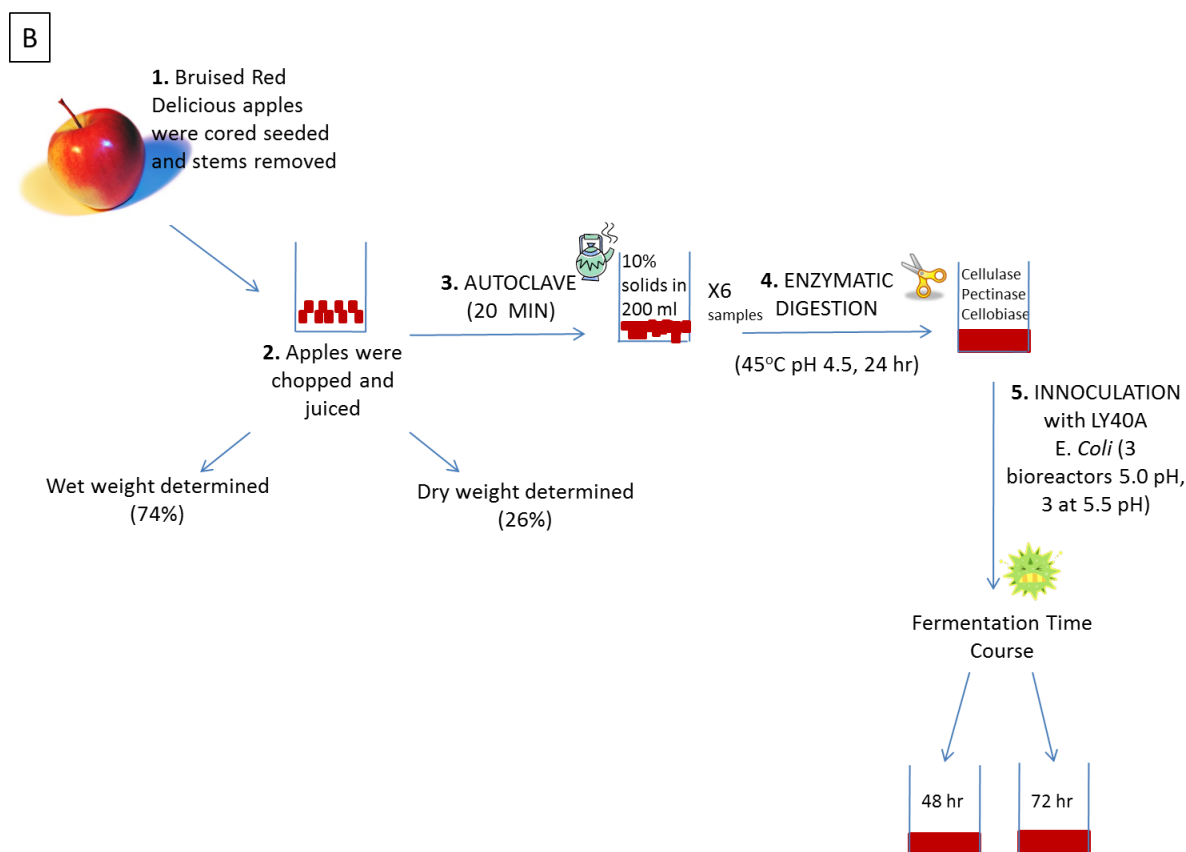
Figure 5.2. Schematic for downstream processing of citrus biomass **(A)** or apple biomass **(B)** to enrich for pectin and apoptotic pectic structure. Supernatants of pectin containing biomass residue were collected during three stages of the bioethanol production process: A) after autoclave pretreatment (tea kettle), B) after autoclave pretreatment and subsequent enzymatic digestion or partial saccharification (tea kettle+scissors) and C and D) after autoclave pretreatment, partial saccharification and fermentation (tea kettle+scissors+ microbe). Pectin was enriched for by incubation with ice cold 85% ethanol for 30 minutes. The precipitated pectin from A, B, and C (*E. coli* fermentation) or D (ADY fermentation) was autoclaved for 20 minutes to enrich for apoptotic pectin structure(s).

In apple biomass, we detected considerable uronic acid in the samples before performing ethanol precipitation to isolate pectin (Figure 5.3). The amount of uronic acid recovered in the ethanol precipitated material was lower than that measured in the original samples. This may have been due to an incomplete precipitation of the uronic acid with the percent ethanol used or because precipitation causes pectin to become difficult to resolubilize. Additionally, there was an apparent increase in the amount of uronic acid present after the ethanol precipitated material was heated by autoclaving, further suggesting that the ethanol precipitated pectin had reduced ability to be resolubilized without additional processing. A similar trend was seen upon analysis of the uronic acid detected in ethanol precipitated and ethanol precipitated and autoclaved citrus biomass (Figure 5.4). While it is difficult to draw conclusions that correlate specific steps in the bioethanol production process with amount of detectable uronic acid, there are some trends worth noting. In the apple biomass original samples, i.e. before ethanol precipitation, the amount of detectable uronic acid decreases with each step in the process, with the 72 h fermentation having the lowest levels (Figure 5.3).

Also, assuming that the autoclaved ethanol precipitated samples were a more accurate measurement of the uronic acid content in the samples than the ethanol precipitated samples for reasons described above, it appears that an early time point of fermentation duration, i.e. the 24 hour inoculations with *E. coli*, have the lowest levels of measurable uronic acid, while the longest inoculation, 72 h, had the highest levels (Figure 5.3). The ethanol precipitated citrus biomass inoculated with *E. coli* followed a similar trend but ethanol-precipitated and then autoclaved citrus biomass exhibited somewhat of an opposite trend regardless of ethanologen (Figure 5.4).
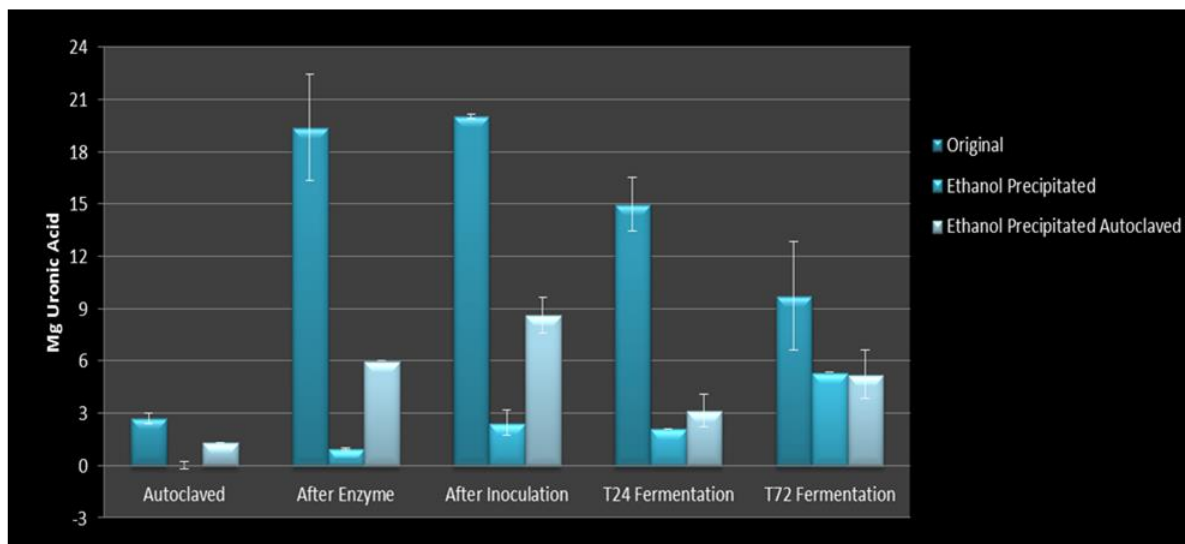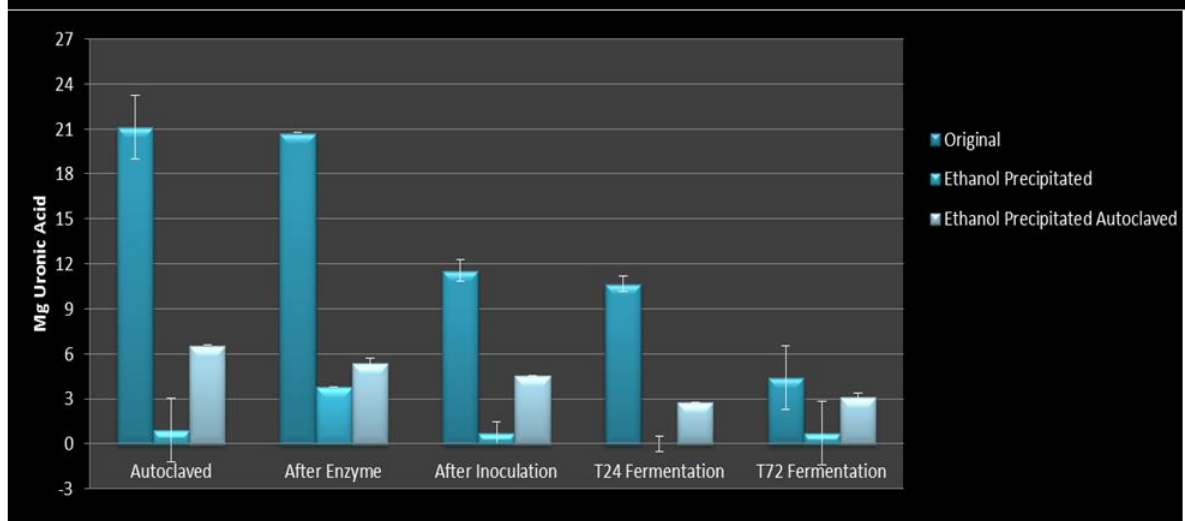
Figure 5.3. Measurement of the uronic acid content of pectin isolated from apple biomass supernatants collected during different stages of the bioethanol production process (i.e. autoclave pretreatment, saccharification (after enzyme) and varying durations of fermentation) (See Figure 5.2B for experimental schematic). Original, ethanol precipitated and ethanol precipitated autoclaved (see legend) describe the degree of downstream processing of the samples to enrich for pectin and an apoptotic structure. (A) Assessment of pectic samples isolated from apple biomass during ethanol production by *E. coli* LY40A. (B) Assessment of pectic samples isolated from apple biomass during ethanol production by Active Dry Yeast (ADY). Uronic acid is a measure of galacturonic acid (GalA) equivalents released from pectic samples after acid hydrolysis. Data represent the amount of uronic acid present in each sample and are the average of duplicate assays.

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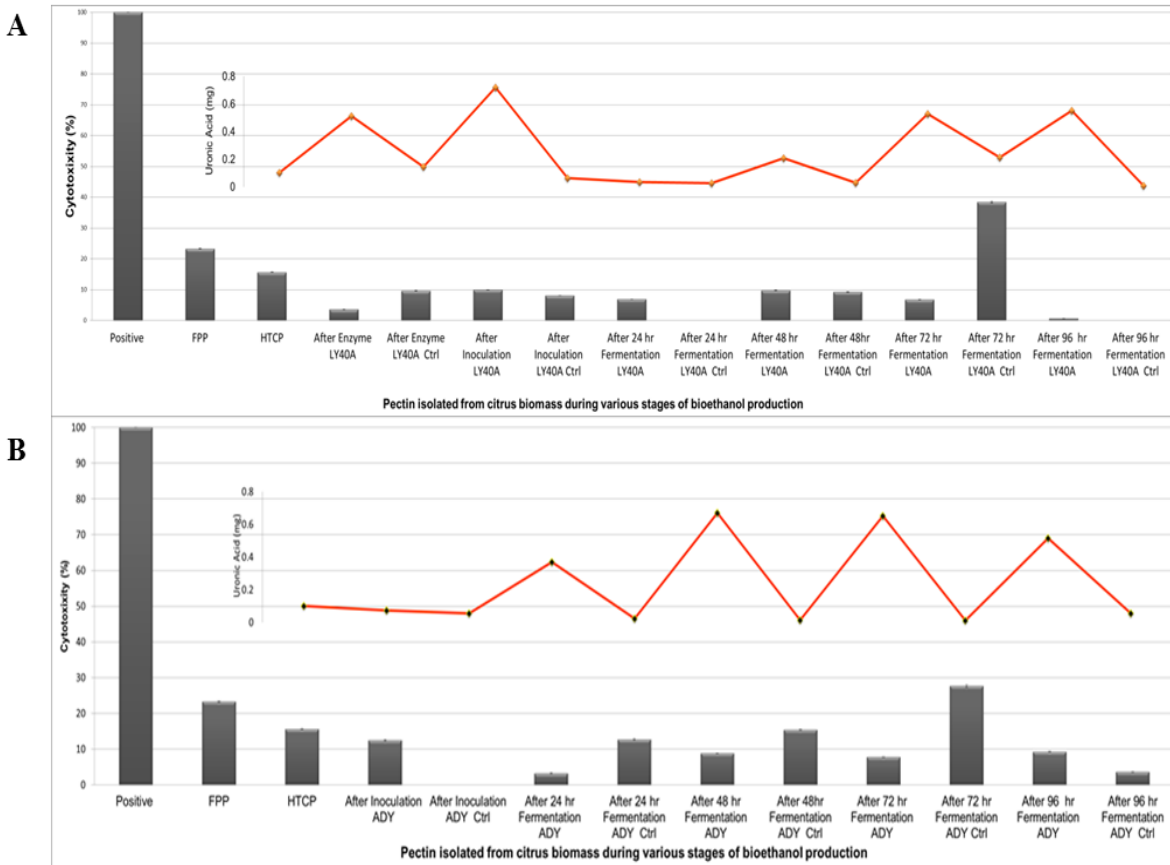
Figure 5.4. Measurement of uronic acid content (line graphs) of pectin isolated from ethanol precipitated and then autoclaved citrus biomass during various stages of bioethanol production and induction of cell cytotoxicity (bar graphs) in LNCaP cells. **(A) Assessment of pectic samples isolated from citrus biomass during ethanol production by *E. coli* LY40A. (B) Assessment of pectic samples isolated from citrus biomass during ethanol production by Active Dry Yeast (ADY).** Uronic acid is a measure of galacturonic acid (GalA) equivalents released from pectic samples after acid hydrolysis. Data represent the amount of uronic acid present in each sample and are indicative of the amount of uronic acid that was applied to LNCaP cells. Data are the average of duplicate assays. As an initial measure of cytotoxic effects of pectin isolated from supernatants of citrus biomass collected during different stages of the bioethanol production process, LNCaP cells were treated with 1 mg/mL of ethanol precipitated pectin or with medium alone (negative control subtracted) for 48 h. Positive controls were cells incubated with medium and treated with lysis buffer after the 48 h incubation. Cytotoxicity is a measurement of lactate dehydrogenase released into the medium from cells during plasma membrane damage. Data are the average of duplicate biological tests (except for controls, reactions containing no citrus biomass) and of duplicate LNCaP cell treatments $\pm$ STDEV.

Defining the bioactivity of pectin isolated from citrus biomass during the bioethanol production

process

The experiments performed with apple biomass were primarily done as preliminary analyses to optimize conditions for bioethanol production from pectin-rich biomass from an industrial source. Since the steam-treated orange peels were obtained from a commercial source after the fruit was processed for juice extraction and were already subjected to steam treatment and thus, more characteristic of a biofuel feedstock on an industrial scale, we utilized this material as the pectic-rich biomass source for our biological assays. In our initial experiments, we assayed for the induction of cell cytotoxicity using ethanol-precipitated pectin and ethanol-precipitated and autoclaved pectin isolated from citrus biomass at various stages of the bioethanol production process. When each of the ethanol precipitated pectic preparations (Figure 5.5A) were incubated with androgen-sensitive lymph node-derived human prostate cancer cells (LNCaP) to test, via a lactate dehydrogenase activity assay, for cytotoxic activity in the pectins present in the supernatant of biofuel residue, we found only modest induction of cytotoxicity in most treatments. T_0 fermentation samples, those taken immediately after adding *E. coli or* ADY, yielded the highest levels of cytotoxicity. However, these results are complicated by the high levels of lactate dehydrogenase activity measured seen in some of the corresponding controls, i.e. the samples taken from bioreactors containing no pectic-rich biomass. Since lactate is a byproduct of fermentation and this assay measures the amount of lactate oxidized by lactate dehydrogenase in the form of NADH produced, we postulate that the high levels of cytotoxicity measured in control samples was due to exogenous lactate present in the treatments applied to the cells. Cells incubated with pectic material isolated by ethanol precipitation and then autoclaved (Figure 5.5 B) yielded even more
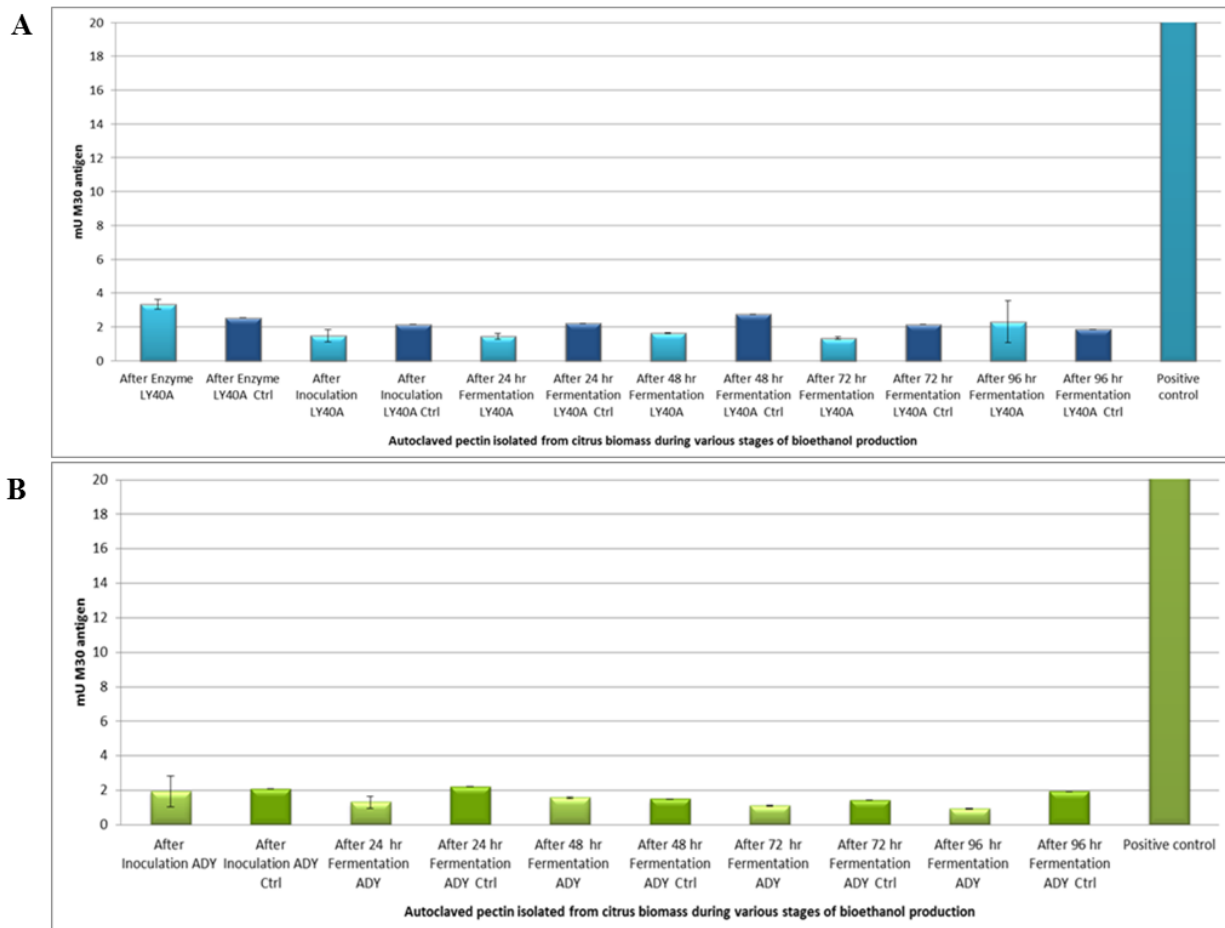
Figure 5.5. Induction of apoptosis by autoclaved pectin isolated from the supernatants of citrus biomass collected during different stages of bioethanol production (i.e. autoclave pretreatment, saccharification or fermentation of varying durations). **(A) Assessment of apoptosis induced by pectin isolated from citrus biomass during ethanol production by *E. coli* LY40A (B) Assessment of apoptosis induced by pectin isolated from citrus biomass during ethanol production by Active Dry Yeast (ADY).** LNCaP cells were treated with 1 mg/mL (DW) of each treatment or with 0.01 mM thapsigargin (Pos) for 48 h. Incubation with medium alone served as the negative control (value subtracted). Controls were samples devoid of biomass and are displayed adjacent to respective treatment. Equal amounts of cell extracts (10 ug protein) were used for measuring apoptosis. Apoptosis was measured using an M30-Apoptosense assay, which measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Data are the average of duplicate apoptosis assays of duplicate cell treatments ±STDEV.

ambiguous results. We conclude that the cellular cytotoxicity assessment using the lactate dehydrogenase assay is an ineffective way of ascertaining biological activity in the samples isolated.

To assay specifically for apoptosis we used the method described in Jackson *et al.* (2007) where apoptosis was quantified using the M30 Apoptosense assay (Peviva), an enzyme-linked immunosorbent assay (ELISA) that measures the generation of an apoptosis-specific neoepitope of cytokeratin-18, which is a substrate of activated caspase. LNCaP cells were treated with 1 mg/mL (DW) of ethanol precipitated and subsequently autoclaved material from each supernatant. Cells incubated in media devoid of pectin served as negative controls and cells treated with thapsigargin, a compound that induces apoptosis by creating endoplasmic reticulum stress and thereby raising cytosolic calcium concentration, served as a positive control. Figure 5.5 shows that, among the pectins tested, only those isolated from citrus biomass after partial saccharification induced apoptosis in both LNCaP cells (Figure 5.5a). This is evidence for soluble apoptotic activity from autoclaved steam-treated orange peel (prior to microbe treatment). These results are consistent with our prior results (Jackson *et al.*, 2007). Overall, however, there is no indication that products of fermentation of steam-treated orange peel by either *E.coli* or yeast, yield a soluble fraction with high levels of apoptotic activity against human prostate cancer cells.

3. Discussion

Pectins are one of the most structurally complex classes of polysaccharides in nature, and it is perhaps due to this complexity that they serve a multitude of functions during plant growth and development. Depending on the feedstock, processing, and desired end products, pectin can be viewed either as a hindrance to biomass degradability, or a source of fermentable sugars, or as

a potentially valuable co-product of biofuel production.

The specific goal of this project was to test the hypothesis that anticancer pectins are produced as a byproduct of the bioethanol production process. Prior work showing that high pressure heat treatment generates pectin that has the ability to kill human prostate cancer cells (Jackson *et al.* 2007) supported a hypothesis that heat treatments used during the biomass pre-treatment step may generate pectins which, depending upon the exact pretreatment and enzyme steps used in the process, could have apoptotic activity on human prostate cancer cells. Such pectins could provide a value-added product from the fermentation process. To determine whether biomedically-active pectic polysaccharides are present in, or generated by, the process of producing ethanol from lignocellulosic biomass, we evaluated the apoptotic activity on LNCaP cells of pectic fragments released during pre-treatment, enzymatic digestion, and fermentation of pectin-rich biomass. While our preliminary data (Jackson *et al.* 2007; Jackson, Suarez and Mohnen unpublished) suggested that pressurized batch hot water pretreatment developed by the Peterson lab to improve digestibility of plant biomass by enzymes during the bioethanol production process may generate apoptotic pectins, we obtained no evidence for a pectin-induced cell cytotoxicity on LNCaPs when the cells were treated with different preparations of pectin released from the bioethanol production of citrus waste. Perhaps a modification of the pectin extraction methods or utilization of a different source of pectic-rich biomass would yield different results. This possibly would require further studies.

4. Materials and Methods

Materials

Steam treated Valencia orange peels (industry partner) and apples (Kroger grocery store) were sources of pectin-rich biomass. The LNCaP prostate cancer cell line, obtained from

American Type Culture Collection (Rockville, MD) is an androgen responsive prostate cancer cell line. Fetal bovine serum (FBS) was from Thermo Scientific Hyclone (Logan, UT). Antimicrobial media supplement (Penicillin/streptomycin/fungizone) was from Corning Mediatech (Manassas, VA) and citrus pectin (P-9135) from Sigma-Aldrich (St Louis, MO). Bio-Rad Protein Assay dye reagent concentrate was purchased from Bio-Rad (Hercules, CA). M-30 Apoptosense ELISA was from Peviva AB (Sweden). All other chemicals, unless otherwise stated, were from Fisher Scientific. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with 2% (wt/vol) glucose for ethanologenic strains. Antibiotics were used at the following concentrations unless otherwise stated: chloramphenicol (Cm) 40 mg/liter, ampicillin (Ap) 50 mg/liter, kanamycin (Kn) 40 mg/liter, erythromycin (Em) 150 mg/liter, and spectinomycin (Spc) 50 mg/liter (all chemicals were obtained from Sigma Chemical Co., St. Louis, MO).

Preparation of pectin-rich biomass from apples

Five Red Delicious apples (Kroger grocery) were stored at 0°C for 33 days then cored and the seeds and stem removed. Juice was extracted and the wet weight was determined using a Denver Instrument (Denver, CO) IR 35 moisture analyzer.

Preparation of pectin-rich biomass from citrus

Frozen steam treated orange peels (1000g) were defrosted and wet weight was determined using a Denver Instrument (Denver, CO) IR 35 moisture analyzer.

Partial fractionation of pectin-rich biomass by heat treatment and saccharification with

pectinases

The juiced apple residue and orange peels, were separated, mechanically chopped into small pieces using a Grindomix 200 food processor (Retsch, Inc., Newtown, PA) at 10,000 rpm for 10 sec. The residues from apple biomass were divided into 6 test bioreactors (three for fermentation with *E. coli* strain LY40A at pH 5.0; three for fermentation with *E. coli* strain LY40A at pH 5.5). Each bioreactor contained 10% AP (apple pomace) solids, 100 ml of 2x Luria-Bertani (LB) medium and water to a final volume of 200 mL. The heat-treated residues from citrus were divided into 6 bioreactors per fermentation group (Group1 for fermentation with *E.* coli strain LY40A at pH 5.5; Group 2 for fermentation with active dry yeast (ADY) at 5.0). Each bioreactor contained10% solids from citrus biomass, 100 ml 2x Luria-Bertani (LB) medium, and water to a final volume of 200 mL. Controls for the citrus samples included samples containing no pectin-rich biomass. There were no negative controls, i.e. bioreactors devoid of biomass, for the apple pomace group. The processed fruit residues were autoclaved at 123.2 8°C at 17.2–21.7 psi for 20 min, prior to inoculation with a mixture of filter-sterilized enzymes. Aliquots (10 mL) were collected, centrifuged and the resulting supernatants filter-sterilized and stored at -20°C. Bioreactors were placed into a water bath at 45°C and stirred with magnetic stirrers. The pH was adjusted to 4.5. For the samples that were to be fermentated by *E. coli*, partial saccharification was conducted for 24 h using cellulase (Novazyme CP; Genencor, Copenhagen, Denmark), pectinase (Pectinex P2736) from *Aspergillus niger* (Novozymes, Franklinton, NC) and cellulobiase at concentrations of 15 filter paper units (FPU) of cellulase/g (dry weight), 60 polygalacturonase units (PGU) of pectinase/g (dry weight), and 50 cellobiose units (CBU) of cellobiase/g (dry weight). Following enzymatic digestion, 10 mL aliquots from each sample were collected, centrifuged at 1400 rpm for 10 min, and the supernatant filter-

sterilized and stored at -20°C until further analysis.

Fermentations of pectin-rich biomass

The pH of half of the bioreactors was raised to 5.0 and half to 5.5 with 10 M KOH, while the temperature was decreased to 35°C. Fermentations were performed as previously described (Edwards 2011). A sufficient volume of *E. coli* cells, strain LY40A, to achieve a final OD_{550} of 1 was centrifuged and resuspended in 3 mL of 1x LB. *E. coli* strain LY40A (isolated from *E.coli* KO11; more ethanol tolerant than KO11 (Yomano et al. 1998)) or ADY (active dry yeast) along with appropriate antibiotics were added to respective bioreactors and conditions maintained for the duration of the fermentation. Fermentations continued for 96 h with samples collected every 24 h. At each time point, samples taken from bioreactors incubating at the same pH were combined and the collected samples were centrifuged at 1400 rpm for 10 min. The supernatant was then filter sterilized and put into 50 mL falcon tubes and stored at -20°C. Ethanol production was quantified using gas chromatography (GC) as described in Doran-Peterson *et al.* (2009). Fermentation supernatant samples were filtered with a 0.22-μm filter prior to analysis. Ethanol concentrations were normalized based on control fermentations conducted without the addition of pure sugar.

Ethanol precipitation of pectin

To isolate and enrich for pectic material amongst the other sugars and salts present, the supernatants collected from the autoclave treatment, saccharifications and fermentations described above, were subjected to ethanol precipitation. Ice cold ethanol was added to each sample, at 0°C, to a final concentration of 85% ethanol. Samples were left for at least 30

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minutes at 4^oC and then centrifuged at 1400 rpm, 4^oC for 6 min. Supernatants were frozen and lyophilized. The precipitated, pelleted pectin was dissolved in deionized water, heated at 123.28^oC at 17.2–21.7 psi for 30 minutes, frozen, and lyophilized. Aliquots of the lyophilized supernatants and redissolved pellets (before lyophilization) were reserved for uronic acid determination. Both lyophilized pellets and lyophilized supernatants were prepared for incubation with human prostate cancer cells (LNCaP) to assay for the presence of apoptotic pectic activity.

Uronic Acids Assay

Uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (1973). Samples were hydrolyzed in 0.3 mL 0.5% (w/v) $Na_2B_4O_7$ in concentrated H_2SO_4 at 100^oC for 5 min. Five microliters of 0.15% m-hydroxybiphenyl dissolved in 0.5% NaOH was added, and the samples were mixed and incubated at room temperature for 30 min. Absorbance was measured at 540 nm. Galacturonic acid (GalA) was used as a standard.

Measurement of cytotoxicity by the lactate dehydrogenase activity assay

Any potential cytotoxicity of pectin isolated for testing with LNCaP cells was assayed by measuring the release of the lactate dehydrogenase (LDH) into the culture medium from dead or dying cells. After 48 h incubation of LNCaP cell with each treatment at a concentration of 1 mg/mL the level of LDH enzyme in the culture supernatants was measured. A colorimetric kit from BioVision was used that yields a product detected at 450 nm following the manufacturer's protocol (BioVision-Milpitas, California). Enzyme assays were done in duplicate and the values averaged.

Cell culture, treatments and quantification of apoptosis

LNCaP (Lymph node derived human prostate cancer) cells were grown in RPMI-1640 medium supplemented with 25 mM HEPES, 2.0 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 U/ml Penicillin, 0.05 mg/ml Streptomycin, and 0.25 μg/ml Fungizone in the presence of 5% CO_2 at 37°C. Cells were maintained in logarithmic growth phase by routine passage every 10-12 days (LNCaP). Cells were plated at a density of 2 x 10^5 cells per well in 6-well culture plates and allowed to adhere to the culture dish for 24 hr. The medium was removed and replaced with medium containing filter-sterilized ethanol-precipitated pectin (0.20 μm nylon filters; Fisher Scientific) isolated from various steps of the bioethanol production process. Thapsigargin (0.01mM) (Sigma, St. Louis, MO) was used as a postitive control and media alone as a negative control. Cells were harvested after 48 hr incubation in the presence of 5% CO_2 at 37°C then lysed with ice cold lysis buffer (10 mM Tris-HCL, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40) and soluble protein was collected by centrifugation at 4°C. Protein concentration was determined in triplicate using a Bradford/Bio-Rad Protein Assay (Bio-Rad Protein Assay Dye Reagent Concentrate). Apoptotic activity was assayed using the M-30 Apoptosense ELISA (see below).

Apoptosense assay

Cells grown in culture plates and treated as described above were harvested and total protein extracted. Cell lysates were assayed for the presence of the apoptosis-specific cytokeratin-18 (K18) neoepitope (K18Asp396-NE) (generated by cleavage of cytokeratin-18 by caspases activated in response to treatment) using the M30-Apoptosense ELISA (Peviva AB,

Sweden).   In brief, protein extract (10 μg) was added to 96-well plates coated with mouse monoclonal M30 antibody. Horseradish peroxidase tracer solution was added to the wells, and the plate was incubated with agitation for 4 hr at room temperature. Color was developed by adding tetramethyl benzidine solution and incubating in darkness for 20 min.   Optical density was determined at 450 nm using Spectra MAX 340 (Molecular Devices, Menlo Park, CA) or Finstruments Model 347 (Vienna, Virginia, USA) microplate readers. The amount of cytokeratin-18 neoepitope was determined based on standard curves generated using standards provided by the manufacturer.

CONCLUSIONS

Prostate cancer is the most common malignancy in American men (*Surveillance, Epidemiology, and End Results Program (SEER),* 1975-2000, Division of Cancer Control and Population Sciences, National Cancer Institute, 2003) and the second leading cause of death from cancer in American men (American Cancer Society, 2004). The goal of many cancer therapies is to induce apoptosis in tumor cells. Usually one of first steps in prostate cancer treatment is androgen depletion therapy (ADT). In hormonally responsive prostate cancer, androgen deprivation therapies induce cell death in androgen-sensitive cells (Colombel and Buttyan, 1995; Buttyan *et al.*, 1997; Perlman *et al.*., 1999; Bruckheimer *et al.*., 1999), while androgen-insensitive cells remain unaffected (Kozlowski and Grayhack, 1991; Santen, 1992; Kreis W, 1995). Androgen deprivation therapy in prostate cancer is rarely curative because the metastatic cancer is heterogenous, consisting of both androgen-dependent and androgen-independent prostate cancer cells. Although, ADT is ineffective against androgen-insensitive cells, the cells are still capable of undergoing apoptosis. Thus, the identification of novel methods to induce apoptosis in prostate cancer cells, irrespective of their androgen response, is of great importance.

Although the etiology of prostate cancer is poorly understood, according to the American Institute of Cancer Research, 30-40% of all cancer cases are preventable by dietary means. A product of plant cell walls and a dietary component of all fruits and vegetables, pectin has been found to suppress colonic tumor incidence in rats (Heitman *et al.*, 1992) and inhibit cancer cell metastasis in mice and rats (Platt and Raz, 1992; Pienta *et al.*,

1995; Nangia-Makker *et al.* 2002).

The interpretation of studies with pectin are complicated by i) the structural complexity of this plant-derived cell wall polysaccharide, ii) the modifications in pectin structure that result from the commercial extraction of pectin from plants, and iii) the further modifications of pectin structure that result from the diverse fragmentation techniques used to produce specialized pectins (e.g. high pH (base) treatment; Platt and Raz, 1992; Pienta *et al.*, 1995; Eliaz, 2001; Nangia-Makker *et al.* 2002). Most of the published reports utilized pectins that are either modified by alterations in pH or by treatment with heat. The goal of the treatments is to fragment pectin structure to facilitate biological effects. Therefore, in our initial studies as a first step to determine whether size and method of preparation affected the biological activity of the pectins, we examined the effects of several commercial pectins, FPP, citrus pectin and PectaSol, on prostate cancer cells. The results showed that only FPP had high apoptosis-inducing activity in prostate cancer cells. CP and PeS did not affect the cells. We hypothesized that, as pectins consist of homogalacturonan and rhamnogalacturonans, these polysaccharides in FPP may have been responsible for its apoptotic effects. Experiments to determine the apoptotic effects of HGA, RGI, and RGII, indicated that the enzymatically separated and purified HG, RG-I, and RG-II components of pectin were not responsible for the apoptosis-inducing activity of FPP. To further examine the structural differences among the tested pectins, the size and glycosyl residue composition of different pectins were compared. These analyses showed no correlation of these parameters among the pectins, even though FPP induced apoptosis while the other pectins did not. Therefore, we concluded that the apoptosis-inducing activity of FPP was related to specific fine structural features of FPP that are not present in PeS and CP.

To further understand the structure-function relationship of apoptotic pectin, FPP was specifically fragmented using endopolygalacturonase (EPGase), which cleaves at contiguous non-esterified GalA residues in HGA. Cleavage of FPP with EPGase did not have a major effect on its apoptotic activity. Thus, two methods were use to remove ester linkages from FPP prior to EPG treatment and thereby, make FPP more susceptible to EPG cleavage: chemical deesterification by mild base treatment and specific enzymatic hydrolysis of methyl esters by treatment with pectin methylesterase (PME). Chemical deesterification of FPP resulted in significant loss of apoptosis activity suggesting that a base sensitive structure, such as an ester, or linkages sensitive to β-elimination under these conditions, is necessary for the apoptotic activity of FPP. However, specific cleavage of methyl esters by pectin methylesterase did not destroy FPP's apoptosis inducing activity, suggesting that linkages other than carboxymethylesters are required for apoptotic function.

PAGE analysis of intact and treated FPP showed that a base-sensitive linkage in a polymeric/oligomeric FPP structure and/or a specific base-sensitive linkage itself is required for the apoptotic activity of FPP. Taken together the results suggest that an ester-based (or related) cross-link in pectin is important for the apoptosis-inducing activity of FPP. More recent purification and enzyme-cleavage efforts to isolate and characterize the apoptotic pectin structure(s) have revealed that key residues and/or linkages are necessary for pectin's biological activity. These studies revealed that while the apoptotic pectin may contain a protein component, it alone does not appear to have provide theapoptosis activity of pectin. Linkages involving terminal galactose residues in the galactan side branches of RG-I and/or a linkages involving terminal galactose residues in the arbabinogalactan side chains of RG-I appear to be essential. Furthermore, activity was abolished by treatment of pectin (HPE) with

RG-I lyase, suggesting that the RG-I backbone is also necessary for the preservation of apoptotic activity. In conclusion, we provide evidence that specific structural characteristics of pectin are responsible for inducing apoptosis in cancer cells *in vitro*. Our results demonstrate that different extraction protocols may alter the structure of pectin and can lead to differences in pectin's apoptosis-inducing activity. This is of particular therapeutic significance, as we have demonstrated that manipulating the structure of pectin results in a compound that is capable of inducing apoptosis and that this structure can be extensively fragmented without destroying apoptotic activity.

Additionally, our results show that *in vitro* LNCaP cells express low levels of the anti-apoptotic proteins TRAF2, IKKγ, pI$_\kappa$Bα, NF$_\kappa$B, andTRADD (TRADD in these studies is likely mediating NF$_\kappa$B activation by binding to TRAF2) and high levels of the pro-apoptotic proteinT-I$_\kappa$Bα when treated with heat modified pectin. When LNCaP cells were incubated with pectin in the absence of androgens the apoptotic activity of pectin was slightly enhanced compared to pectin-induced apoptosis in LNCaP cells grown in the presence of androgens. LNCaP cells treated with serum-deprived medium alone exhibited no apoptosis and cells under the same conditions in the presence of pectin show slightly higher apoptotic activity than that observed in normal testing conditions (with media containing serum). Therefore, pectin appeared to act synergistically with androgens and as well as through a separate mechanism, independent of androgen ablation, perhaps involving growth factor inhibition. When LNCaPs were incubated with 0.1, 1.0 and 3.0 mg/mL heat-modified pectin in the presence of TRAIL, there was an increased apoptotic effect compared to heat-modified by itself. These results suggest, that when treated with heat-modified pectin LNCaP cells exhibited increased sensitivity to death-inducing ligands via the NF$_\kappa$B signaling pathway, perhaps involving sensitization to TRAIL, inhibition of

XIAP-mediated activation of NF$_\kappa$B, and increased sensitivity to androgen withdrawal.

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