PECTIN HYDROGELS OF CURCUMIN AND OBESITY APPLICATIONS

by

JI YEON LEE

(Under the Direction of Louise Wicker)

ABSTRACT

Blockwise de-esterified citrus pectin with targeted degree of esterification (DE) values of 55% (55P) or 35% (35P) formed hydrogels to encapsulate curcumin and were evaluated by *in vitro* dissolution study. Encapsulation efficiency of the modified pectins and commercial low-methoxyl pectin (LMP) were above 99%. Pectin molecular weight and ζ-potential decreased with charge modification. Release rates of curcumin in simulated digestive media were less than 2% in any of the pectin hydrogels. Charge modification of pectin with PME or chemical saponification is a viable method to tailor pectins for phytochemical encapsulation. The study to determine the effects of pectin on inhibition of adipogenesis of 3T3-L1 adipocytes showed high-methoxyl pectin (HMP) at 100 μg/mL had inhibitory effects on lipid content. Pectins did not have significant influence on cell viability; combinations of curcumin and pectin did not have an effect on lipid accumulation.

INDEX WORDS: Pectin, Curcumin, Hydrogel, *In Vitro,* Encapsulation Efficiency, Release Rate, 3T3-L1, Adipogenesis

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DEDICATION

Thank you so much, Mom and Dad, for believing in me and trusting me. This work is dedicated to you guys from your daughter. I love you always.

아버지(이 용록), 어머니 (권 영미) 에게 늘 노력하는 지연이가 이 글과 공부를 바칩니다. 저를 믿어주시고 할수있다라는 말을 해주셔서 항상 고맙고 사랑해요.

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CHAPTER 1

LITERATURE REVIEW

Pectin

Pectin structure

Pectin is an anionically charged complex plant polysaccharide consisting mainly of galacturonic acid residues and neutral sugar side chains found in the middle lamella and cell walls of plants (Voragen et al., 2009). The two main structural features of pectin include a linear backbone of α-(1-4)-D-galacturonic acid residues that forms the homogalacturonan region and the branched rhamnogalacturonan region with neutral sugar side chains (McNeil, Darvill, Fry, & Albersheim, 1984; Voragen, Coenen, Verhoef, & Schols, 2009). Some of the galacturonic acid residues are in forms of methoxyl esters (NCI, 1996). Homogalacturonan accounts for about 60% of total pectin and at carbon six, methyl esterification may occur for the galacturonic acids (Gee et al., 1959). Pectin is mainly extracted from citrus and apple in the food industry and is primarily used as a gelling, thickening, and stabilizing agent (Voragen et al., 2003).

Pectin- DE

Degree of esterification (DE) is a method of classification where pectin can broadly be categorized as a high-methoxyl pectin (HMP) or low-methoxyl pectin (LMP) if DE is greater than 50%, or less than 50%, respectively. DE value is a strong determinant of pectin gelling capabilities, but other factors influence it as well, such as

molecular weight, degree of acetylation, charge density, and charge distribution. Compared to fungal pectinmethylesterase (PME), which de-esterifies pectin randomly, plant PME de-esterifies contiguously the methyl esters on the pectin (Limberg et al., 2000); the pattern of de-esterification influences gelling and other functionalities of pectin. Pectins de-esterified by some plant PMEs have shorter un-esterified blocks of charge due to only a certain removal of methyl esters of a pectin structure, attributed to a different mechanism of action at different pH values (Denes et al., 2000; Willats et al., 2001).

Pectin- Charge

Pectins that have the same DE value can have differing total charge, charge density, and charge distribution if the structure has been modified. There are many different types of PME isozymes that can be purified from plants, especially citrus, that show different characteristics, such as thermally tolerant and sensitive forms of PME or salt-dependent and independent PMEs (Cameron and Grohmann, 1996; Savary et al., 2002). Different isozymes of citrus PME have different modes of action. Pectins that had the same DE but were treated with two isozymes of PME created pectins with different de-esterification pattern, attributed to differences in charge distribution (Kim et al., 2005). De-esterification of pectin with partially purified and crude citrus PMEs has a critical limit of de-esterification and increases the gel strength in a non-linear relationship (Lee et al., 2008). Block-wise distribution of free carboxyl groups in a pectin structure has a higher affinity of calcium binding than random distributions (Thibault and Rinaudo, 1985). The quantitative measurement of the distribution of the blocks of carboxyl groups

is possible by analyzing the methyl-esterified oligomers using a high-performance anion-exchange chromatography, which illuminates the structure-to-function behavior of the action of PME on pectin (Daas et al., 2000).

Plant PME de-esterified pectin and encapsulation

Pectin, especially charge-modified citrus pectin, forms hydrogels that encapsulate bioactive material, acting as a carrier through the gastro-intestinal tract for site-specific targeted delivery, while itself being non-toxic and biodegradable (Jung, Arnold, & Wicker, 2013). LMP gelation occurs when calcium ions and free carboxyl groups of pectin interact (Oakenfull and Scott, 1984; Powell et al., 1982). The junction zones formed by the calcium ions and carboxyl groups in a LMP gel, the 'egg box' model, attributes to LMP's strong gel strength in the presence of calcium (Axelos and Thibault, 1991; Grant et al., 1973). However, pectins modified using different PMEs showed different gel strengths at similar total charge, (Kim and Wicker, 2009; Lee et al. 2008) suggesting that plant PME charge modified pectins with overall higher gel strength than commercial LMP may be a highly effective delivery vehicle. LMP is a favorable drug delivery vehicle due to crosslinking with calcium ions with polymers (Wong et al., 2011). This leads to the charge neutralization of the drug which has a positive effect on the rate of drug release by limiting the interaction with dissolution media (Wong et al., 2011). LMP and charge modified pectin effectively encapsulates a water insoluble drug, indomethacin and protect it against *in-vitro* gastrointestinal conditions (Jung et al., 2013).

Health

Colon cancer

Colon cancer has gained wide attention in diet-affluent countries as the fourth most globally common form of cancer (Boyle and Leon, 2002). There are many contributing factors that affect an individual's chances of getting colon cancer, such as fat content in the body, especially abdomen, alcohol consumption, red and processed meat consumption, and many other probable factors. The latest update by the American Institute for Cancer Research (AICR) reported foods with fiber are one of the strongest factors linked to a decrease in risk of colorectal cancer (Research, 2011).

Pectin as a soluble fiber, increases gastrointestinal transit time, fecal bulk, excretion of bile acid, and production of short-chain fatty acid, by undergoing fermentation in the colon (Lupton, 2000). The short-chain fatty acids (SCFAs) produced from the fermentation of pectin are the primary energy source of colonocytes that lower the pH of the colonic environment (Lupton, 2000; Moore et al., 1998). SCFAs, especially butyrate, exerts cellular effects through the induction of histone hyperacetylation which has a link to the antiproliferative, apoptotic, and differentiating properties of SCFAs (Hinnebusch et al., 2002).The number and incident of colon tumor decreased with citrus pectin and apple pectin in rats (Ohkami et al., 1995; Tazawa et al., 1997).

Microbiota & products on signaling & gastrointestinal health

There are an estimated 100 trillion microbial organisms with more than 1,000 species-level phylotypes found in the human GI tract (Qin et al., 2010; Rajilic-Stojanovic et al., 2007; Savage, 1977). The composition of microbiota is diverse and varies from

individuals due to many factors, such as age, diet, geographic location, antibiotic usage, and presence of diseases (Eckburg et al., 2005). Colonic microflora might have a major function in maintaining a healthy colon which affects the development of colorectal cancer (O'Keefe, 2008). There is emerging evidence that the composition of intestinal microbiota is influenced by the consumption of probiotics, prebiotics such as oligosaccharides, and polyphenols, which have possible beneficial effects on colon cancer (Davis and Milner, 2009). The dietary material undigested and unabsorbed by the small intestine can undergo fermentation by the microflora in the large intestine. Carbohydrate fermentation and proteolytic fermentation are the two major types of anaerobic fermentation in the gut (Manning and Gibson, 2004). Carbohydrate fermentation produces short chain fatty acids, such as butyrate, acetate, and propionate, while proteolytic fermentation yields phenolic compounds, amines, ammonia, N-nitroso compounds, and indoles (Manning and Gibson, 2004).

Prebiotics are nondigestible food ingredients, such as oligosaccharides, which stimulate growth and activity of the microflora and benefits the host (Lim et al., 2005). Dietary fibers which can be fermentable are also considered to be a prebiotic (Lim et al., 2005). Synbiosis describes the synergistic effects of combining prebiotics and probiotics (de Vrese and Schrezenmeir, 2008). Although dietary fibers and polyphenols have beneficial prebiotic effects on the gut microflora, the combination of the two enhances the benefits.

Bacteria generate short-chain fatty acids and other metabolites by fermentation of prebiotic materials, which can act as growth signals and aid in the proliferation of intestinal epithelium (Mai, 2004). These short-chain fatty acids such as butyrate prevent

apoptosis in healthy colonocytes, while inhibiting cell proliferation and angiogenesis in colon carcinoma cells (Basson et al., 2000; Pryde et al., 2002; Wachtershauser and Stein, 2000).

Curcumin

Curcumin

Polyphenols are micronutrients that are secondary metabolites of plants which have a structure of hydroxyl groups on aromatic rings; polyphenols are theorized to have beneficial effects on cancer and cardiovascular disease risk (Manach et al., 2004). The most commonly found polyphenols in the human diet are not active in the body due to low intrinsic activity or poor absorption (Manach et al., 2004). Curcumin is the main polyphenolic component of turmeric, a curry spice commonly used as a food additive, preservative, and colorant. The ground product of dried rhizomes, mainly from *Curcuma longa* L., is known as turmeric. The plant *Curcuma longa* is naturally found in tropical climates such as the Indian subcontinent, with India being the world's primary supplier of turmeric (Ploto, 2003).

Curcumin has gained attention in the last 10-15 years in terms of preclinical and clinical trials due to potential health benefits. There are many studies of potential doses of curcumin in preclinical and clinical studies to determine the toxicity levels of curcumin. The average intake of curcumin for an individual in the Indian population is approximately 100 mg, calculated from an high daily intake of turmeric of about 2,000-2,500 mg (Chainani-Wu, 2003). The FDA has labelled turmeric as Generally Recognized As Safe (GRAS) and the Joint FAO/WHO Expert Committee on Food

Additives has put 0.1-3 g/kg-BW as the acceptable intake level per day (1996). Based on a compilation of multiple studies, levels of 4,000-8,000 mg curcumin for clinical trials are recommended (Basnet and Skalko-Basnet, 2011).

Although there are many health benefits associated with curcumin, it has a low bioavailability after oral administration due to possible rapid metabolism in the intestines. A study reported that approximately 35% of orally administered curcumin was excreted in feces unchanged while the 65% of remaining curcumin was excreted in the form of metabolites of curcumin (Ravindranath and Chandrasekhara, 1981). Therefore, delivery systems are needed for the protection of the phenolic compound.

Clinical trials of anti-oxidant and anti-inflammatory activity of curcumin

Curcumin is an effective antioxidant and anti-inflammatory agent (Anand et al., 2007). Recent studies show that inflammation affects tumor progression and cancers arise from inflammation sites. A randomized controlled trial conducted using a supplement of 480 mg of curcumin and 20 mg of quercetin on patients with cadaveric renal transplants undergoing dialysis, showed that the patients given the drugs had significantly improved graft functions compared to the placebo group and that the patients also experienced less tremor and neurotoxicity (Shoskes et al., 2005). Another study that demonstrated the anti-oxidant properties of curcumin was a randomized controlled study with chronic tropical pancreatitis patients who were orally given either 500 mg of curcumin with 5 mg of piperine or placebo to evaluate the levels of malondialdehyde (MDA) and glutathione (GSH) in the serum, indicators of lipid peroxidation and anti-oxidant potential,

respectively. Patients who were treated with curcumin and piperine had significantly decreased levels of MDA, while GSH levels were unaffected (Durgaprasad et al., 2005).

In terms of anti-inflammatory effects of oral curcumin, there are many studies that document beneficial results. In a randomized double-blind study, patients with ulcerative colitis (UC) were either given 1,000 mg of oral curcumin twice a day with other standard treatments of UC or a placebo with only the standard treatments of UC. In the 6-month period, there was a significant reduction rate of relapse for the patients treated with curcumin, at 4.65%, compared to the control, at 20.51% (Hanai et al., 2006). The anti-inflammatory activity of curcumin was consistently observed in multiple studies on postoperative patients, rheumatoid arthritis patients, chronic anterior uveitis patients, and other inflammatory related conditions (Deodhar et al., 1980; Lal et al., 1999; Satoskar et al., 1986).

More recent studies of the anti-inflammatory effects of curcumin are also prevalent. A study on curcumin-loaded solid lipid nanoparticles intraperitoneally administered (30 mg/kg) on mice showed marked alleviation of the sepsis-induced damage to organs, including kidney, liver, and heart (Wang et al., 2015). In another study, intrathecal curcumin decreased arthritic pain by inhibiting glial activation and the production of inflammatory mediators in the spinal cord of rats (Chen et al., 2015). Curcumin transdermal gel also showed anti-inflammatory effects by down-regulating IFNγ production which inhibits TPA-induced Th1 inflammation in mice (Sun et al., 2015).

Colon site directed delivery

The colon has a large absorption capacity with the primary role of compacting feces by removing water and electrolytes (Watts and Lllum, 1997). The absorption capability is a beneficial aspect to the drugs that specifically target the colon for maximum effects. Also, the colonic media has a neutral pH of approximately 6.4 to 7.0 at different regions, compared to the highly acidic conditions of the stomach (Singh, 2007). This allows the colon to be an ideal target for drug delivery due to minimal acidic and enzymatic degradation. The colon is also an appropriate site of delivery for some drugs and bioactives due to the long transit time (Dumitriu and Chornet, 1998)

An ideal colon specific drug delivery system does not degrade until the drug reaches the colon so absorption in the stomach and small intestine must be avoided for both materials (Akala et al., 2003). Chemotherapeutic drugs that are administered via oral route rather than by injection are the preferred method of delivery to patients with colorectal cancer, due to the improvement of quality of life and cost-effectiveness of treatment since patients can spend less time at the hospital (Sakamoto et al., 2006).

There are five colon-specific drug delivery technologies which include azo-polymer systems, time-dependent systems, pH-dependent systems, pressure dependent systems, and microbial triggered systems (Singh, 2007; Sinha and Kumria, 2003; Vandamme et al., 2002; Watts and Lllum, 1997). In this pectin-curcumin *in-vitro* simulation research, pH-dependent and time-dependent systems were of importance. The pH of the small intestine and the large intestine do not considerably differ so there might be difficulty determining which site the drug is actually being released, if pH is the main factor of the system (Wong et al., 2011). Time-dependent systems takes the gastro-

intestinal transit time into consideration to calculate the drug delivery, although a downside of this method is that time can vary greatly among individuals (Gazzaniga et al., 2006). More specifically however, there are two common approaches to delivery of drugs to the colon. These include a system of releasing preloaded drugs according to a calculated time it takes to reach the colon and a system that releases the drugs due to the response to the environmental conditions, such as pH and enzymes (Kost and Langer, 2001; Liu et al., 2003).

Quantification of Curcumin

There are different methods of quantifying curcumin. The most common method in practice is using HPLC, but some others include UV/Visible spectrophotometry, fluorescence spectroscopy, and FTIR. Table 1.1 shows a list of examples from recent literature of different methods to determine the amount of curcumin.

Obesity

Obesity

Another area of interest in relation with curcumin and pectin is the possible role in regulating obesity. Obesity is a major health issue of concern for billions of individuals worldwide. Accumulation of excess body fat has adverse health effects, specifically in relation to type 2 diabetes mellitus, coronary heart disease, certain cancers, sleep apnea, and osteoarthritis (Kopelman, 2000). Many other factors affect an individual's susceptibility to obesity, such as genetics and environmental factors, but the disproportion of energy intake and energy expenditure mainly results in obesity

(Lichtman et al., 1992). Obesity is characterized by a state of mild inflammation and an increase of inflammation-related adipokines parallel to the expansion of adipose tissue (Trayhurn, 2005). Despite intensive research, current efforts to prevent or reduce obesity by diet, exercise, education, surgery and drug therapies are failing to provide effective long-term solutions to this epidemic. Obesity is primarily caused by pathological expansion of adipose tissue, yet there are no current therapies for obesity with adipocyte as a target.

The biological functions of adipose tissue can be summarized in three main topics. Adipocytes are primarily involved in lipid metabolism and the storage of triacylglycerol to be used when the body is deficient in energy. Also, adipocytes release fatty acids and glycerol for use by major organs since fatty acids are the secondary source of fuel for organs, with glucose being primary. Lastly, adipocytes play a key role in the synthesis and secretion of peptides which include hormones, cytokines and other important proteins necessary for body function (Trayhurn, 2005).

Adipocyte life cycle

Adipogenesis is defined as a process of differentiating preadipocytes into mature adipocytes which includes lipid synthesis. In this process, adipocytes markedly increase *de novo* synthesis of lipids and acquire sensitivity to insulin in addition to increasing glucose transporters (Gregoire, 2001). Preadipocytes can become mature adipocytes any time in the cell life under the appropriate conditions. *In vitro*, cell morphology changes from elongated to spherical form and gradually fill with lipid droplets during the process

of adipogenesis. This process takes about 8 – 10 days. Mature adipocytes can be induced to either undergo apoptosis or lipolysis (Andersen et al., 2010).

The adipocyte life cycle starts with differentiation of adipocytes from either committed embryonic stem cells or adipose-derived stem cells in adipose tissue or mesenchymal stem cells in bone marrow. Fibroblasts, macrophages, monocytes, preadipocytes, and other cell types comprise an adipose tissue, with mature adipocytes derived from mesenchymal stem cells composing approximately one third of adipose tissue (Geloen et al., 1989). The general trend of the adipocyte life cycle is the alteration of cell shape, growth arrest, clonal expansion, and changes in gene expression. These changes lead to an accumulation of lipids and eventual cell death (Gregoire, 2001).

The first step in the life cycle is a growth phase followed by growth arrest and clonal expansion (Gregoire, 2001). It is at this stage that the preadipocytes are induced to differentiate into mature adipocytes *in vitro*. Differentiation is normally induced by supplementing the cell culture media with dexamethasone, 1-methyl-3-isobutylxanthin (IBMX) and insulin. These agents modulate both the expression of more than 100 adipocyte-specific transcription factors. These rapidly induced proteins promote the differentiation of adipocytes (Sadowski et al., 1992).

Growth arrest is a process where preadipocytes withdraw from the cell cycle before converting to adipose. At least one round of DNA replication is necessary after growth arrest stage and thus require adipogenic signals to continue with differentiation (MacDougald and Lane, 1995). The important players of adipocyte differentiation are peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) (Rosen et al., 1999). C/EBPβ and C/EBPδ drive PPARγ in the early

stages of differentiation that later positively affects the expression of PPARγ and C/EBPα (Rosen et al., 2002) The morphology of cells also changes as differentiation occurs, with cells changing from a fibroblast shape to a spherical shape.

During the final stages of differentiation, there is an increase in levels of enzymes related to triacylglycerol synthesis and degradation resulting from the activation of transcriptional cascade (Gregoire, 2001). In this terminal phase, activity of several enzymes like adipocyte-specific fatty acid binding protein, FAT/CD36, a fatty acid transporter and perilipin, a lipid droplet-associated protein involved in lipid synthesis are upregulated (Gregoire et al., 1998).When the white adipocytes expand and mature into adult fat cells, fat cells increase in size and number, whereas the ability to generate new fat cells is restricted. Contrary to former understanding of the number of adipocytes, new research shows that a process of apoptosis can occur to cause death of a cell (Prins and O'Rahilly, 1997). Apoptosis is programmed cell death and inducing apoptosis to decrease the number of mature adipocytes has been used as an approach to decrease adipose tissue mass (Kim et al., 2006). Adipocytes cleared through apoptosis trigger no inflammatory response making this approach a clean process. Lipolysis on the other hand is defined as a process of mobilization of lipid stored in the adipocytes into extracellular environment. Lipolytic agents were also investigated for anti-obesity effects (Park et al., 2007).

Phytochemicals and obesity

Obesity and related disorders have been treated with herbal medicine for centuries in Eastern medicine, but in the last 10 years there has been a surge of studies in the scientific literature on the research between obesity and herbal compounds. All over the

world, customers are spending astonishing amount of money on over-the-counter weight controlling products that have not been fully tested *in vitro* or in clinical trials to show a clear beneficial effect. Organic chemists use natural products as important sources of drug synthesis ideas and there has been a history of successful identification of natural products' abilities for the biochemical actions in the treatment of many diseases (Beghyn et al., 2008; Calixto et al., 2004; Cowan, 1999).

Table 1.1. Research papers on different methodology to quantify curcumin.

HPLC

Title	Author, Year, Journal, Citation	Encapsulating method	Curcumin detection	Detector & Column	EE%	Notes
A biodegradable hydrogel system containing curcumin encapsulated in micelles for cutaneous wound healing	Gong, ChangYang 2013 Biomaterials (Gong et al., 2013)		HPLC	Waters 2996 detector C18 (4.6 × 150 mm–5 μm, Sunfire Analysis column)	98.40 ± 0 .81%	Method was used to extract curcuminoids from turmeric powder Best extracting solvent was dichloromethane
Separation and identification of curcuminoids in turmeric powder by HPLC using phenyl column	Ali, Imran 2014 Royal Society of Chemistry (Ali et al., 2014a)		HPLC	Absorbanc e detector (Sapphire 600 UV-vis.) Sunniest PhE (phenyl ethyl) RP column (250 x 4.6 mm, 5.0 μm)		Separated within 10.5 min using acetonitrile–methanol–water (40 : 20 : 40, v/v) as the mobile phase with 1.0 mL min flow rate and 360 nm detection
The formulation and delivery of	Wang, Ping	curcumin and SLN-	HPLC at 328 nm	C18 column		Retention time was 5.3 min

curcumin with solid lipid nanoparticles for the treatment of on non-small cell lung cancer both in vitro and in vivo	2013 Materials Science and Engineering (Wang et al., 2013)	curcumin were prepared in PBS (with help of ethanol)		(25 cm × 4. 6 mm, 5 μm)		Mobile phase consisted of a gradient between buffer A (acetonitrile) and buffer B (5% acetonitrile, 1% TFA)
Curcumin-loaded hydrogel na noparticles: application in anti-malarial therapy and toxicological evaluation	Dandekar, Prajakta P. 2010 Journal of Pharmaceutical Sciences (Dandekar et al., 2010)	Solvent emulsion-evaporation technique HPMC + curcumin (dissolved in methanol & dicholoromethane) and PVP & Pluronic F68 (dissolved in water) were emulsified and organic phase was evaporated to form nanoparticles	HPLC		72.34 ± 0.34%	Nanoparticles were freeze-dried= 0°C for 5 h → 10°C for 2.5 h and 15*C for 2 h 25°C for 2.5 h → final samples stored at 4°C

Hydrogel based oil encapsulation for controlled release of curcumin by using a ternary system of chitosan, kappa-carrageenan, and carboxymethylcellulose sodium salt	Nakagawa, K. 2013 LWT - Food Science and Technology (Nakagawa et al., 2013)	Curcumin in triolein o/w emulsions	HPLC at 254 nm	UV-Spectrometer	89–99%	
SPE-HPLC Quantification of Curcumin in Different Cultivars and Organs of Ginger, Zingiber officinale Roscoe	Wu, Bing 2008 Natural Product Research & Development (Wu et al., 2008)		HPLC	Solid Phase Extraction (SPE) column for purification of curcumin RP column (UV abs. at 420 nm) for quantitative analysis		Results: ginger is not a good source of curcumin compared to *Curcuma* species

UV/Vis Spectrophotometry

Title	Author, Year, Journal, Citation	Encapsulating method	Curcumin detection	Detector & Column	EE%	Notes
Quantitative Estimation of Curcumin in Ayurvedic Proprietary Medicine By U.V. Spectrophotometry	Patil Snehal, J. 2012 Journal of Pharmacy Research (Patil Snehal et al., 2012)		UV/Vis spectrophotometer (quartz cell) at 263 nm			Standard stock solution: 100 µg/mL of curcumin but it had to be diluted to 4-36 µg/mL to get a calibration curve with low enough absorbances
Mucoadhesive microparticulate drug delivery system of curcumin against Helicobacter pylori infection: Design, development and optimization	Ali, M. S. 2014 Journal of Advanced Pharmaceutical Technology & Research (Ali et al., 2014b)		UV/Vis spectrophotometer at 424 nm		28-63%	Obeyed Beer Lambert's law in 2-20 µg/mL
Spectrometric study on the binding of	Zhou, Haibo		UV spectroph			Had 25°C water circulating through cuvette holder

curcumin with AOT: Effect of micelle-to-vesicle transition	2014 Food Chemistry (Zhou et al., 2014)		otometer at 425 nm			
Physico-chemical state influences in vitro release profile of curcumin from pectin beads	Nguyen, An Thi-Binh 2014 Colloids and Surfaces B: Biointerfaces (Nguyen et al., 2014)		UV at 429 nm		72.4-78.3%	Low methoxyl pectin with different surfactants Pectin hydrogels
Development and Characterization of Curcumin Loaded Silver Nanoparticle Hydrogels for Antibacterial and Drug Delivery Application	Ravindra, S. 2012 Journal of Inorganic and Organometallic Polymers and Materials (Ravindra et al., 2012)	50 mg of hydrogels in 20 ml of curcumin solution (5 mg/20 ml, 4:6 acetone:distilled water)	UV–Vis Spectrophotometer at 491.2 nm FTIR used for characterization of hydrogel		31-63%	

Other

Title	Author, Year, Journal, Citation	Encapsulating method	Curcumin detection	Detector & Column	EE%	Notes
Utilization of solid lipid nanoparticles for enhanced delivery of curcumin in cocultures of HT29-MTX and Caco-2 cells	Guri, Anilda 2013 The Royal Society of Chemistry (Guri et al., 2013)	emulsions	Fluorescence spectrometer Excitation and emission wavelengths of 430 and 549 nm			Fluorescence coefficient of curcumin was ~$2x10^{10}$ $M^{-1}cm^{-1}$ Unentrapped curcumin was separated from the SLN dispersions using ultracentrifugation
Encapsulation of curcumin in self-assembling peptide hydrogels as injectable drug delivery vehicles	Altunbas, Aysegul 2011 Biomaterials (Altunbas et al., 2011)	2% curcumin in DMSO solutions DMSO solution + cell culture mix= added to peptide solution to yield hydrogels	Fluorescence spectrophotometry Emission spectra of 450 to 700 nm excitation wavelengt			

			h of 420 nm			
Curcumin-containing chitosan nanoparticles as a potential mucoadhesive delivery system to the colon	Chuah, Lay Hong 2011 Pharmaceutical Development and Technology (Chuah et al., 2013)		FTIR spectrometer Data were acquired between 2000 cm^{-1} and 600 cm^{-1}			Potassium bromide (KBr) disks containing the material of interest were prepared at a weight ratio of 98:2 of KBr

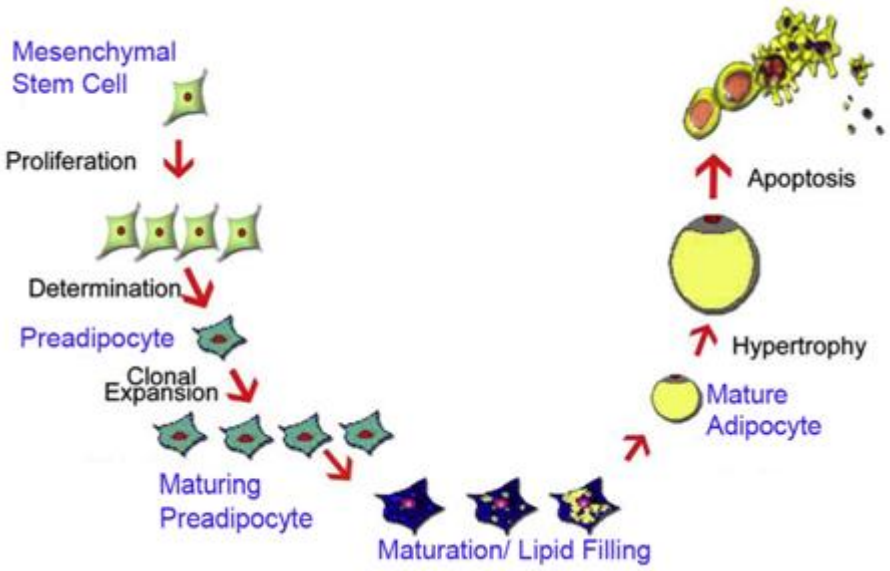


Fig. 1.1. Diagram of an adipocyte life cycle. (Rayalam et al., 2008)

References

1996. Clinical development plan: curcumin. J Cell Biochem Suppl 26, 72-85.

Akala, E.O., Elekwachi, O., Chase, V., Johnson, H., Lazarre, M., Scott, K., 2003. Organic redox-initiated polymerization process for the fabrication of hydrogels for colon-specific drug delivery. Drug Dev Ind Pharm 29, 375-386.

Ali, I., Haque, A., Saleem, K., 2014a. Separation and identification of curcuminoids in turmeric powder by HPLC using phenyl column. Analytical Methods 6, 2526-2536.

Ali, M.S., Pandit, V., Jain, M., Dhar, K.L., 2014b. Mucoadhesive microparticulate drug delivery system of curcumin against Helicobacter pylori infection: Design, development and optimization. J Adv Pharm Technol Res 5, 48-56.

Altunbas, A., Lee, S.J., Rajasekaran, S.A., Schneider, J.P., Pochan, D.J., 2011. Encapsulation of curcumin in self-assembling peptide hydrogels as injectable drug delivery vehicles. Biomaterials 32, 5906-5914.

Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B.B., 2007. Bioavailability of Curcumin: Problems and Promises. Molecular Pharmaceutics 4, 807-818.

Andersen, C., Rayalam, S., Della-Fera, M.A., Baile, C.A., 2010. Phytochemicals and adipogenesis. BioFactors (Oxford, England) 36, 415-422.

Axelos, M., Thibault, J., 1991. The chemistry of low-methoxyl pectin gelation. Academic Press: New York.

Basnet, P., Skalko-Basnet, N., 2011. Curcumin: An Anti-Inflammatory Molecule from a Curry Spice on the Path to Cancer Treatment. Molecules 16, 4567-4598.

Basson, M.D., Liu, Y.W., Hanly, A.M., Emenaker, N.J., Shenoy, S.G., Gould Rothberg, B.E., 2000. Identification and comparative analysis of human colonocyte short-chain fatty acid response genes. J Gastrointest Surg 4, 501-512.

Beghyn, T., Deprez-Poulain, R., Willand, N., Folleas, B., Deprez, B., 2008. Natural compounds: leads or ideas? Bioinspired molecules for drug discovery. Chem Biol Drug Des 72, 3-15.

Boyle, P., Leon, M.E., 2002. Epidemiology of colorectal cancer. Br Med Bull 64, 1-25.

Calixto, J.B., Campos, M.M., Otuki, M.F., Santos, A.R., 2004. Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med 70, 93-103.

Cameron, R.G., Grohmann, K., 1996. Purification and Characterization of a Thermally Tolerant Pectin Methylesterase from a Commercial Valencia Fresh Frozen Orange Juice. Journal of Agricultural and Food Chemistry 44, 458-462.

Chainani-Wu, N., 2003. Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). J Altern Complement Med 9, 161-168.

Chen, J.-J., Dai, L., Zhao, L.-X., Zhu, X., Cao, S., Gao, Y.-J., 2015. Intrathecal curcumin attenuates pain hypersensitivity and decreases spinal neuroinflammation in rat model of monoarthritis. Sci. Rep. 5.

Hinnebusch, B.F., Meng, S., Wu, J.T., Archer, S.Y., Hodin, R.A., 2002. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. The Journal of Nutrition 132, 1012-1017.

Chuah, L.H., Billa, N., Roberts, C.J., Burley, J.C., Manickam, S., 2013. Curcumin-containing chitosan nanoparticles as a potential mucoadhesive delivery system to the colon. Pharm Dev Technol 18, 591-599.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12, 564-582.

Daas, P.J.H., Voragen, A.G.J., Schols, H.A., 2000. Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. Carbohydrate research 326, 120-129.

Dandekar, P.P., Jain, R., Patil, S., Dhumal, R., Tiwari, D., Sharma, S., Vanage, G., Patravale, V., 2010. Curcumin-loaded hydrogel nanoparticles: application in anti-malarial therapy and toxicological evaluation. Journal Of Pharmaceutical Sciences 99, 4992-5010.

Davis, C.D., Milner, J.A., 2009. Gastrointestinal microflora, food components and colon cancer prevention. J Nutr Biochem 20, 743-752.

de Vrese, M., Schrezenmeir, J., 2008. Probiotics, Prebiotics, and Synbiotics, in: U. Stahl, U.B. Donalies, E. Nevoigt (Eds.), Food Biotechnology. Springer Berlin Heidelberg, 1-66.

Denes, J.M., Baron, A., Renard, C.M., Pean, C., Drilleau, J.F., 2000. Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. Carbohydrate research 327, 385-393.

Deodhar, S.D., Sethi, R., Srimal, R.C., 1980. Preliminary study on antirheumatic activity of curcumin (diferuloyl methane). Indian J Med Res 71, 632-634.

Dumitriu, S., Chornet, E., 1998. Inclusion and release of proteins from polysaccharide-based polyion complexes. Advanced drug delivery reviews 31, 223-246.

Durgaprasad, S., Pai, C.G., Vasanthkumar, Alvres, J.F., Namitha, S., 2005. A pilot study of the antioxidant effect of curcumin in tropical pancreatitis. Indian J Med Res 122, 315-318.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., Relman, D.A., 2005. Diversity of the human intestinal microbial flora. Science (New York, N.Y.) 308, 1635-1638.

Gazzaniga, A., Maroni, A., Sangalli, M.E., Zema, L., 2006. Time-controlled oral delivery systems for colon targeting. Expert Opin Drug Deliv 3, 583-597.

Gee, M., Reeve, R.M., McCready, R.M., 1959. Measurement of Plant Pectic Substances, Reaction of Hydroxylamine with Pectinic Acids. Chemical Studies and Histochemical Estimation of the Degree of Esterification of Pectic Substances in Fruit. Journal of agricultural and food chemistry 7, 34-38.

Geloen, A., Roy, P.E., Bukowiecki, L.J., 1989. Regression of white adipose tissue in diabetic rats. Am J Physiol 257, E547-553.

Gong, C., Wu, Q., Wang, Y., Zhang, D., Luo, F., Zhao, X., Wei, Y., Qian, Z., 2013. A biodegradable hydrogel system containing curcumin encapsulated in micelles for cutaneous wound healing. Biomaterials 34, 6377-6387.

Grant, G.T., Morris, E.R., Rees, D.A., Smith, P.J.C., Thom, D., 1973. Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Letters 32, 195-198.

Gregoire, F.M., 2001. Adipocyte differentiation: from fibroblast to endocrine cell. Exp Biol Med (Maywood) 226, 997-1002.

Gregoire, F.M., Smas, C.M., Sul, H.S., 1998. Understanding adipocyte differentiation. Physiol Rev 78, 783-809.

Guri, A., Gulseren, I., Corredig, M., 2013. Utilization of solid lipid nanoparticles for enhanced delivery of curcumin in cocultures of HT29-MTX and Caco-2 cells. Food Funct 4, 1410-1419.

Hanai, H., Iida, T., Takeuchi, K., Watanabe, F., Maruyama, Y., Andoh, A., Tsujikawa, T., Fujiyama, Y., Mitsuyama, K., Sata, M., Yamada, M., Iwaoka, Y., Kanke, K., Hiraishi, H., Hirayama, K., Arai, H., Yoshii, S., Uchijima, M., Nagata, T., Koide, Y., 2006. Curcumin

maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial. Clin Gastroenterol Hepatol 4, 1502-1506.

Hinnebusch, B.F., Meng, S., Wu, J.T., Archer, S.Y., Hodin, R.A., 2002. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. The Journal of Nutrition 132, 1012-1017.

Jung, J., Arnold, R.D., Wicker, L., 2013. Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. Colloids Surf B Biointerfaces 104, 116-121.

Kim, H.-K., Nelson-Dooley, C., Della-Fera, M.A., Yang, J.-Y., Zhang, W., Hartzell, D.L., Hamrick, M.W., Baile, C.A., 2006. Genistein Decreases Food Intake, Body Weight and Fat Pad Weight and Causes Adipose Tissue Apoptosis in Ovariectomized Female Mice. J Nutr 136, 409-414.

Kim, Y., Teng, Q., Wicker, L., 2005. Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. Carbohydrate research 340, 2620-2629.

Kim, Y., Wicker, L., 2009. Valencia PME isozymes create charge modified pectins with distinct calcium sensitivity and rheological properties. Food Hydrocolloids 23, 957-963.

Kopelman, P.G., 2000. Obesity as a medical problem. Nature 404, 635-643.

Kost, J., Langer, R., 2001. Responsive polymeric delivery systems. Advanced Drug Delivery Reviews 46, 125-148.

Lal, B., Kapoor, A.K., Asthana, O.P., Agrawal, P.K., Prasad, R., Kumar, P., Srimal, R.C., 1999. Efficacy of curcumin in the management of chronic anterior uveitis. Phytother Res 13, 318-322.

Lee, H., Rivner, J., Urbauer, J.L., Garti, N., Wicker, L., 2008. De-esterification pattern of Valencia orange pectinmethylesterases and characterization of modified pectins. Journal of the Science of Food and Agriculture 88, 2102-2110.

Lichtman, S.W., Pisarska, K., Berman, E.R., Pestone, M., Dowling, H., Offenbacher, E., Weisel, H., Heshka, S., Matthews, D.E., Heymsfield, S.B., 1992. Discrepancy between self-reported and actual caloric intake and exercise in obese subjects. N Engl J Med 327, 1893-1898.

Lim, C.C., Ferguson, L.R., Tannock, G.W., 2005. Dietary fibres as "prebiotics": implications for colorectal cancer. Molecular nutrition & food research 49, 609-619.

Limberg, G., Korner, R., Buchholt, H.C., Christensen, T.M., Roepstorff, P., Mikkelsen, J.D., 2000. Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from A. niger. Carbohydrate research 327, 293-307.

Liu, L., Fishman, M.L., Kost, J., Hicks, K.B., 2003. Pectin-based systems for colon-specific drug delivery via oral route. Biomaterials 24, 3333-3343.

Lupton, J.R., 2000. Is fiber protective against colon cancer? Where the research is leading us. Nutrition (Burbank, Los Angeles County, Calif.) 16, 558-561.

MacDougald, O.A., Lane, M.D., 1995. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64, 345-373.

Mai, V., 2004. Dietary modification of the intestinal microbiota. Nutr Rev 62, 235-242.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. The American journal of clinical nutrition 79, 727-747.

Manning, T.S., Gibson, G.R., 2004. Microbial-gut interactions in health and disease. Prebiotics. Best practice & research. Clinical gastroenterology 18, 287-298.

Moore, M.A., Park, C.B., Tsuda, H., 1998. Soluble and insoluble fiber influences on cancer development. Crit Rev Oncol Hematol 27, 229-242.

Nakagawa, K., Sowasod, N., Tanthapanichakoon, W., Charinpanitkul, T., 2013. Hydrogel based oil encapsulation for controlled release of curcumin by using a ternary system of chitosan, kappa-carrageenan, and carboxymethylcellulose sodium salt. LWT - Food Science and Technology 54, 600-605.

NCI, D., 1996. Clinical development plan: Curcumin. J Cell Biochem S 26, 72-85.

Nguyen, A.T.-B., Winckler, P., Loison, P., Wache, Y., Chambin, O., 2014. Physico-chemical state influences in vitro release profile of curcumin from pectin beads. Colloids And Surfaces. B, Biointerfaces.

O'Keefe, S.J., 2008. Nutrition and colonic health: the critical role of the microbiota. Current opinion in gastroenterology 24, 51-58.

Oakenfull, D., Scott, A., 1984. Hydrophobic Interaction in the Gelation of High Methoxyl Pectins. Journal of Food Science 49, 1093-1098.

Ohkami, H., Tazawa, K., Yamashita, I., Shimizu, T., Murai, K., Kobashi, K., Fujimaki, M., 1995. Effects of apple pectin on fecal bacterial enzymes in azoxymethane-induced rat colon carcinogenesis. Jpn J Cancer Res 86, 523-529.

Park, H.J., Hausman, D., Della-Fera, M.A., Rayalam, S., Yang, J.Y., Ambati, S., Baile, C.A., 2007. Genistein inhibits adipogenesis and increases lipolysis in primary human adipocytes. Faseb Journal 21, A363-A363.

Patil Snehal, J., Salunkhe Vijay, R., Alai Manoj, H., Kadam Trupti, S., Chaturvedi Neha, P., 2012. Quantitative Estimation of Curcumin in Ayurvedic Proprietary Medicine By U.V. Spectrophotometry. Journal of Pharmacy Research 5, 3047.

Ploto, 2003. Turmeric: Post-Production Management for Improved Market Access for Herbs and Spices-Turmeric. Rome: Food and Agriculture Organization of the United Nations (FAO).

Powell, D., Morris, E., Gidley, M., Rees, D., 1982. Conformations and interactions of pectins: II. Influence of residue sequence on chain association in calcium pectate gels. Journal of molecular biology 155, 517-531.

Prins, J.B., O'Rahilly, S., 1997. Regulation of adipose cell number in man. Clin Sci (Lond) 92, 3-11.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 217, 133-139.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S.D., Wang, J., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59-65.

Rajilic-Stojanovic, M., Smidt, H., de Vos, W.M., 2007. Diversity of the human gastrointestinal tract microbiota revisited. Environmental microbiology 9, 2125-2136.

Research, A.I.f.C., 2011. AICR/WCRF Continuous Update Project Report: Colorectal Cancer, 2011.

Ravindra, S., Mulaba-Bafubiandi, A., Rajinikanth, V., Varaprasad, K., Narayana Reddy, N., Mohana Raju, K., 2012. Development and Characterization of Curcumin Loaded Silver

Nanoparticle Hydrogels for Antibacterial and Drug Delivery Applications. J Inorg Organomet Polym 22, 1254-1262.

Ravindranath, V., Chandrasekhara, N., 1981. In vitro studies on the intestinal absorption of curcumin in rats. Toxicology 20, 251-257.

Rayalam, S., Della-Fera, M.A., Baile, C.A., 2008. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 19, 717-726.

Research, A.I.f.C., 2011. AICR/WCRF Continuous Update Project Report: Colorectal Cancer, 2011.

Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., Spiegelman, B.M., 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16, 22-26.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., Mortensen, R.M., 1999. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 4, 611-617.

Sadowski, H.B., Wheeler, T.T., Young, D.A., 1992. Gene expression during 3T3-L1 adipocyte differentiation. Characterization of initial responses to the inducing agents and changes during commitment to differentiation. J Biol Chem 267, 4722-4731.

Sakamoto, J., Oba, K., Matsui, T., Kobayashi, M., 2006. Efficacy of Oral Anticancer Agents for Colorectal Cancer. Dis Colon Rectum 49, S82-S91.

Satoskar, R.R., Shah, S.J., Shenoy, S.G., 1986. Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. Int J Clin Pharmacol Ther Toxicol 24, 651-654.

Savage, D.C., 1977. Microbial ecology of the gastrointestinal tract. Annual review of microbiology 31, 107-133.

Savary, B.J., Hotchkiss, A.T., Cameron, R.G., 2002. Characterization of a Salt-Independent Pectin Methylesterase Purified from Valencia Orange Peel. Journal of Agricultural and Food Chemistry 50, 3553-3558.

Shoskes, D., Lapierre, C., Cruz-Correa, M., Muruve, N., Rosario, R., Fromkin, B., Braun, M., Copley, J., 2005. Beneficial effects of the bioflavonoids curcumin and quercetin on early function in cadaveric renal transplantation: a randomized placebo controlled trial. Transplantation 80, 1556-1559.

Singh, B.N., 2007. Modified-release solid formulations for colonic delivery. Recent Pat Drug Deliv Formul 1, 53-63.

Sinha, V.R., Kumria, R., 2003. Microbially triggered drug delivery to the colon. Eur J Pharm Sci 18, 3-18.

Sun, J., Zhao, Y., Jin, H., Hu, J., 2015. Curcumin relieves TPA-induced Th1 inflammation in K14-VEGF transgenic mice. International Immunopharmacology 25, 235-241.

Tazawa, K., Okami, H., Yamashita, I., Ohnishi, Y., Kobashi, K., Fujimaki, M., 1997. Anticarcinogenic action of apple pectin on fecal enzyme activities and mucosal or portal prostaglandin E2 levels in experimental rat colon carcinogenesis. J Exp Clin Cancer Res 16, 33-38.

Thibault, J.F., Rinaudo, M., 1985. Interactions of mono- and divalent counterions with alkali- and enzyme-deesterified pectins in salt-free solutions. Biopolymers 24, 2131-2143.

Trayhurn, P., 2005. Endocrine and signalling role of adipose tissue: new perspectives on fat. Acta Physiol Scand 184, 285-293.

Vandamme, T.F., Lenourry, A., Charrueau, C., Chaumeil, J.C., 2002. The use of polysaccharides to target drugs to the colon. Carbohydrate Polymers 48, 219-231.

Voragen, A.G., Voragen, F., Schols, H., Visser, R., 2003. Advances in pectin and pectinase research. Springer Science & Business Media.

Voragen, A.J., Coenen, G.-J., Verhoef, R., Schols, H., 2009. Pectin, a versatile polysaccharide present in plant cell walls. Struct Chem 20, 263-275.

Wachtershauser, A., Stein, J., 2000. Rationale for the luminal provision of butyrate in intestinal diseases. European journal of nutrition 39, 164-171.

Wang, J., Wang, H., Zhu, R., Liu, Q., Fei, J., Wang, S., 2015. Anti-inflammatory activity of curcumin-loaded solid lipid nanoparticles in IL-1β transgenic mice subjected to the lipopolysaccharide-induced sepsis. Biomaterials 53, 475-483.

Wang, P., Zhang, L., Peng, H., Li, Y., Xiong, J., Xu, Z., 2013. The formulation and delivery of curcumin with solid lipid nanoparticles for the treatment of on non-small cell lung cancer both in vitro and in vivo. Materials Science and Engineering: C 33, 4802-4808.

Watts, P.J., Lllum, L., 1997. Colonic Drug Delivery. Drug Development and Industrial Pharmacy 23, 893-913.

Willats, W.G., Orfila, C., Limberg, G., Buchholt, H.C., van Alebeek, G.J., Voragen, A.G., Marcus, S.E., Christensen, T.M., Mikkelsen, J.D., Murray, B.S., Knox, J.P., 2001. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem 276, 19404-19413.

Wong, T.W., Colombo, G., Sonvico, F., 2011. Pectin matrix as oral drug delivery vehicle for colon cancer treatment. AAPS PharmSciTech 12, 201-214.

Wu, B., Ni, H., Li, H.-h., Li, L., 2008. SPE-HPLC Quantification of Curcumin in Different Cultivars and Organs of Ginger, Zingiber officinale Roscoe. Natural Product Research & Development 20.

Zhou, H., Yang, Q., Wang, X., 2014. Spectrometric study on the binding of curcumin with AOT: effect of micelle-to-vesicle transition. Food Chem 161, 136-141.

CHAPTER 2

ENCAPSULATION AND *IN VITRO* STABILITY OF CURCUMIN IN PECTIN HYDROGELS

To be submitted to Food & Function.

Abstract

Pectinmethylesterase (PME) was used to blockwise de-esterify high methoxyl citrus pectin (HMP) to degree of esterification (DE) values of 55% (55P) or 35% (35P) while high methoxyl citrus pectin of 72% DE (72P) was a control. Curcumin was encapsulated at 100 mg or 400 mg per 50 mL of 2% pectin dispersion. Hydrogels were formed by dispersing pectin/curcumin mixture into 300 mM $CaCl_2$ solution. *In-vitro* dissolution study was carried out with 100 mg of freeze-dried hydrogels in 900 mL 0.1M HCl, pH 1.2, for 2 h, 37°C, followed by 900 mL 0.2 M phosphate buffer, pH 6.5, for 3 h. Encapsulation efficiency (EE) of the modified pectins, 55P and 35P and the control low methoxyl pectin (LMP) were above 99%. Molecular weight decreased from 208 to 120 kdalton and ζ-potential decreased from -22 to -47 mV ($p<0.05$) with modification. Release rates of curcumin in simulated digestive media were less than 2% in any pectin hydrogel. Commercial LMP lost shape after 2 h in pH 1.2 solution. Commercial LMP and PME charge modified pectin protect curcumin from release under *in-vitro* simulation, especially for higher doses of curcumin. Correlation coefficients of 0.65 or -0.69 were observed between encapsulation efficiency and molecular weight and ζ-potential, respectively; no strong correlation was observed for release rates. Charge modification of pectin with PME or chemical saponification is a viable method to tailor pectins for phytochemical encapsulation. The similarities in EE and release rates between pectins suggests that other methods, such as physical adsorption of curcumin to pectin are involved in encapsulation.

Keywords: Pectin, Curcumin, PME modification, Hydrogel, *In Vitro*, Encapsulation Efficiency, Release Rate

Introduction

Pectin is an anionically charged complex plant polysaccharide found in the middle lamella and cell walls of plants that is mainly extracted from citrus and apple in the food industry and primarily used as a gelling, thickening, and stabilizing agent (Visser, Schols, & Voragen, 2003). The two main structural features of pectin include a linear backbone of α-(1-4)-D-galacturonic acid residues that forms the homogalacturonan region and the branched rhamnogalacturonan region with neutral sugar side chains (McNeil, Darvill, Fry, & Albersheim, 1984; Voragen, Coenen, Verhoef, & Schols, 2009).

Pectins that have the same DE value can have differing total charge, charge density, and charge distribution. There are many different types of PME isozymes that can be isolated from plants, especially citrus, that show different characteristics, such as thermally tolerant and sensitive forms of PME or salt-dependent and independent PMEs (Cameron and Grohmann, 1996; Savary et al., 2002). Different isozymes of citrus PME have different modes of action. Pectins that had the same DE but were treated with two isozymes of PME, created pectins with different physico-chemical properties, attributed to differences in charge distribution (Kim et al., 2005). De-esterification of pectin with partially purified and crude citrus PMEs has a critical limit of de-esterification and increases gel strength in a non-linear relationship (Lee et al., 2008). Block-wise distribution of free carboxyl groups in a pectin structure has a higher affinity of calcium binding than random distributions (Thibault and Rinaudo, 1985). The quantitative measurement of the distribution of the blocks of carboxyl groups is possible by analyzing the methyl-esterified oligomers using a high-performance anion-exchange chromatography, which illuminates the structure-to-function behavior of the action of PME on pectin (Daas et al., 2000).

Pectin, especially charge-modified citrus pectin, forms hydrogels that encapsulate bioactive material, acting as a carrier through the gastro-intestinal tract for site-specific targeted delivery, while itself being non-toxic and biodegradable (Jung, Arnold, & Wicker, 2013). LMP gelation occurs when calcium ions and free carboxyl groups of pectin interact (Oakenfull and Scott, 1984; Powell et al., 1982). Junction zones formed by calcium ions and carboxyl groups in adjacent pectin chains, known as the 'egg box' model, forms strong LMP gels (Axelos and Thibault, 1991; Grant et al., 1973). However, pectins modified using different PMEs showed different gel strengths at similar total charge, (Kim and Wicker, 2009; Lee et al. 2008) suggesting that plant PME charge modified pectins with overall higher gel strength than commercial LMP may be a highly effective delivery vehicle. LMP is a favorable drug delivery vehicle due to crosslinking with calcium ions with polymers (Wong et al., 2011). This leads to the charge neutralization of the drug which has a positive effect on the rate of drug release by limiting the interaction with dissolution media (Wong et al., 2011). LMP and charge modified pectin effectively encapsulates a water insoluble drug, indomethacin, and protects it against *in-vitro* gastrointestinal conditions (Jung et al., 2013).

There is emerging evidence that the composition of intestinal microbiota is influenced by the consumption of probiotics, prebiotics such as oligosaccharides, and polyphenols, which have possible beneficial effects on colon cancer (Davis and Milner, 2009). Bacteria generate short-chain fatty acids and other metabolites by fermentation of prebiotic materials which can act as growth signals and aid in the proliferation of intestinal epithelium (Mai, 2004). The short-chain fatty acids produced as end products of polysaccharide fermentation can lower the pH in the colon, which has beneficial effects to reduce incidence of colon cancer (Sinha & Kumria, 2003). These short-chain fatty acids such as butyrate prevent apoptosis in healthy colonocytes, while

inhibiting cell proliferation and angiogenesis in colon carcinoma cells (Basson, Liu, Hanly, Emenaker, Shenoy, & Gould Rothberg, 2000; Pryde, Duncan, Hold, Stewart, & Flint, 2002; Wachtershauser & Stein, 2000).

The colon is an appropriate site of delivery for some drugs and bioactives since the degradation of the drug/bioactive through the gastro-intestinal tract is decreased due to neutral pH, longer transit time, low proteolytic enzyme activity, and greater responsiveness to drug absorption (Dumitriu & Chornet, 1998). In order to have colon-targeted site-specific drug delivery, the drug must not be released prematurely in the upper gastrointestinal tract (Wong, Colombo, & Sonvico, 2011). The two approaches to delivery of drugs to the colon include a system of releasing preloaded drugs according to a calculated time required to reach the colon and a system that releases the drugs due to the response to the environmental conditions, such as pH and enzymes (Kost & Langer, 2001; Liu, Fishman, Kost, & Hicks, 2003).

Polyphenols are micronutrients that are secondary metabolites of plants which have a structure of hydroxyl groups on aromatic rings and beneficial effects on cancer and cardiovascular diseases (Manach et al., 2004). The most commonly found polyphenols in the human diet are not active in the body due to low intrinsic activity or poor absorption and high metabolism (Manach et al., 2004). Curcumin, a polyphenol in turmeric, a curry spice, has anti-oxidative and anti-inflammatory activities which have potential roles in cancer treatment, but has low solubility and bioavailability, a common issue with polyphenols (Bansal, Vadhanam, & Gupta, 2011; Basnet & Skalko-Basnet, 2011). Since studies using mice have shown approximately 75% of orally-administered curcumin are excreted as feces and very low amounts are detectable in plasma, alternative methods to incorporate curcumin continue to be investigated (Wahlstrom & Blennow, 1978). The average daily intake of curcumin is

approximately 100 mg in the Indian population, where turmeric is consumed frequently (Chainani-Wu, 2003). Based on the potential toxicity of curcumin, clinical trials recommend curcumin concentrations between 4000-8000 mg for maximum effect, but lower concentrations are recommended for non-pharmaceutical uses (Basnet & Skalko-Basnet, 2011).

In previous studies, LMP was an effective encapsulating hydrocolloid for hydrogel based drug delivery. Given that plant PME modified pectins have higher gel strengths than commercial LMPs, it is feasible that charge modified pectins may be more effective to encapsulate non-water soluble phytochemicals like curcumin. The objective of this study was to determine the encapsulation efficiency of curcumin entrapped in charge modified pectin hydrogels and to determine the release rate under simulated gastric *in-vitro* conditions. In this study, commercial citrus pectin was charge modified to different DE values and compared to commercial LM pectin. Encapsulation efficiency and release rate for the different pectins were reported.

Materials and Methods

Materials

Valencia orange pulp was donated by Citrus World (Lake Wales, FL). HMP and LMP was donated by CP Kelco (Copenhagen, Denmark). Curcumin (≥65%), calcium chloride, sulfuric acid, and meta-hydroxydiphenyl were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Sodium tetraborate was purchased from Fisher Scientific (Fair Lawn, NJ). Sulfamic acid was purchased from Acros Organics (Waltham, MA). Sodium phosphate dibasic, sodium phosphate monobasic, and sodium nitrate were purchased from J.T. Baker (Phillipsburg, NJ). Miracloth was purchased from EMD Millipore (Billerica, MA).

PME preparation

The crude extract was prepared as described by Wicker et al. (Wicker, Vassallo, & Echeverria, 1988). Valencia orange pulp was combined with 4 parts (w/v) 0.1 M NaCl, 0.25 M Tris buffer at pH 8 and homogenized (Pro 300A, Proscientific Inc., Monroe, Conn., U.S.A.) for 1 min at 4°C. The pH was readjusted to 8 and centrifuged at 8000g, 4°C, for 20 min (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, Ga., U.S.A.). After filtering through Miracloth, the supernatant was collected and stored at 40°C, and was used as the crude Valencia PME extract. The PME activity of the crude extract was determined with a pH stat titrator (Brinkmann, Westbury, NY) at 30°C. The enzyme was added to 1% HMP with 0.1 M NaCl at pH 7.5. The definition of a unit of PME activity was the microequivalent of ester hydrolyzed/min at 30°C.

Pectin modification

HMP was de-esterified at 30°C using the crude extract of Valencia orange PME using a slightly altered method originally derived from Kim et al. (Kim, Teng, & Wicker, 2005). HMP mixture (1%, w/v) with 0.1 M NaCl was adjusted to pH 7.5 with 0.5 M NaOH. The pH was maintained with 0.5 M NaOH for the duration of the estimated time near 30 min to achieve the target DE values of 55% and 35% after the addition of a calculated activity of crude PME. The amount of crude PME added to achieve a target DE value of 55% and 35% was varied so that the time of de-esterification was close to 30 min. At the end of the reaction time, pH was immediately dropped to 5 with HCL and the dispersions were transferred to boiling 95% ethanol and boiled for 10 min to inactivate PME. The modified pectins were cooled to room temperature and washed with acetone. The precipitate was collected by filtration with MiraCloth, transferred

to glass dish, covered with MiraCloth and dried under the fume hood for 2-3 days. After drying, the modified pectins were finely ground using a coffee grinder (Mr. Coffee, Cleveland, OH). A control pectin at 72% DE was also prepared using the same procedures described above except no enzyme was added, but pH was adjusted and held at pH 7.5 for 30 min.

HPSEC-MALS-RI

High performance size exclusion chromatography (HPSEC) system was used to measure the molecular weight (MW) of pectins using a Dawn Heleos-II multi-angle light scattering (MALS) detector and an Optilab T-rEX differential refractive index (RI) detector (Wyatt Technologies, Santa Barbara, CA). The HPSEC system also included an Infinity 1260 isocratic pump with an inline degasser (Agilent Technologies, Santa Clara, CA), an autosampler (1100 Series G1313A ALS, Agilent/HP, Inc., San Diego, CA), and PL-Aquagel-OH mix column (300 x 7.5 mm) with PL-Aquagel-OH guard column (50 x 7.5 mm) (Agilent Technologies, Santa Clara, CA). The eluent buffer was 100 mM sodium nitrate in 10 mM sodium phosphate buffer, pH 7, with 0.02% sodium azide; the eluent buffer was used to disperse the pectin samples at 2 mg/mL and 50 µL were injected and the flow rate was set at 0.5 mL/min. Prior to analysis, pectin dispersions were filtered through 13 mm 0.45 µm polyethersulfone (PES) filter (Whatman, Maidstone, UK). ASTRA software Version 6.1.1.17 (Wyatt Technology Corporation, Santa Barbara, CA) was used for data analysis.

Uronic Acid Assay

The total pectin from alcohol insoluble solid (AIS) as galacturonic acid was assayed colorimeterically by a method derived from Filisetti-Cozzi et al. as described by Yoo et al.

(Filisetti-Cozzi & Carpita, 1991; Yoo, Fishman, Hotchkiss Jr, & Lee, 2006). Aliquots of 400 μL of pectin (10 μg/mL) were combined with 40 μL of 4 M sulfamic acid/potassium sulfamate solution (pH 1.6) and 2.4 mL of 75 mM sodium tetraborate in concentrated sulfuric acid, while thoroughly mixing by vortex after each addition. The hydrolysate concentration was 2 mg/mL. The samples were heated in a 100°C water bath for 20 min then immediately transferred to an ice bath at 4°C for 10 min. An aliquot of 80 μL of meta-hydroxydiphenyl solution was added to each sample. For a control, 80 μL of 0.5% NaOH (w/v) was added instead of meta-hydroxydiphenyl. The contents were vigorously mixed and the absorbance at 525 nm was read within a 3-5 min time. The uronic acid content of each sample was calculated relative to a D-galacturonic acid standard. Uronic acid assays were done in duplicates.

Zeta (ζ)-potential

A particle size analyzer with a BI-Zeta option (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mV diode laser (90 angles) and a BI-9000AT correlator was used to measure the particle size and ζ-potential of the pectin samples. Pectin samples (0.5 mg/mL) were prepared in 0.01 M sodium phosphate buffer, pH 7 for both the particle size and ζ-potential measurements (Kim and Wicker 2009). Prior to analysis, pectin samples were filtered through a 25 mm, 5 μm polyvinylidene fluoride (PVDF) filter (Whatman, Maidstone, UK). The particle size measurements were taken in duplicates with 5 runs with 2 min between each run. The ζ-potential value was calculated from the effective diameter of the particles in solution by the cumulative fit of the autocorrected intensity obtained by the intensity fluctuation of the scattered light from the 90-Plus particle sizing software (version 3.37, Brookhaven Instruments, Worcestershire, UK). All experiments were carried out at 25°C with the laser beam at 659.0 nm, and 1.330 as the

refractive index. The ζ-potential measurements were taken in duplicates with 5 runs of 2 min each and 5 s between each run.

Curcumin encapsulated pectin hydrogel formation

Curcumin at 0 mg, 100 mg, or 400 mg, was added to 50 ml of 2% (wt%) pectins, 72P, 55P, 35P, or LM pectin. The pectin-curcumin mixtures were stirred for 2 h at room temperature and hydrated overnight at 4°C. The hydrogel formation was carried out at room temperature. The hydrated mixtures were deposited into 200 ml of stirring, 300 mM $CaCl_2$ using a peristaltic pump with 0.40 mm inner diameter tubing (P-1 peristaltic pump, Pharmacia Ltd., Sweden). The beads were stirred for 15 min before still equilibration at room temperature for 4 h. The beads were separated from the $CaCl_2$ solution by MiraCloth and the remaining $CaCl_2$ solution was reserved for further analysis of curcumin released during the equilibration process. The hydrogels were freeze-dried for 24 h at -40°C at <150 millitorr vacuum in a freeze drier (Unitop 600L, VirTis Company, Gardiner, NY).

***In vitro* dissolution study**

The conditions for the *in vitro* gastro-intestinal simulation were adapted from a previous study (Shavi, Nayak, Averineni, Arumugam, Meka, Nayanabhirama, et al., 2009). An aliquot of 100 mg of freeze-dried curcumin-enclosed pectin hydrogel beads was added to 900 ml of 0.1 N HCl, pH 1.2. The acid stage of dissolution was carried out for 2 h at 37°C to simulate the gastric conditions. After the beads were submerged in HCl solution for 2 h, 1 ml of the solution was withdrawn for analysis of the amount of curcumin released from the hydrogels. The supernatant was combined with ethanol to a final concentration of 53% ethanol to maintain solubility of

curcumin while avoiding precipitation of pectin. The mixture was assayed for curcumin at 420-nm by a UV-spectrophotometer (Genesys 20, Thermo Scientific, USA), at 420 nm (Tønnesen et al., 1986). The HCl was decanted and replaced with 900 ml of 0.2 M monobasic and dibasic sodium phosphate, pH 6.5, to the hydrogel beads. The dissolution was continued for 3 h at 37°C to simulate intestinal conditions. After 3 h, 1 ml of the solution was withdrawn for further analysis in the same method as mentioned above. During the *in vitro* dissolution study, samples were continuously agitated at speed setting of 1.5 on an orbital shaker (PR 70, Hoefer Scientific, San Francisco, CA) placed inside an incubator, 37°C (Isotemp, Fischer Scientific, USA). The *in vitro* study was conducted in triplicates.

Encapsulation Efficiency (EE)

The amount of curcumin that was released into the $CaCl_2$ solutions during the hydrogel formation period were measured by UV-spectrophotometer at 420 nm. The encapsulation efficiency was calculated using an equation derived from Teng et al. (Teng, Li, & Wang, 2014).

$$EE = 100 - \frac{amount\ of\ unencapsulated\ curcumin}{total\ amount\ of\ curcumin} \times 100$$

The amount of unencapsulated curcumin is the amount measured in the $CaCl_2$ solutions and the total amount of curcumin is the amount of curcumin added to the pectin dispersion, either 2 mg/mL or 8 mg/mL. A standard curve of the amount of curcumin (μg) plotted against absorbance values at 420 nm was constructed to estimate the curcumin.

Statistical analysis

The results were expressed as the mean and standard deviation. Multivariate correlation analysis and student's *t*-test was used for statistical analysis of the pectin samples and $p<0.05$ was

considered significant. All statistical analyses were performed with JMP Pro software (version 11; SAS Institute, Cary, NC).

Results and Discussion

Characterization of commercial and charge-modified pectins

The particle size, ζ-potential, UA, Mw, polydispersity, and RMS of HMP, LMP, 72P, 55P, and 35P are reported (Table 2.1). Particle size, ζ-potential, GalA, and Mw of all pectins are significantly different ($p<0.05$). The particle size of charge-modified pectins is two to three times larger than the commercial pectins. Among the modified pectins, 35P at 3170 nm are significantly ($p<0.05$) larger than 72P and 55P, at 1886 nm and 1659 nm, respectively.

The ζ-potential is negative for all pectin samples and decreases with the decrease in DE of pectins. The 35P pectin has the lowest ζ-potential at -47 mV while HMP has the highest value, at -22 mV. All PME modified pectins have a lower ζ-potential than the commercial pectins. Negative values of ζ-potential are due to the overall net surface charge of the pectin polymer. The precursor of the modified pectins, HMP, had a ζ-potential of -22 mV while the control pectin, 72P, which did not undergo PME treatment, but underwent the same modification process, was significantly more negative at -26 mV ($p<0.05$). This decrease in ζ-potential most likely reflects some chemical saponification as a result of adjusting and holding pectin pH at 7.5 and 30°C for 30 min. Commercial LMP and 35P, with the same targeted DE, have significantly different ζ-potentials; 35P modified pectin has greater negative surface charge at -47 mV. The ζ-potential measurements provides information on the surface charge of colloids (Dalgleish and Hallett, 1995; Nakamura et al., 2003). The 35P pectin with the longest citrus PME exposure, has

the lowest ζ-potential. The reported ζ-potential of pectins modified by partially purified citrus PME to about 62% DE was -30 to -40 mV depending on type of isozyme (Kim and Wicker, 2009). In this study, with a crude extract of citrus PME and lower targeted DE, the ζ-potential was lower and ranged from -43 to -47 mV. The similar ζ-potential at large DE differences may be related to the critical limit of de-esterification as described by Lee et al. (Lee et al., 2008). Under the conditions of this study, block co-polymers (Daas et al., 2000) was the likely mechanism of de-esterification of 55P and 35P.

The Mw of the commercial HMP and the three modified pectins were larger than LMP (Table 1). A significant ($p<0.05$) decrease in the Mw of the two control pectins was observed. HMP and 72P had Mw of 208 kDa and 173Da, respectively. Also, the Mw of the PME modified pectins was 154 kDa, and 120 kDa for 55P and 35P, respectively. Previous studies from this laboratory showed no significant decrease in Mw with PME modification (Kim et al., 2005; Lee et al., 2008). The difference shown between the Mw of this particular study and previous studies is most likely due to difficulty in controlling hydrolysis during the pH adjustment stage of PME modification, where the pH of the commercial HMP were raised from around 3.5 to 7.5. There is a large potential for localized high pH environments due to poor mixing during PME modification, especially when conducted in large batches. Other laboratories studying Mw of PME modified pectin have reported no change (Hotchkiss et al., 2002) or a decrease (Cameron et al., 2008; Fishman et al., 2000) in Mw. The HMP pectin had an average Mw of 208k Da; the commercial LMP had a lower Mw of 88 kDa (Table 2.1). The decrease in Mw is most likely due to hydrolysis during the chemical saponification process (Voragen et al., 2003). Notably, the 72P pectin had a lower ($p<0.05$) Mw of 173 kDa; the decrease in Mw from 208 kDa was most likely due to chemical hydrolysis. Likewise, among the modified pectins,

55P and 35P had Mw values of 154 and 120 kDa, respectively. The lower Mw is in contrast to previous work which reported no loss in Mw with PME modification (Hunter and Wicker, 2005; Kim and Wicker, 2009; Lee et al., 2008). A slight decrease in Mw with PME modification was reported (Cameron et al., 2008; Fishman et al., 2000). The differences between studies may be due to experimental variation and the difficulty in maintaining pH, mixing and avoiding localized regions of saponification on laboratory scale procedures.

RMS conformation plot slope is a good indicator of a shape of a polymer (Wyatt, 1993). A RMS conformation plot value closer to 0 indicates spherically branched and more compact shapes, values of 0.33 describing a spherical shape, 0.50-0.60 indicates a random coil, and 1.0 indicates a rigid rod (Beri et al., 1993). HMP and LMP have more spherical shapes while the modified 72P, 55P, and 35P have a spherically branched, compact shape. 72P, 55P, and 35P are not significantly different from one another ($p<0.05$). It is notable that HMP and 72P do not have a similar polymeric shape, nor do LMP and 35P. The standard deviation for LMP is very high.

HMP, LMP, and 55P pectins have similar polydispersity indices ranging from 1.7-1.9. The 72P and 35P pectins have polydispersity indices over 2.2 ($p<0.05$). The polydispersity index is calculated as the ratio of the weight average molecular weight (Mw) and the number average molecular weight (Mn). Pectin is a polydisperse molecule and polydispersity values have consistently been greater than 1 in studies from different groups (Fishman et al., 2000; Kim and Wicker, 2009).

The elution profiles using HPSEC-MALS-RI on the commercial and modified pectins are depicted in Fig. 2.2. The signal for light scattering (LS) and refractive index (RI) show peaks elution at 6.5-8.5 mL for all except HMP which continued to elute until 9.5 mL. For all pectins, the higher RI signal relative to the LS signal near 10 mL indicates a larger amount of low

molecular weight polymers and the higher LS signal relative to RI signal of eluant between about 6-8 mL indicates a greater amount of higher molecular weight polymers. Overall, the LS and RI signals do not overlap and the peaks are not symmetrical, consistent with the high polydispersity values.

The GalA content was different ($p<0.05$) for all pectins. The GalA content for HMP, 72P, 55P, and 35P were expected to be similar in value since they all derived from the same pectin source. LMP was sourced from a different pectin lot, and the measured GalA is lower than expected. The GalA content for commercial pectin should be greater than 650 μg/mg (Voragen et al., 2009).

Encapsulation efficiency (EE) and release rates of curcumin

The encapsulation efficiency and release rate data are reported in Table 2.2. The EE of all pectins and the two concentrations of curcumin, 100 mg and 400 mg, showed values greater than 99%. There were no significant differences in EE among the samples ($p>0.05$). Compared to other literature values, this EE is very high. The $CaCl_2$ concentration of 300 mM probably contributed to high encapsulation efficiency as a previous study showing a protective effect with higher concentrations of $CaCl_2$ was shown regardless of pectin types (Jung et al., 2013). A higher Ca^{2+} level might form stronger hydrogels by increasing the number of crosslinks within the matrix of the polymer (Ashford et al., 1994). A similar study by Nguyen et al. using LMP and surfactants to encapsulate curcumin showed EE values ranging from 72.4-78.3%. A higher EE of indomethacin with PME modified pectin than with commercial LMP was observed (Jung et al. 2013). The EE of commercial LMP ranged from 26.5%-54.8% while the EE of modified pectin had a range of 37%-81.1% in that study.

The curcumin release rates were measured at pH 1.2 solution of HCl and at pH 6.5 solution of sodium phosphate (Table 2.2). Gastric emptying time and small intestinal transit time were considered for the 2 h and 3 h time selection for *in vitro* simulation (Krishnaiah et al., 2002). HMP and 72P results are not included in the results due to the inability of high % DE pectins to form a gel under these conditions. The curcumin release rates from all pectin samples were less than 0.3% at pH 1.2. For both pH 1.2 and pH 6.5 stages, pectin hydrogels with 400 mg curcumin had lower release rates than those with 100 mg of curcumin. Overall, the percent release of curcumin from the hydrogels of modified pectins were similar to that observed for commercial LMP, although the average RR at pH 6.5 had high standard deviations. Similar low release rate results at pH 1.2 and 6.5 were observed by Jung et al., (2013) but charge modified pectin showed lower RR than commercial LMP in that study (Jung et al., 2013).

Although quantitatively no differences were seen between the release rates of commercial and modified pectins, qualitative results show that the pectin beads for 35P did not dissolve at pH 1.2 nor pH 6.5, while both 55P and LMP had complete dissolution of hydrogel structure at the end of pH 6.5 incubation. At the end of pH 1.2 phase, LMP beads lost the shape and were fragile, while modified pectins were more rigid in structure.

Multivariate correlation analysis of the different pectin characteristics

Multivariate correlation analysis of the different pectin characteristics against EE and RR of curcumin under *in vitro* conditions are shown in Table 2.3 and 2.4, respectively. A correlation coefficient close to +1 indicates a strong, positive relationship between the two characteristics, while a value close to -1 indicates a strong, negative relationship. Encapsulation efficiency and ζ-potential have a negative correlation at -0.6857, indicating that the encapsulation efficiency of

pectin increases as the ζ-potential decreases. The EE and MW show a positive correlation at +0.6553, indicating that larger polymers are favorable for higher encapsulation. The measured pectin characteristics had only a weak correlation coefficient with RR; the correlation coefficient was highest at -0.2253 for particle size and %RR in HCl. The amount of curcumin added had a negative correlation coefficient of -0.8885 for %RR in phosphate buffer. A lower curcumin release rate at higher curcumin loading was observed in HCl and in phosphate buffer. Since the ζ-potential and EE had a relatively low correlation coefficient of -0.6857, other factorsth than surface charge must contribute to encapsulation of curcumin. Encapsulation by non-specific adsorption to pectin may be a secondary encapsulation mechanism. The ζ-potential and Mw has a correlation of -0.9256. This is consistent with results of this study that show pectins with a lower Mw also have more negative values for ζ-potential. The ζ-potential is also strongly and negatively associated with particle size at -0.8911. Charge modified pectins had an extremely large particle size compared to the commercial pectins and lower ζ-potential, as well.

Conclusions

In this study, a high encapsulation efficiency of curcumin in LMP or PME modified pectin hydrogels was observed. A total release rate of less than 1.5% was observed no matter the concentration of curcumin or type of pectin used, indicating that pectin hydrogels are a highly effective encapsulation technology. All pectins, 55P, 35P, and LMP, had very high EE of >99%, which shows that low DE pectins and charge modification produces a strong hydrogel for drug encapsulation. Charge modified pectin is likely an effective delivery vehicle for encapsulation of hydrophobic phytochemicals or drugs.

Table 2.1. Characterization of Commercial pectins (HMP and LMP) and Charge-modified Pectins (72P, 55P, 35P)[1]

Sample[2]	Particle size (nm)[3]	ζ-potential (mV)[3]	UA (µg/mg)[3]	Mw (kDa)[3]	Mw/Mn[3]	RMS[3]
HMP	799^{a}±11	-22^{a}±4.1	171^{a}±24	208^{a}±8.2	1.9^{a}±0.42	0.30^{a}±0.07
LMP	849^{b}±98	-30^{b}±2.9	481^{b}±15	88^{b}±6.0	1.7^{a}±0.12	0.21^{ab}±0.13
72P	1886^{c}±0.1	-26^{c}±1.2	657^{c}±18	173^{c}±22.1	2.2^{b}±0.1	0.12^{b}±0.01
55P	1659^{d}±0.0	-43^{d}±0.9	566^{d}±8	154^{d}±5.5	1.9^{a}±0.03	0.17^{b}±0.06
35P	3170^{e}±0.1	-47^{e}±1.4	982^{e}±10	120^{e}±16.7	2.3^{b}±0.18	0.12^{b}±0.04

[1]Particle size, ζ-potential, uronic acid (UA), weight average molecular weight (Mw), polydispersity, and conformation plot slope (RMS) values for corresponding

[2] High-methoxyl pectin (HMP), low-methoxyl pectin (LMP), charge modified pectins with % DE of 72%, 55%, and 35% (72P, 55P, 35P)

[3] Means in columns with superscripts with different letters are significantly different ($p<0.05$)

Table 2.2. Encapsulation Efficiency (EE) and Percent Release Rates (RR) in pH 1.2 and 6.5

Pectin samples[1]	**% EE**[3]	**% RR in HCl (pH 1.2)**[2, 3]	**% RR in Na_4PO_3 (pH 6.5)**[2, 3]
55P 100 mg	99.9^{a}	$0.20^{a}\pm0.01$	$1.02^{a}\pm0.64$
55P 400 mg	100^{a}	$0.06^{b}\pm0.00$	$0.19^{c}\pm0.14$
35P 100 mg	99.9^{a}	$0.20^{a}\pm0.01$	$0.89^{ab}\pm0.63$
35P 400 mg	100^{a}	$0.06^{b}\pm0.00$	$0.21^{bc}\pm0.22$
LMP 100 mg	99.7^{a}	$0.28^{c}\pm0.03$	$0.50^{abc}\pm0.18$
LMP 400 mg	99.9^{a}	$0.08^{d}\pm0.01$	$0.21^{bc}\pm0.08$

[1] High-methoxyl pectin (HMP), low-methoxyl pectin (LMP), charge modified pectins with % DE of 72%, 55%, and 35% (72P, 55P, 35P). The different concentrations under pectin samples (100 mg and 400 mg) indicate the amount of curcumin originally added to the pectin dispersions.

[2] The release rate calculations for pH 1.2 and pH 6.5 solutions were determined after incubation of hydrogels for 2 h or 3 h, respectively

[3] Means in columns with superscripts with different letters are significantly different ($p<0.05$)

Table 2.3. Multivariate Correlation Analysis of Encapsulation Efficiency (EE) and Pectin Characteristics[1]

	Curcumin added (mg)	**Encapsulation Efficiency (%)**	**Particle size (nm)**	**Zeta-potential (mV)**	**Molecular weight (kDa)**
Curcumin added (mg)	1.0000				
EE (%)	0.6537	1.0000			
Particle size (nm)		0.6358	1.0000		
Zeta-potential (mV)		-0.6857	-0.8911	1.0000	
Molecular weight (kDa)		0.6553	0.9966	-0.9256	1.0000

[1] The values close to +1 or -1 represent correlation coefficient values that indicate a strong correlation between the two categories.

Table 2.4. Multivariate correlation analysis of Release Rates (RR) and Pectin Characteristics[1]

	Curcumin added (mg)	**RR in pH 1.2 (%)**	**RR in pH 6.5 (%)**	**Particle size (nm)**	**Zeta-potential (mV)**	**Molecular weight (kDa)**
Curcumin added (mg)	1.0000					
RR in pH 1.2 (%)	0.0474	1.0000				
RR in pH 6.5 (%)	-0.8885	-0.1296	1.0000			
Particle size (nm)		-0.2253	0.1979	1.0000		
Zeta-potential (mV)		-0.0780	-0.2874	-0.8911	1.0000	
Molecular weight (kDa)		-0.1736	0.2175	0.9966	-0.9256	1.0000

[1] The values close to +1 or -1 represent correlation coefficient values that indicate a strong correlation between the two categories.

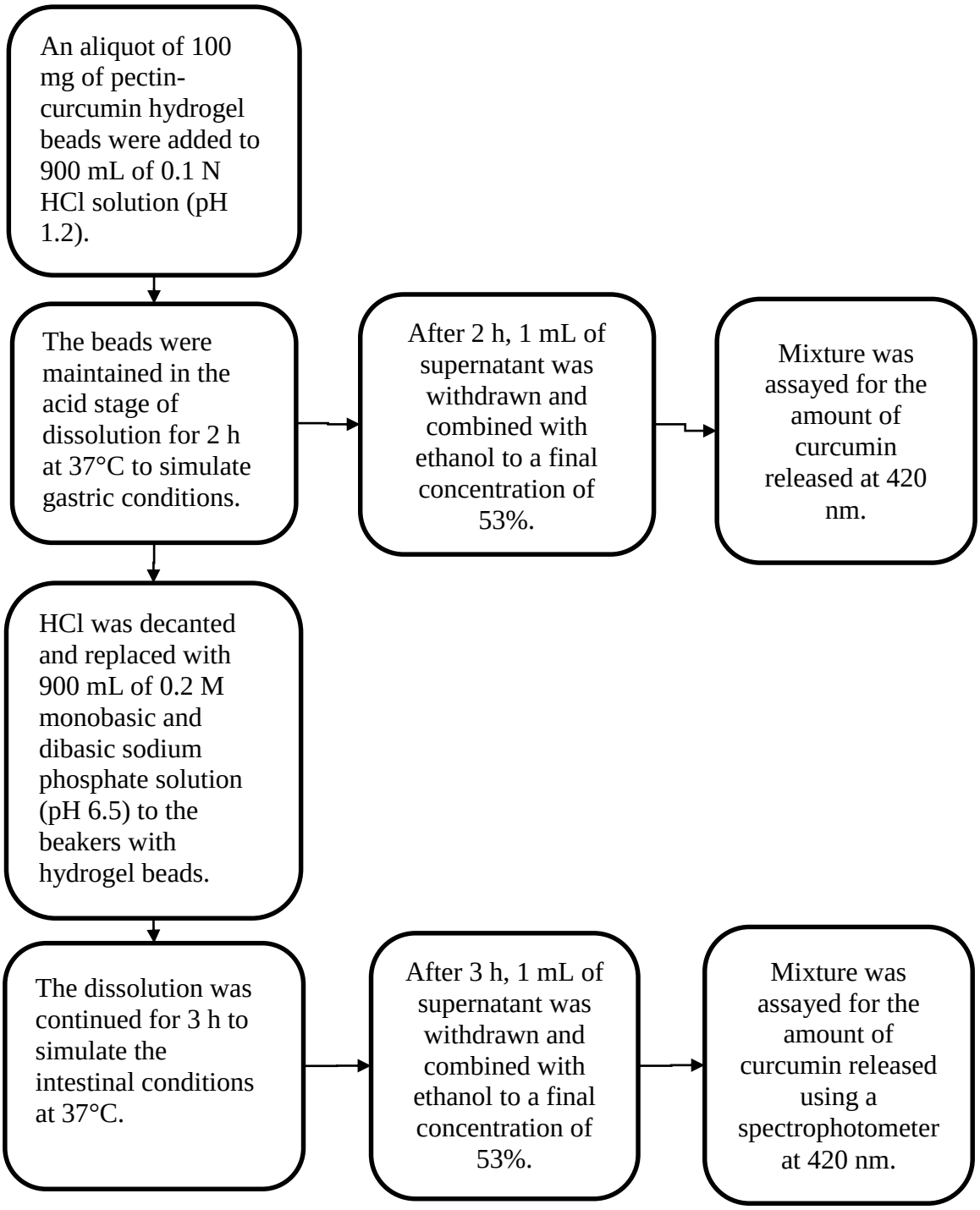
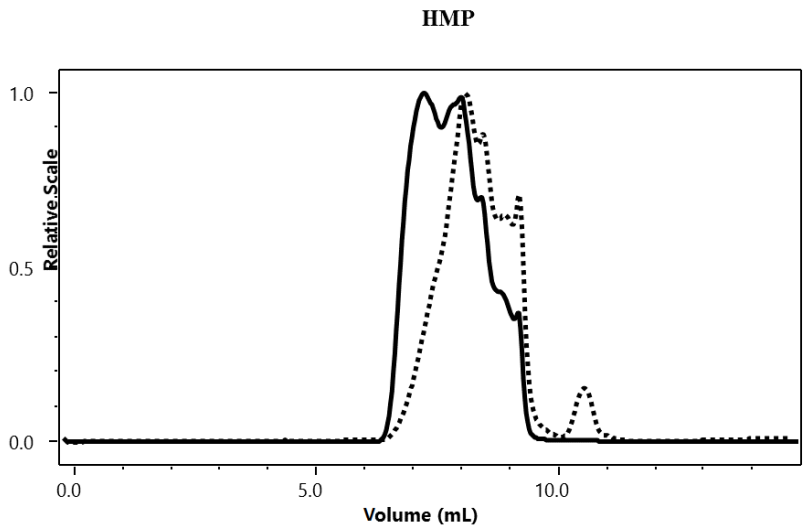


Fig. 2.1. Flow diagram for the steps in *in-vitro* gastro-intestinal simulation.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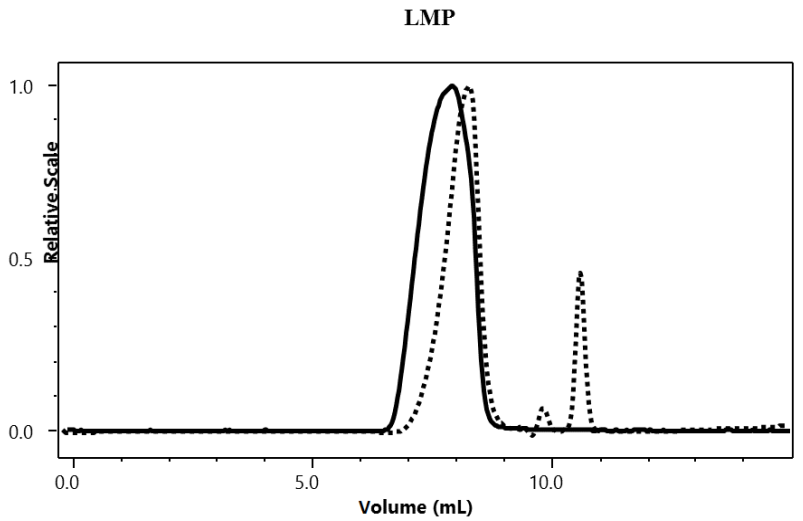
Volume (mL)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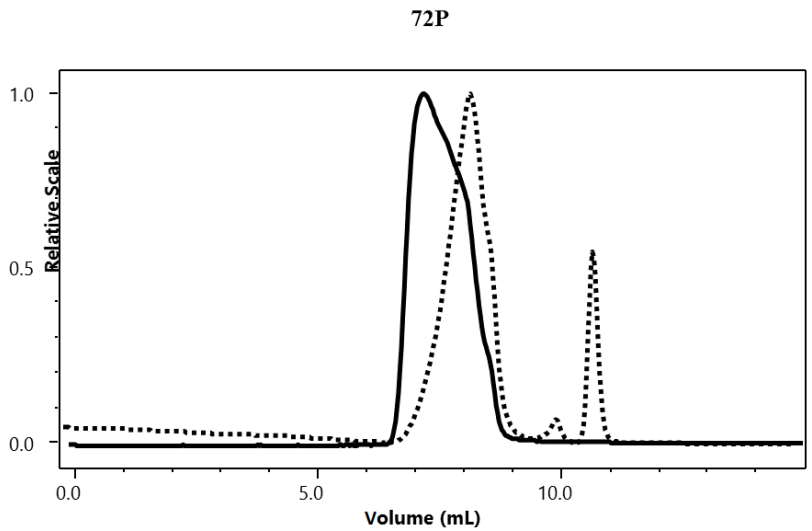
72P
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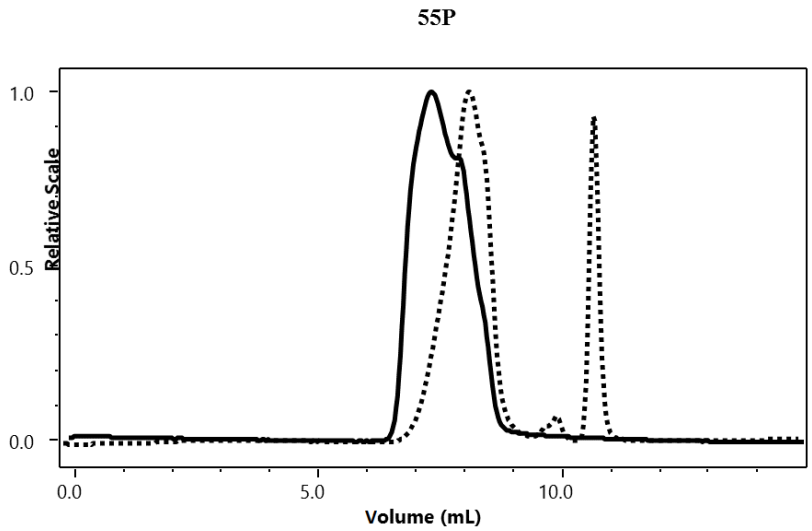
Volume (mL)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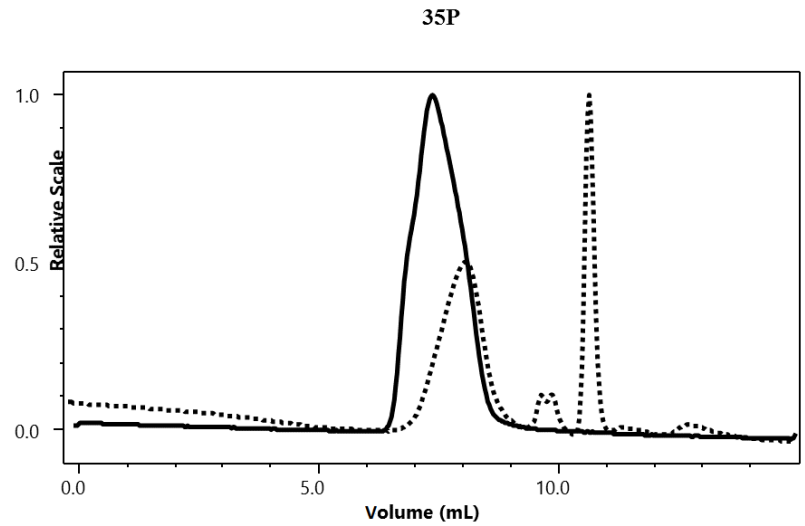


Figure 2.2. Representative elution profiles of HMP, LMP, 72P, 55P, and 35P monitored by multi angle light scattering (MALS) and differential refractive index (RI). Solid line indicates light scattering (LS) signal while the dotted line indicates refractive index (RI) signal.

References

Agilent, 2015. Polymer Molecular Weight Distribution and Definitions of MW Averages, April 30, 2015 ed. Agilent Technologies.

Ashford, M., Fell, J., Attwood, D., Sharma, H., Woodhead, P., 1994. Studies on pectin formulations for colonic drug delivery. Journal of Controlled Release 30, 225-232.

Axelos, M., Thibault, J., 1991. The chemistry of low-methoxyl pectin gelation. Academic Press: New York.

Bansal, S. S., Vadhanam, M. V., & Gupta, R. C. (2011). Development and in vitro-in vivo evaluation of polymeric implants for continuous systemic delivery of curcumin. *Pharm Res, 28*(5), 1121-1130.

Basnet, P., & Skalko-Basnet, N. (2011). Curcumin: An Anti-Inflammatory Molecule from a Curry Spice on the Path to Cancer Treatment. *Molecules, 16*(6), 4567-4598.

Basson, M. D., Liu, Y. W., Hanly, A. M., Emenaker, N. J., Shenoy, S. G., & Gould Rothberg, B. E. (2000). Identification and comparative analysis of human colonocyte short-chain fatty acid response genes. *J Gastrointest Surg, 4*(5), 501-512.

Beri, R.G., Walker, J., Reese, E.T., Rollings, J.E., 1993. Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique. Carbohydrate research 238, 11-26.

Cameron, R.G., Grohmann, K., 1996. Purification and Characterization of a Thermally Tolerant Pectin Methylesterase from a Commercial Valencia Fresh Frozen Orange Juice. Journal of Agricultural and Food Chemistry 44, 458-462.

Cameron, R.G., Luzio, G.A., Goodner, K., Williams, M.A.K., 2008. Demethylation of a model homogalacturonan with a salt-independent pectin methylesterase from citrus: I. Effect of pH on

demethylated block size, block number and enzyme mode of action. Carbohydrate Polymers 71, 287-299.

Chainani-Wu, N. (2003). Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). *J Altern Complement Med, 9*(1), 161-168.

Daas, P.J., Voragen, A.G., Schols, H.A., 2000. Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. Carbohydrate research 326, 120-129.

Dalgleish, D.G., Hallett, F.R., 1995. Dynamic light scattering: applications to food systems. Food Research International 28, 181-193.

Davis, C. D., & Milner, J. A. (2009). Gastrointestinal microflora, food components and colon cancer prevention. *J Nutr Biochem, 20*(10), 743-752.

Dumitriu, S., & Chornet, E. (1998). Inclusion and release of proteins from polysaccharide-based polyion complexes. *Advanced drug delivery reviews, 31*(3), 223-246.

Fishman, M.L., Chau, H.K., Hoagland, P., Ayyad, K., 2000. Characterization of pectin, flash-extracted from orange albedo by microwave heating, under pressure. Carbohydrate research 323, 126-138.

Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Anal Biochem, 197*(1), 157-162.

Grant, G.T., Morris, E.R., Rees, D.A., Smith, P.J.C., Thom, D., 1973. Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Letters 32, 195-198.

Hotchkiss, A.T., Jr., Savary, B.J., Cameron, R.G., Chau, H.K., Brouillette, J., Luzio, G.A., Fishman, M.L., 2002. Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. J Agric Food Chem 50, 2931-2937.

Hunter, J.L., Wicker, L., 2005. De-esterification of pectin by alkali, plant and fungal pectinmethylesterases and effect on molecular weight. Journal of the Science of Food and Agriculture 85, 2243-2248.

Jung, J., Arnold, R.D., Wicker, L., 2013. Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. Colloids Surf B Biointerfaces 104, 116-121.

Kim, Y., Teng, Q., Wicker, L., 2005. Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. Carbohydrate research 340, 2620-2629.

Kim, Y., Wicker, L., 2009. Valencia PME isozymes create charge modified pectins with distinct calcium sensitivity and rheological properties. Food Hydrocolloids 23, 957-963.

Kost, J., & Langer, R. (2001). Responsive polymeric delivery systems. *Adv Drug Deliv Rev, 46*(1-3), 125-148.

Krishnaiah, Y.S., Satyanarayana, V., Dinesh Kumar, B., Karthikeyan, R.S., 2002. In vitro drug release studies on guar gum-based colon targeted oral drug delivery systems of 5-fluorouracil. Eur J Pharm Sci 16, 185-192.

Laurent, M. A., & Boulenguer, P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food Hydrocolloids*, *17*(4), 445-454.

Lee, H., Rivner, J., Urbauer, J.L., Garti, N., Wicker, L., 2008. De-esterification pattern of Valencia orange pectinmethylesterases and characterization of modified pectins. Journal of the Science of Food and Agriculture 88, 2102-2110.

Limberg, G., Körner, P., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., Mikkelsen, J. D., Limberg, G., Körner, P., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000). Analysis of different de-esterification mechanisms for pectin by

enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from A. Niger. *Carbohydr Res, 327,* 293-307.

Liu, L., Fishman, M. L., Kost, J., & Hicks, K. B. (2003). Pectin-based systems for colon-specific drug delivery via oral route. *Biomaterials, 24*(19), 3333-3343.

Mai, V. (2004). Dietary modification of the intestinal microbiota. *Nutr Rev, 62*(6 Pt 1), 235-242.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. The American journal of clinical nutrition 79, 727-747.

McNeil, M., Darvill, A. G., Fry, S. C., & Albersheim, P. (1984). Structure and Function of the Primary Cell Walls of Plants. *Annual Review of Biochemistry, 53*(1), 625-663.

Nakamura, A., Furuta, H., Kato, M., Maeda, H., Nagamatsu, Y., 2003. Effect of soybean soluble polysaccharides on the stability of milk protein under acidic conditions. Food Hydrocolloids 17, 333-343.

Powell, D., Morris, E., Gidley, M., & Rees, D. (1982). Conformations and interactions of pectins: II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of molecular biology, 155*(4), 517-531.

Oakenfull, D., Scott, A., 1984. Hydrophobic Interaction in the Gelation of High Methoxyl Pectins. Journal of Food Science 49, 1093-1098.

Pryde, S. E., Duncan, S. H., Hol, G. L., Stewart, C. S., & Flint, H. J. (2002). The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett, 217*(2), 133-139.

Savary, B.J., Hotchkiss, A.T., Cameron, R.G., 2002. Characterization of a Salt-Independent Pectin Methylesterase Purified from Valencia Orange Peel. Journal of Agricultural and Food Chemistry 50, 3553-3558.

Shavi, G. V., Nayak, U., Averineni, R. K., Arumugam, K., Meka, S. R., Nayanabhirama, U., & Sureshwar, P. (2009). Multiparticulate drug delivery system of aceclofenac: development and in vitro studies. *Drug Dev Ind Pharm, 35*(2), 252-258.

Sinha, V. R., & Kumria, R. (2003). Microbially triggered drug delivery to the colon. *Eur J Pharm Sci, 18*(1), 3-18.

Teng, Z., Li, Y., & Wang, Q. (2014). Insight into curcumin-loaded beta-lactoglobulin nanoparticles: incorporation, particle disintegration, and releasing profiles. *J Agric Food Chem, 62*(35), 8837-8847.

Thibault, J.F., Rinaudo, M., 1985. Interactions of mono- and divalent counterions with alkali- and enzyme-deesterified pectins in salt-free solutions. Biopolymers 24, 2131-2143.

Tønnesen, H., Karlsen, J., van Henegouwen, G., 1986. Studies on curcumin and curcuminoids VIII. Photochemical stability of curcumin. Z Lebensm Unters Forch 183, 116-122.

Visser, R., Schols, H., & Voragen, F. (2003). Advances in pectin and pectinase research / edited by Fon

Voragen, A.G., Voragen, F., Schols, H., Visser, R., 2003. Advances in pectin and pectinase research. Springer Science & Business Media.

Voragen, A.J., Coenen, G.-J., Verhoef, R., Schols, H., 2009. Pectin, a versatile polysaccharide present in plant cell walls. Struct Chem 20, 263-275.

Voragen, Hank Schols, and Richard Visser. In): Dordrecht ; Boston : Kluwer Academic Publishers, c2003.

Wachtershauser, A., & Stein, J. (2000). Rationale for the luminal provision of butyrate in intestinal diseases. *Eur J Nutr, 39*(4), 164-171.

Wahlstrom, B., & Blennow, G. (1978). A study on the fate of curcumin in the rat. *Acta*

Pharmacol Toxicol (Copenh), 43(2), 86-92.

Wicker, L., Vassallo, M. R., & Echeverria, E. J. (1988). Solubilization of Cell Wall Bound, Thermostable Pectinesterase from Valencia Orange. *Journal of Food Science, 53*(4), 1171-1174.

Wong, T. W., Colombo, G., & Sonvico, F. (2011). Pectin matrix as oral drug delivery vehicle for colon cancer treatment. *AAPS PharmSciTech, 12*(1), 201-214.

Wyatt, P.J., 1993. Light scattering and the absolute characterization of macromolecules. Analytica chimica acta 272, 1-40.

Yoo, S.-H., Fishman, M. L., Hotchkiss Jr, A. T., & Lee, H. G. (2006). Viscometric behavior of high-methoxy and low-methoxy pectin solutions. *Food Hydrocolloids, 20*(1), 62-67.

CHAPTER 3

CURCUMIN AND PECTIN EFFECT ON ADIPOGENESIS OF 3T3-L1 ADIPOCYTES

To be submitted to Food & Function.

Abstract

This study was to determine the effects of pectin and curcumin on inhibition of adipogenesis of 3T3-L1 adipocytes. Lipid content, cell viability, and lipolysis were measured after treatment of adipocytes with different types and doses of pectin, as well as the combination with curcumin, a polyphenol with inhibitory effects on adipogenesis. Low methoxyl pectin (LMP), high methoxyl pectin (HMP), charge modified citrus pectins with % degree of esterification (DE) of 35% (35P), and sugar beet pectin (SBP) were used as treatments at concentrations of 10 to 1000 μg/mL. Results show that LMP, SBP, and 35P do not have an effect on adipogenesis and does not have lipolytic activity on mature 3T3-L1 adipocytes. HMP decreased adipogenesis at 100 μg/mL. Pectins do not have a significant influence on cell viability. The combinations of curcumin and pectin do not have an effect on lipid accumulation.

Keywords: Pectin, Curcumin, Adipogenesis, 3T3-L1, Cell Viability, Lipid

Introduction

Currently there are very few FDA approved weight loss drugs in the market in the United States and present therapeutic strategies for obesity are proving to be not very effective. Since obesity is a complex disease, monotherapy could lead to activation of multiple compensatory mechanisms that might overcome the inhibition of one pathway. Thus, a multi-target approach seems a logical and an ideal way to develop strategies for prevention and treatment of complex diseases like obesity.

Obesity is a major issue of concern for billions of individuals worldwide. Obesity adversely affects health, most specifically type 2 diabetes mellitus, coronary heart disease, certain cancers, sleep apnea, and osteoarthritis (Kopelman, 2000). Other factors that affect susceptibility to obesity are genetics and environmental factors, but the disproportion of energy intake and energy expenditure strongly influences obesity (Lichtman et al., 1992). Global projections indicate that by 2030 there will be more than 2.16 billion overweight and 1.12 billion obese individuals (Kelly et al., 2008). Despite intensive research, current efforts to prevent or reduce obesity by diet, exercise, education, surgery and drug therapies are failing to provide effective long-term solutions to this epidemic. Obesity is primarily caused by pathological expansion of adipose tissue, yet there are no viable therapies for obesity with adipocyte as a target.

The adipocyte life cycle starts with differentiation of adipocytes from either committed embryonic stem cells or adipose-derived stem cells in adipose tissue or mesenchymal stem cells in bone marrow. The first step in the life cycle is a growth phase followed by growth arrest and clonal expansion (Gregoire, 2001). It is at this stage that the preadipocytes are induced to differentiate into mature adipocytes *in vitro*. Differentiation, in culture, is normally induced by

supplementing the cell culture media with dexamethasone, 1-methyl-3-isobutylxanthin (IBMX) and insulin. These agents modulate both the expression of more than 100 adipocyte-specific transcription factors. These rapidly induced proteins promote the differentiation of adipocytes (Sadowski et al., 1992). During the terminal phase of differentiation, activity of several enzymes like adipocyte-specific fatty acid binding protein, FAT/CD36, a fatty acid transporter and perilipin, a lipid droplet-associated protein involved in lipid synthesis are upregulated (Gregoire et al., 1998).

Adipogenesis can be defined as a process of differentiating preadipocytes into mature adipocytes which includes lipid synthesis. In this process, adipocytes markedly increase *de novo* synthesis of lipids and acquire sensitivity to insulin in addition to increasing glucose transporters (Gregoire, 2001). Preadipocytes can become mature adipocytes any time in the cell life under the appropriate conditions. *In vitro,* cell morphology changes from elongated to spherical form and gradually fill with lipid droplets during the process of adipogenesis. This process takes about 8 – 10 days. Mature adipocytes can be induced to either undergo apoptosis or lipolysis (Andersen et al., 2010).

Apoptosis is programmed cell death, and inducing apoptosis to decrease the number of mature adipocytes, has been used to decrease adipose tissue mass (Kim et al., 2006). Adipocytes cleared through apoptosis trigger no inflammatory response making this approach a clean process. Lipolysis on the other hand is defined as a process of mobilization of lipid stored in the adipocytes into extracellular environment. Lipolytic agents were also investigated for the anti-obesity effects (Park et al., 2007).

Obesity and related disorders have been treated with herbal medicine for centuries in Eastern medicine, but in the last 10 years there has been a surge of studies in the scientific

literature on the research between obesity and herbal compounds. All over the world, customers are spending astonishing amount of money on over-the-counter weight controlling products that have not been fully tested *in vitro* or in clinical trials to show a clear beneficial effect. Organic chemists use natural products as important sources of drug synthesis ideas and there has been a history of successful identification of natural product ability for the biochemical actions in the treatment of many diseases (Beghyn et al., 2008; Calixto et al., 2004; Cowan, 1999).

Curcumin modulates multiple cellular signaling targets related to proliferation of adipocytes that can have therapeutic effects on various diseases such as diabetes, obesity, and cancer (Aggarwal and Shishodia, 2006). AMP-activated kinase (AMPK) is an emerging factor for potential control of obesity as a sensor of cellular energy status regards to apoptosis in adipocytes (Dagon et al., 2006; Misra, 2008). Peroxisome-proliferator-activated receptor (PPAR)-γ is a key molecule in the insulin resistance and obesity by promoting adipocyte differentiation (Leff et al., 2004). Curcumin activates AMPK by controlling PPAR-γ, which inhibits the differentiation of adipocytes, as well as modulates AMPK/MAP kinases and cyclooxygenase (COX)-2 in cancer cells to regulate apoptosis (Lee et al., 2009). Curcumin interacts with proteins in adipocytes where it down regulates the inflammatory cytokines, resistin and leptin, and upregulates adiponectin, which are associated with obesity (Shehzad et al., 2011). Curcumin also enhances the beneficial antitumor and apoptotic effects of cisplatin, a chemotherapy drug, on cancer cells such as ovarian carcinoma cells (Chan et al., 2003).

Curcumin inhibits Wnt/β-catenin signaling pathway which decreases adipocyte differentiation. Curcumin also inhibits the expression of adipocyte specific transcription factors, C/EBPs, PPARγ, SREBP-1c and fatty acid synthase in adipocytes (Aggarwal, 2010). Curcumin affects fatty acid oxidation and down regulation of PPARγ by activating AMP activated protein

kinase (AMPK), which regulates glucose uptake and beta oxidation of fatty acids. Curcumin also inhibits adipocyte differentiation by inhibiting c-Jun NH2 terminal kinase (JNK) (Aggarwal, 2010).

At ≥5 μM and ≥20 μM, curcumin decreased proliferation of preadipocytes and lipid accumulation in 3T3-L1 cell line, respectively (Ejaz et al., 2009). Curcumin inhibits differentiation of 3T3-L1 preadipocytes dose-dependently (0-30 μM) (Kim et al., 2011). Another study showed the dose-dependent negative response of adipose tissue weight and body weight with curcumin on rats fed dietary supplemented curcumin (Asai and Miyazawa, 2001).

Epidemiologically, there is an inverse relationship with dietary fiber intake and body weight (Davis et al., 2006). A reason for this trend could be due to the ability of dietary fibers to increase satiety. Among dietary fibers, soluble, viscous, and gel-forming fibers are the most efficient in increasing satiety due to delayed mastication, extended stay in stomach digesta, and slowing of nutrient absorption (Kristensen and Jensen, 2011). Although pectin increases satiety in some cases, LM pectins show a consistent induced satiety at all concentrations while HM pectins showed inconsistent effects, possibly due to varying viscosities (Logan et al., 2015). With increased amount of pectin in the diet (0-10% w/w apple pectin), rats showed decrease in food intake, body weight gain and body fat content (Adam et al., 2015).

Elucidating clear information on pectin and obesity is difficult and the effect pf pectin and adipocytes is currently lacking. The effect of pear pomace water extract on 3T3-L1 preadipocytes showed inhibition of adipogenesis and induction of apoptosis (Rhyu et al., 2014). Based on the description of the preparation of the pear extract, it most likely contains polyphenolics, as well as pectin components. There are a few findings on fucoidan, a sulfated polysaccharide derived from brown seaweeds, and effects on decreasing lipid accumulation and down regulation of adipocyte

markers, thus inhibiting adipocyte differentiation (100 and 200 μg/ml) (Kim et al., 2010; Kim et al., 2009). Commercial pectins, charged-modified citrus pectin, and sugar beet pectins have unique structures and might decrease adipogenesis and viability in 3T3-L1 mouse adipocyte cell line. Pectin in combination with curcumin, which has beneficial effects on 3T3-L1 adipocytes, might induce synergistic decrease in adipogenesis and viability in the same cell culture model. The aim of this research was to demonstrate the anti-adipogenic effects of different pectins in 3T3-L1 cell culture model system and to investigate the synergistic effects of pectins with curcumin on decreasing adipogenesis and viability in 3T3-L1 cell culture model.

Materials and Methods

Materials

The pectins used in this study are high-methoxyl pectin (HMP), low-methoxyl pectin (LMP), sugar beet pectin (SBP), and 35P, which are described previously in the thesis (Lee, 2015). Dulbecco's modified Eagle's medium (DMEM), newborn calf serum, fetal bovine serum (FBS), penicillin streptomycin, and dimethyl sulfoxide (DMSO) were purchased from Life Technologies (Grand Island, NY). Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and trypsin-EDTA were purchased from Sigma-Aldrich Inc. (St. Louis, MO). AdipoRed™ Adipogenesis Assay Reagent was purchased from Lonza, Inc. (Basel, Switzerland). CellTiter 96® Aqueous One Solution Cell Proliferation Assay was purchased by Promega (Madison, WI). Lipolysis Assay kit was purchased from Zen-Bio, Inc, (Research Triangle Park, NC).

Cell culture

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and were induced to allow maturation into adipocytes by differentiation. The cells were plated in either 96-well or 12-well plates and cultured in DMEM with 10% bovine calf serum until confluent. Two days after the cells reached confluence (Day 0), the media was removed and differentiation media consisting of DMEM containing 10% fetal bovine serum (FBS), 167 nmol/L insulin, 0.5 μmol/L IBMX, and 1 μmol/L dexamethasone was added. On day 2, the differentiation media was replaced with DMEM and 10% FBS media with 167 nmol/L insulin. Two days later (Day 4), the insulin media was replaced with a 10% FBS/DMEM media and incubated for 4 additional days. By day 8, >90% of cells were mature adipocytes with accumulated fat droplets. All media contained 100 U/ml of penicillin, 100 μg/ml of streptomycin and 1% (v:v) 100 mmol/L pyruvate. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Pectin solutions were made by dissolving pectin in deionized water and hydrated overnight and stored at 4˚C. Curcumin solutions were made by solubilizing curcumin in DMSO and stored at -20˚C.

Cell viability

3T3-L1 pre-adipocytes were seeded (15,000 cells/cm^2) into either 96 or 12 well culture plates, grown to confluence, and differentiated as described above. Mature adipocytes were treated with test compounds for 24 or 48 hours. Immediately following treatment, cell viability was tested using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay by Promega (Madison, WI) according to manufacturer's protocols. Following 1 hour incubation, absorbance

of metabolically active cells was measured at 560/590 nm in a Synergy HT (BioTek Instruments, Inc).

Lipid content determination

Commercially available AdipoRed assay reagent (Lonza, Inc) was used to quantify the lipid content of the confluent cells according to the manufacturer's instructions. Two day post confluent, 3T3-L1 adipocytes were induced to differentiate as described earlier. Test compounds were added to the differentiation media and cells were incubated with test compounds for 8 days. After Day 8, the media was discarded and cells were washed with phosphate-buffered saline (PBS). For a 96-well plate, the wells were then filled with 200 µl of PBS and 5 µl of AdipoRed reagent. After 10 min incubation in the dark at room temperature, the fluorescent signal was measured on a plate reader with excitation at 485 nm and emission at 590 nm. The experiments were performed with at least 6 replicates per treatment and repeated three times.

Lipolysis assay

To determine the extent of lipolysis induced by test compounds, mature adipocytes were treated with test compounds for 6 or 24 h, and free glycerol released was assayed by using Lipolysis Assay kit for 3T3-L1 adipocytes (Zen-Bio, Inc, Research Triangle Park, NC) following manufacturer's instructions. The experiment was repeated two times with at least three replicates.

Statistical analysis

The results were expressed as the mean ± standard error (SE). One way ANOVA was used for statistical analysis of the data and $p<0.05$ was considered significant. All statistical analyses were performed with JMP Pro software (version 11; SAS Institute, Cary, NC).

Results and Discussion

Lipid content determination

The 3T3-L1 preadipocytes were treated with pectin and/or curcumin while undergoing differentiation into mature adipocytes and the amount of lipid accumulated at the end of the cell maturation period was measured and compared to a control with no treatment. The percentage of lipid accumulation in adipocytes after treatment with pectin and curcumin is depicted in Fig. 3.1. For the individual pectins, only cells treated with 100 μg/mL of HMP showed a significant decrease ($p<0.05$) in lipid content at 40% decrease compared to the control. At concentrations of 10 and 100 μg/mL SBP, there was no significant ($p<0.05$) effect on adipocyte differentiation and the percent lipid content. At concentrations of 10, 100, or 500 μg/mL of 35P, there was no significant ($p>0.05$) effect by charge modified pectin. Similarly, at 10 or 100 μg/mL, LMP had no significant effect on lipid content.

The combined effects of curcumin and pectins on adipogenesis were studied (Fig. 3.1). Individually, curcumin at 6 μg/mL or HMP at 100 μg/mL showed a significant ($p<0.05$) decrease in lipid accumulation from the control. However, the combination of curcumin at 6 μg/mL and pectin at 100 μg/mL showed no significant differences from the control. Also, 35P and LMP at

100 μg/mL had no significant effect on lipid content. The combination of curcumin with 35P or LMP with curcumin also had no significant effect on lipid content.

There is no clear dose-dependent effect of various pectins on the lipid content of adipocytes and adipogenesis was decreased only by HMP. HMP may decrease lipid content of adipocytes by interaction with the cell membrane and might adhere to the surface of the cell. Pectins affect cell-cell signaling that affects plant cell wall homeostasis (Wolf et al., 2012). The mechanism of HMP influence on adipogenesis may be analogous to the anti-cancer effect of pectin due to binding to the carbohydrate recognition domain of Galectin 3, an extra- and intra-prometastatic protein (Maxwell et al. 2012). The influence of Gal-3 depends on the type and stage of cancer and ranges from inhibition of cell death, regulation of cell growth, to differentiation and apoptosis. Pectin exerts influence without absorption by cells. The molar mass of pectins in this study range from 88 to 208 kDaltons. Molecules greater than 30 kDa are not well absorbed by the body (Fuchs and Fuchs, 2004). The average large molecular weight of pectin molecules prevent effective absorption by the cells. However, pectin are polydisperse in molecular and there was a fraction of HMP with an Mw average of 17 kDa; 35P also had fractions with Mw values of 13 kDa. Other than LMP, there are smaller molecular weight components pectin fractions samples that might affect adipogenesis by entering the cell membrane. In the study on the effects of pear pomace water extract on 3T3-L1 preadipocytes, pear pomace water extract, which most likely contains pectin components, showed an inhibition of adipogenesis and induction of apoptosis (Rhyu et al., 2014). However, without more information on the nature of the pear extract, the effect cannot be linked directly to pectin.

Additionally, pectin may have decreased the pH of the cell media. The pKa of pectin, specifically galacturonate residues, is approximately 3.4 (Plaschina et al., 1978). HMP, with a

high degree of esterification is less charged and the methoxyl group contributes to hydrophobic interactions, compared to LM or charge modified pectins. Notably, adipogenesis results were inconsistent even with the same pectin samples and doses. Variation in results at same pectins and same doses was observed in use of 96-well plates and 12-well plates, which contain a total volume of 200 μL/well and 1 mL/well, respectively. Different wells gave inconsistent results, possibly due to surface tension and the change in the viscosity of the media associated with pectin.

A visible gel layer was formed on top of the cell surface, especially for pectins at concentrations of 100 μg/mL and higher. Effects of pectin might not have been seen due to the lack of incorporation of pectin into the cell media. Another possible reason for the results shown might be attributed to a gel layer formed by the pectins after treatment to the adipocytes. The gel layer formed on top of the cell surface is most likely due to the viscous nature of pectin. In this study, pectins up to 1% concentration were used as treatment. At this concentration, pectin has nearly Newtonian like flow behavior (n=0.96) and an apparent viscosity concentration coefficient value for an exponential model of 0.0106 Pa s at 40°C (Marcotte et al., 2001). Qualitatively, SBP had less of the gel layer, but there are no significant differences ($p<0.05$) of SBP to the control on lipid content. SBP is unique from citrus pectin and is acetylated, contains ferulic acid, has higher protein, and has emulsifying activity (Levigne et al., 2002). The lack of a significant effect on lipid content by SBP suggests that the gel layer does not influence variability in adipogenesis.

Cell viability

The percent of cells alive after differentiation and treatments, reported as percent viability, are shown in Fig. 3.2. LMP, HMP, and 35P do not show significant difference ($p<0.05$) of live cell count from the control at different treatment doses. At concentrations of 10 and 100 μg/mL SBP, there was no significant ($p<0.05$) effect on cell viability. Curcumin-treated cells had a significant drop in cell viability percent with 25 μg/mL treatment, while 6 μg/mL did not have an effect on the viability ($p<0.05$). Curcumin at a high dose is toxic to cells and thus killed the adipocytes during the process of differentiation.

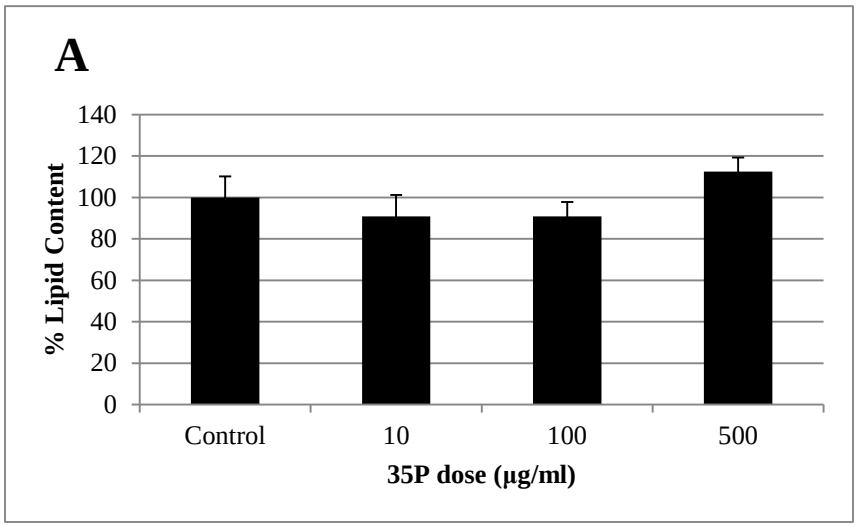
Lipolysis

The lipolysis data for the different pectins are depicted in Fig. 3.3. Lipolysis is the breakdown on triglycerides in adipocytes resulting in a release of glycerol and fatty acids and only occurs in mature adipocytes (Frayn et al., 2003; Hauner et al., 2001). The control was 3T3-L1 adipocytes that were differentiated with no treatment of pectin. Cells were induced to differentiate and then treated with pectin for 6 h and 24 h. The results for isoproterenol show there was about a 12% decrease in the breakdown of triglycerides, compared to the control. This is reasonable because isoproterenol is a positive control for lipolysis. No significant ($p<0.05$) effect lipolysis was observed in cells treated with pectin for 6 h and 24 h, pectins have.

Conclusion

LMP, SBP, and 35P do not have an effect on adipogenesis, while HMP showed a decrease in adipogenesis. Pectin does not have lipolytic effect on mature 3T3-L1 adipocytes.

Curcumin at a high dose of 25 μg/mL kills adipocytes during differentiation, but pectins do not have a significant influence on viability ($p<0.05$). At this point, there is insufficient data to make a strong conclusion about the effects of pectin on adipogenesis. Although a combined effect of curcumin, a phytochemical, and pectin, a dietary fiber, was seen as a possibility, there was only some effect on adipogenesis by HMP. This research was one of the first to study the effects of pectin on adipogenesis; therefore, a continued, in-depth research is necessary to further understand how pectin interacts with 3T3-L1 adipocytes. This approach can result in synergistic anti-obesity effects by not only increasing pectin-induced satiety but also increasing bio-availability of anti-obesity phytochemicals.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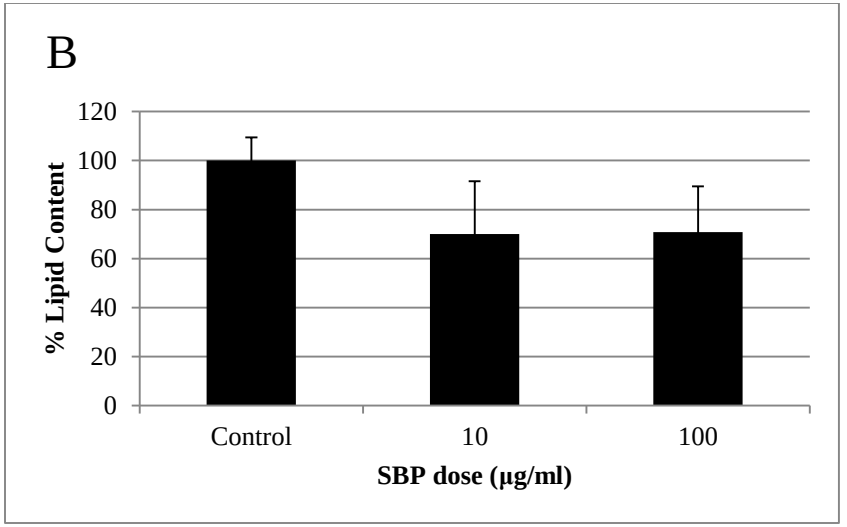
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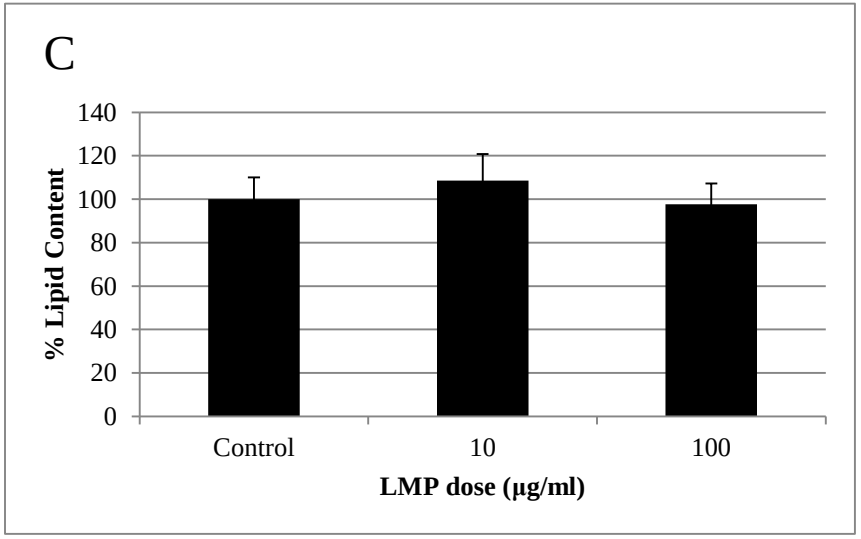
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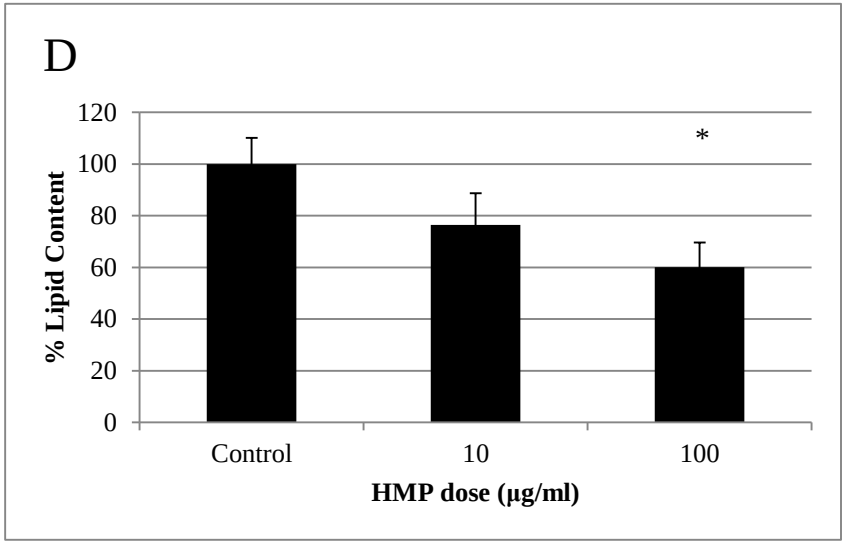
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100
HMP dose (µg/ml)

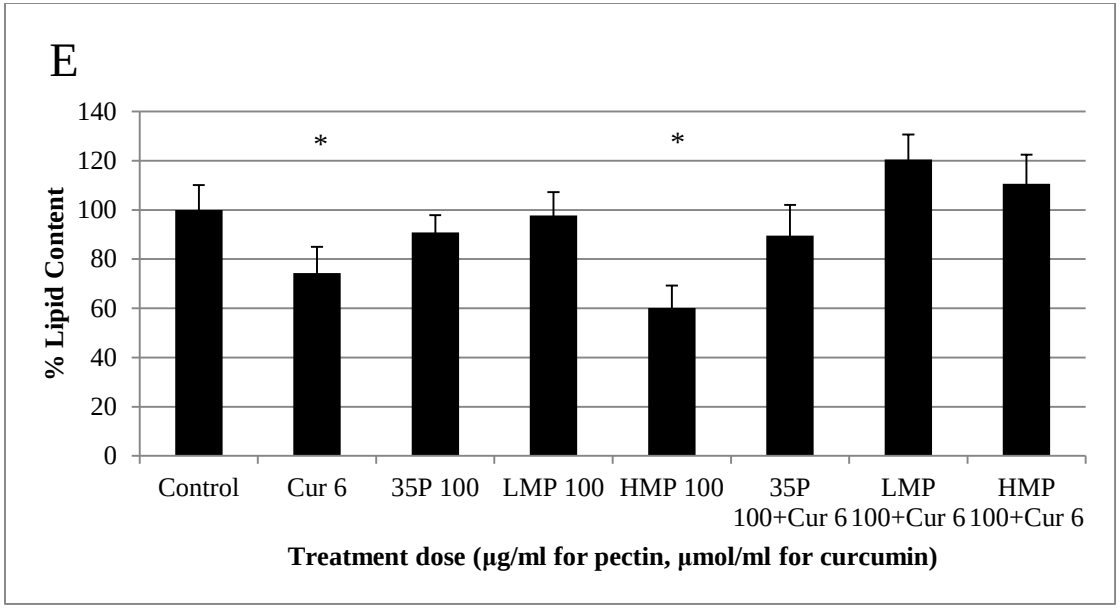
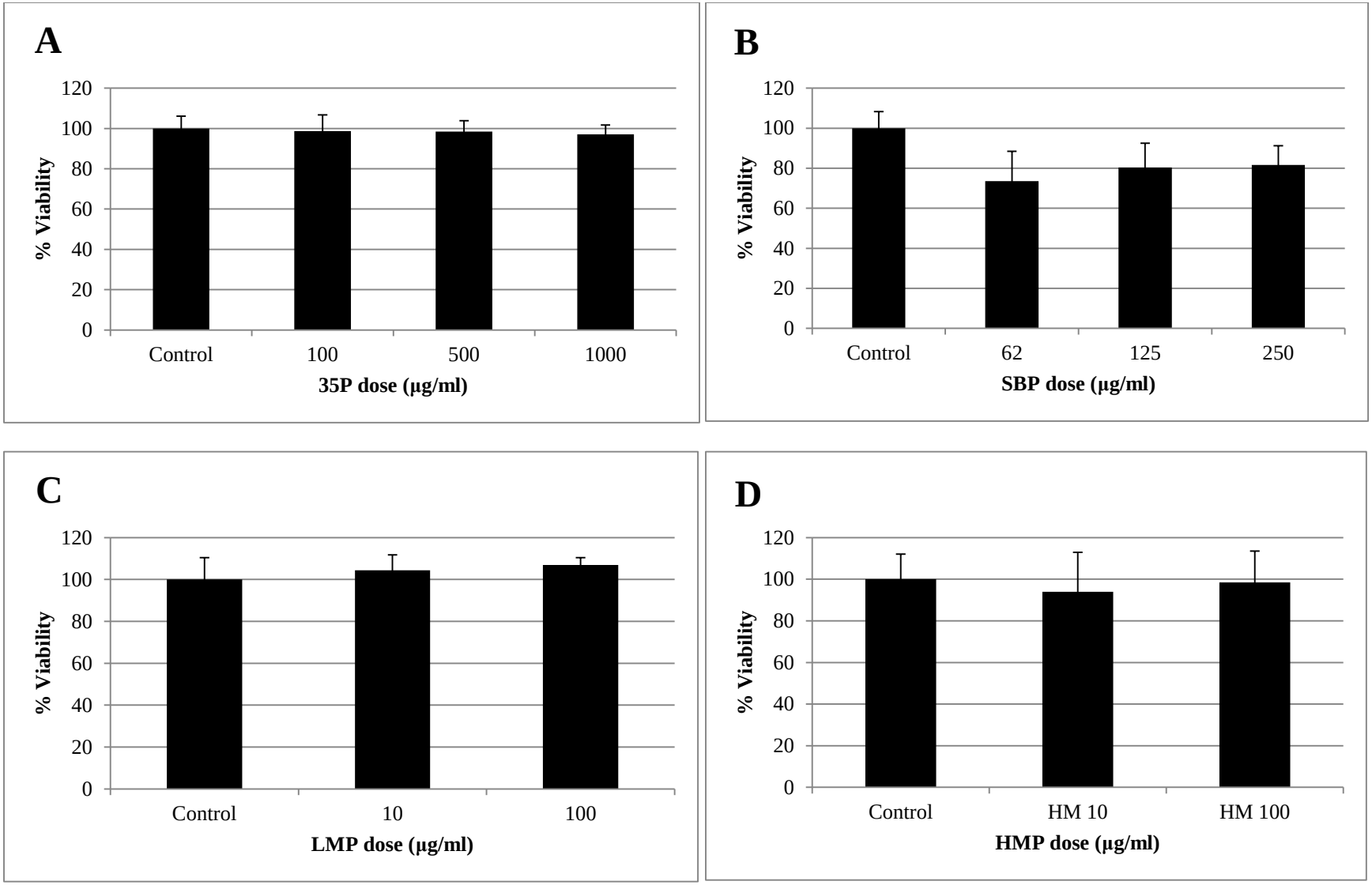


Fig. 3.1. Bar graph of the percentage of lipid accumulation in 3T3-L1 adipocytes after culturing and treatment with pectin and curcumin. The symbol * above the bars indicate significant difference from the control ($p<0.05$). (A) Percent lipid content of adipocytes treated with 35P. 35P stands for charge-modified pectin with % DE of 35%. (B) Percent lipid content of adipocytes treated with sugar beet pectin (SBP). (C) Percent lipid content of adipocytes treated with low-methoxyl pectin (LMP). (D) Percent lipid content of adipocytes treated with high-methoxyl pectin (HMP). (E) Percent lipid content of adipocytes treated with curcumin, LMP and HMP, and combinations of pectin with curcumin.


A
120
100
80
60
40
20
0
% Viability
Control
100
500
1000
35P dose (µg/ml)
B
120
100
80
60
40
20
0
% Viability
Control
62
125
250
SBP dose (µg/ml)
C
120
100
80
60
40
20
0
% Viability
Control
10
100
LMP dose (µg/ml)
D
120
100
80
60
40
20
0
% Viability
Control
HM 10
HM 100
HMP dose (µg/ml)

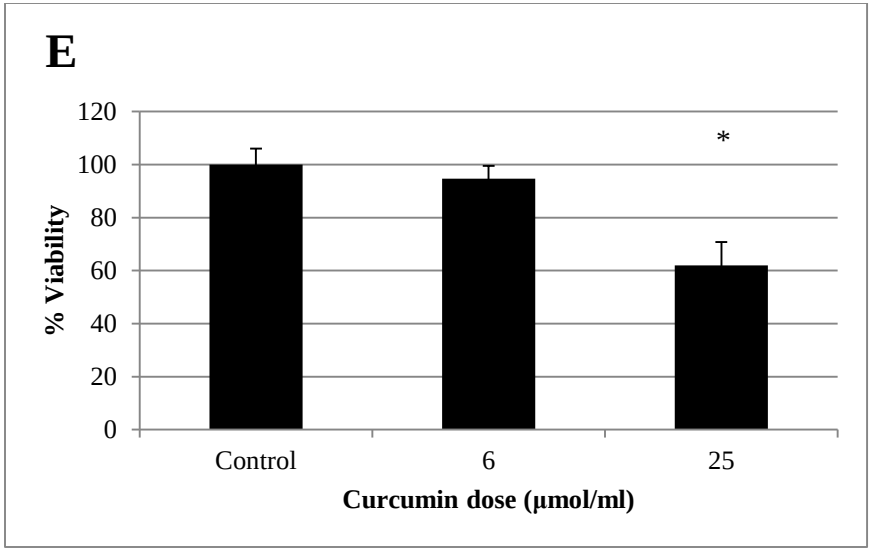


Fig. 3.2. Bar graph of the percentage of live 3T3-L1 adipocytes after culturing and treatment with pectin and curcumin. The symbol * above the bars indicate significant difference from the control ($p<0.05$). (A) Percent viability of adipocytes treated with 35P. (B) Percent viability of adipocytes treated with SBP. (C) Percent viability of adipocytes treated with LMP. (D) Percent viability of adipocytes treated with HMP. (E) Percent viability of adipocytes treated with curcumin.

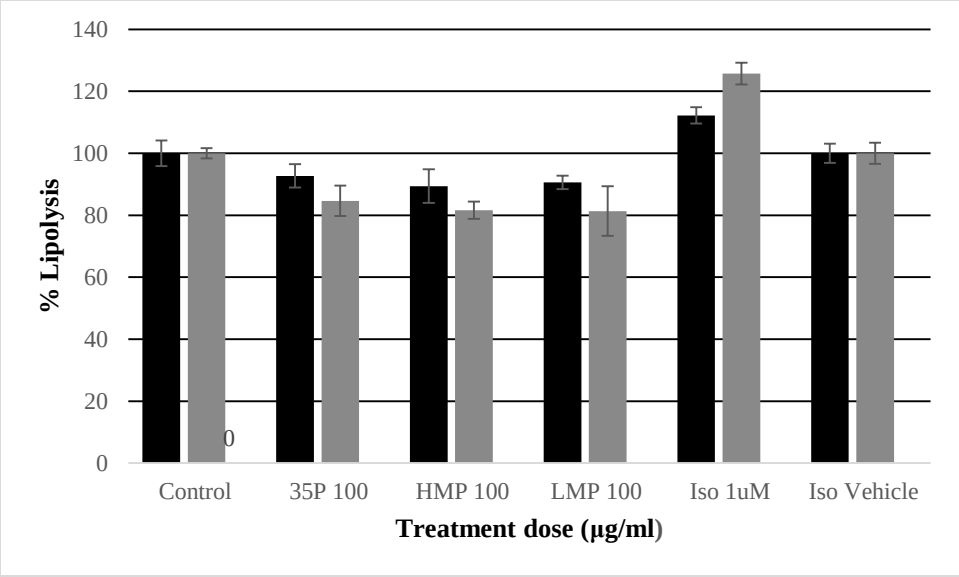


Fig. 3.3. Percent lypolysis of 3T3-L1 adipocytes with different pectin treatments. The bars in black represent adipocytes treated with representative samples for 6 h while the grey bars represent adipocytes treated with representative samples for 12 h. Iso stands for isoproterenol, which is the positive control for lypolysis and the Iso Vehicle stands for the vehicle control of isoproterenol in DMSO.

References

Adam, C.L., Williams, P.A., Garden, K.E., Thomson, L.M., Ross, A.W., 2015. Dose-Dependent Effects of a Soluble Dietary Fibre (Pectin) on Food Intake, Adiposity, Gut Hypertrophy and Gut Satiety Hormone Secretion in Rats. PloS one 10, 1-14.

Aggarwal, B.B., 2010. Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. Annu Rev Nutr 30, 173-199.

Aggarwal, B.B., Shishodia, S., 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 71, 1397-1421.

Andersen, C., Rayalam, S., Della-Fera, M.A., Baile, C.A., 2010. Phytochemicals and adipogenesis. BioFactors (Oxford, England) 36, 415-422.

Asai, A., Miyazawa, T., 2001. Dietary curcuminoids prevent high-fat diet-induced lipid accumulation in rat liver and epididymal adipose tissue. J Nutr 131, 2932-2935.

Beghyn, T., Deprez-Poulain, R., Willand, N., Folleas, B., Deprez, B., 2008. Natural compounds: leads or ideas? Bioinspired molecules for drug discovery. Chem Biol Drug Des 72, 3-15.

Calixto, J.B., Campos, M.M., Otuki, M.F., Santos, A.R., 2004. Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med 70, 93-103.

Chan, M.M., Fong, D., Soprano, K.J., Holmes, W.F., Heverling, H., 2003. Inhibition of growth and sensitization to cisplatin-mediated killing of ovarian cancer cells by polyphenolic chemopreventive agents. J Cell Physiol 194, 63-70.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12, 564-582.

Dagon, Y., Avraham, Y., Berry, E.M., 2006. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes. Biochem Biophys Res Commun 340, 43-47.

Davis, J.N., Hodges, V.A., Gillham, M.B., 2006. Normal-weight adults consume more fiber and fruit than their age- and height-matched overweight/obese counterparts. J Am Diet Assoc 106, 833-840.

Ejaz, A., Wu, D., Kwan, P., Meydani, M., 2009. Curcumin Inhibits Adipogenesis in 3T3-L1 Adipocytes and Angiogenesis and Obesity in C57/BL Mice. The Journal of Nutrition 139, 919-925.

Frayn, K.N., Karpe, F., Fielding, B.A., Macdonald, I.A., Coppack, S.W., 2003. Integrative physiology of human adipose tissue. Int J Obes Relat Metab Disord 27, 875-888.

Fuchs, N.K., Fuchs, N., 2004. Modified Citrus Pectin (MCP): A Super Nutraceutical. Basic Health Publications, Inc.

Gregoire, F.M., 2001. Adipocyte differentiation: from fibroblast to endocrine cell. Exp Biol Med (Maywood) 226, 997-1002.

Gregoire, F.M., Smas, C.M., Sul, H.S., 1998. Understanding adipocyte differentiation. Physiol Rev 78, 783-809.

Hauner, H., Skurk, T., Wabitsch, M., 2001. Cultures of human adipose precursor cells. Methods Mol Biol 155, 239-247.

Kelly, T., Yang, W., Chen, C.S., Reynolds, K., He, J., 2008. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond) 32, 1431-1437.

Kim, C.Y., Le, T.T., Chen, C., Cheng, J.-X., Kim, K.-H., 2011. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. The Journal of Nutritional Biochemistry 22, 910-920.

Kim, H.-K., Nelson-Dooley, C., Della-Fera, M.A., Yang, J.-Y., Zhang, W., Hartzell, D.L., Hamrick, M.W., Baile, C.A., 2006. Genistein Decreases Food Intake, Body Weight and Fat Pad

Weight and Causes Adipose Tissue Apoptosis in Ovariectomized Female Mice. J Nutr 136, 409-414.

Kim, K.-J., Lee, O.-H., Lee, B.-Y., 2010. Fucoidan, a sulfated polysaccharide, inhibits adipogenesis through the mitogen-activated protein kinase pathway in 3T3-L1 preadipocytes. Life Sciences 86, 791-797.

Kim, M.-J., Chang, U.-J., Lee, J.-S., 2009. Inhibitory Effects of Fucoidan in 3T3-L1 Adipocyte Differentiation. Mar Biotechnol 11, 557-562.

Kopelman, P.G., 2000. Obesity as a medical problem. Nature 404, 635-643.

Kristensen, M., Jensen, M.G., 2011. Dietary fibres in the regulation of appetite and food intake. Importance of viscosity. Appetite 56, 65-70.

Lee, J.Y., 2015. Pectin Hydrogels of Curcumin and its Obesity Applications, Food Science and Technology. University of Georgia.

Lee, Y.K., Lee, W.S., Hwang, J.T., Kwon, D.Y., Surh, Y.J., Park, O.J., 2009. Curcumin Exerts Antidifferentiation Effect through AMPKα-PPAR-γ in 3T3-L1 Adipocytes and Antiproliferatory Effect through AMPKα-COX-2 in Cancer Cells. Journal of agricultural and food chemistry 57, 305-310.

Leff, T., Mathews, S.T., Camp, H.S., 2004. Review: peroxisome proliferator-activated receptor-gamma and its role in the development and treatment of diabetes. Exp Diabesity Res 5, 99-109.

Levigne, S., Ralet, M.-C., Thibault, J.-F., 2002. Characterisation of pectins extracted from fresh sugar beet under different conditions using an experimental design. Carbohydrate Polymers 49, 145-153.

Lichtman, S.W., Pisarska, K., Berman, E.R., Pestone, M., Dowling, H., Offenbacher, E., Weisel, H., Heshka, S., Matthews, D.E., Heymsfield, S.B., 1992. Discrepancy between self-reported and actual caloric intake and exercise in obese subjects. N Engl J Med 327, 1893-1898.

Logan, K., Wright, A.J., Goff, H.D., 2015. Correlating the structure and in vitro digestion viscosities of different pectin fibers to in vivo human satiety. Food Funct 6, 63-71.

Marcotte, M., Taherian Hoshahili, A.R., Ramaswamy, H.S., 2001. Rheological properties of selected hydrocolloids as a function of concentration and temperature. Food Research International 34, 695-703.

Maxwell, E.G., Belshaw, N.J., Waldron, K.W., Morris, V.J., 2012. Pectin – An emerging new bioactive food polysaccharide. Trends in Food Science & Technology 24, 64-73.

Misra, P., 2008. AMP activated protein kinase: a next generation target for total metabolic control. Expert Opin Ther Targets 12, 91-100.

Park, H.J., Hausman, D., Della-Fera, M.A., Rayalam, S., Yang, J.Y., Ambati, S., Baile, C.A., 2007. Genistein inhibits adipogenesis and increases lipolysis in primary human adipocytes. Faseb Journal 21, A363-A363.

Plaschina, I.G., Braudo, E.E., Tolstoguzov, V.B., 1978. Circular-dichroism studies of pectin solutions. Carbohydrate research 60, 1-8.

Rhyu, J., Kim, M.S., You, M.-K., Bang, M.-A., Kim, H.-A., 2014. Pear pomace water extract inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes. Nutrition Research and Practice 8, 33-39.

Sadowski, H.B., Wheeler, T.T., Young, D.A., 1992. Gene expression during 3T3-L1 adipocyte differentiation. Characterization of initial responses to the inducing agents and changes during commitment to differentiation. J Biol Chem 267, 4722-4731.

Shehzad, A., Ha, T., Subhan, F., Lee, Y.S., 2011. New mechanisms and the anti-inflammatory role of curcumin in obesity and obesity-related metabolic diseases. European journal of nutrition 50, 151-161.

Wolf, S., Mravec, J., Greiner, S., Mouille, G., Hofte, H., 2012. Plant cell wall homeostasis is mediated by brassinosteroid feedback signaling. Current biology : CB 22, 1732-1737.

APPENDIX

Release rate calculations:

1. Get absorbance values of curcumin released in pH 1.2 or pH 6.5 solutions from spectrophotometer at 420 nm

2. Subtract the control hydrogel absorbances with no curcumin from 100 mg and 400 mg values

3. Substitute absorbance values into the linear equation from the standard curve to get the µg/mL of curcumin in the solutions

4. Multiple values by 4 to factor in the dilution (ratio of 95% ethanol)

5. Calculate the amount of actual curcumin used (µg) in freeze-dried beads used in-vitro

a. Amount of curcumin added to initial pectin dispersion before hydrogel formation multiplied by the amount of freeze-dried beads used (100 mg)

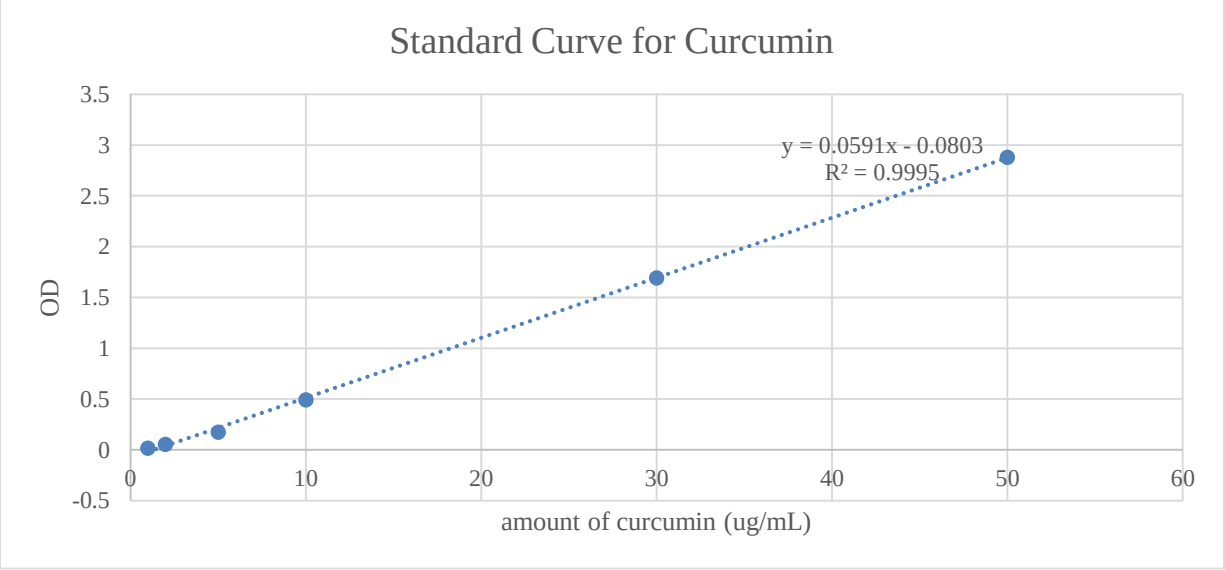
b. Divide that value by the total amount of beads after freeze-drying

c. Multiple by 1000 to convert mg into µg

6. Subtract value from step 4 from µg of actual curcumin used in-vitro

7. Divide value from Step 4 by step 6

8. Multiple by 100 to get release rate



Column1	OD of CaCl2	curcumin in CaCl2 (ug/mL)
72% no c	0.006	1.460
72% 100mg	0.187	4.523
72% 400mg	0.566	10.936
55% no c	0	1.359
55% 100mg	0.02	1.697
55% 400mg	0.025	1.782
35% no c	0.008	1.494
35% 100mg	0.018	1.663
35% 400mg	0.032	1.900
LM no c	0.006	1.460
LM 100mg	0.045	2.120
LM 400mg	0.062	2.408

TRIAL 1	beads inta	OD after 2	corrected	ug/mL cur	DF factore	beads intact after 3h?			OD after 3	corrected	ug/mL cur	DF factore
55% no c	yes	0.001				no			0.274			
55% 100m	yes	0.013	0.012	1.58	6.31	no			0.614	0.34	7.11	28.45
55% 400m	yes	0.003	0.002	1.41	5.64	no			0.705	0.431	8.65	34.61
35% no c	yes	0.005				yes			0.149			
35% 100m	yes	0.007	0.002	1.48	5.91	yes			0.74	0.591	11.36	45.43
35% 400m	yes	0.004	-0.001	1.43	5.71	yes			0.797	0.648	12.32	49.29
LM no c	yes	0.003				no			0.431			
LM 100mg	no	0.004	0.001	1.43	5.71	no			0.574	0.143	3.78	15.11
LM 400mg	yes	0.028	0.025	1.83	7.33	no			0.703	0.272	5.96	23.84
TRIAL 2	beads inta	OD after 2	corrected	ug/mL cur	DF factore	beads intact after 3h?			OD after 3	corrected	ug/mL cur	DF factore
55% no c	yes	0.005				no			0.02			
55% 100m	yes	0.003	-0.002	1.41	5.64	no			0.655	0.635	12.10	48.41
55% 400m	yes	0.006	0.001	1.46	5.84	no			0.152	0.132	3.59	14.37
35% no c	yes	0				no			0.028			
35% 100m	yes	0.009	0.009	1.51	6.04	yes			0.341	0.313	6.65	26.62
35% 400m	yes	0.002	0.002	1.39	5.57	yes			0.095	0.067	2.49	9.97
LM no c	yes	0.003				no			0.03			
LM 100mg	yes	0.006	0.003	1.46	5.84	no			0.065	0.035	1.95	7.80
LM 400mg	yes	0.007	0.004	1.48	5.91	no			0.126	0.096	2.98	11.93
TRIAL 3	beads inta	OD after 2	corrected	ug/mL cur	DF factore	beads intact after 3h?			OD after 3	corrected	ug/mL cur	DF factore
55% no c	yes	0.012				no			0.007			
55% 100m	yes	0.007	-0.005	1.48	5.91	no			0.099	0.092	2.92	11.66
55% 400m	yes	0.005	-0.007	1.44	5.77	no			0.04	0.033	1.92	7.67
35% no c	yes	0.005				yes			0.003			
35% 100m	yes	0.014	0.009	1.60	6.38	yes			0.043	0.04	2.04	8.14
35% 400m	yes	0.013	0.008	1.58	6.31	yes			0.033	0.03	1.87	7.47
LM no c	yes	0.007				no			0.011			
LM 100mg	yes	0.019	0.012	1.68	6.72	no			0.067	0.056	2.31	9.23
LM 400mg	yes	0.011	0.004	1.54	6.18	no			0.128	0.117	3.34	13.35
Average	ug/mL cur	SD		ug/mL cur	SD							
55% no c												
55% 100m	5.95	0.34		29.51	18.40							
55% 400m	5.75	0.10		18.88	14.02							
35% no c												
35% 100m	6.11	0.24		26.73	18.65							
35% 400m	5.86	0.10		22.24	5.86							
LM no c												
LM 100mg	6.09	0.55		10.71	3.88							
LM 400mg	6.47	0.75		16.38	6.51							

TRIAL 1	DF factored in	DF factored in	total cur ir	% released in HCl		total cur ir	% released in Na4PO3		HCl			Na4PO3	
55% no c									mean	SD		mean	SD
55% 100mg	6.314720812	28.44670051	2912.73	0.216797345		2912.73	0.976633699	55% 100m	0.20	0.01		1.02	0.64
55% 400mg	5.637901861	34.60575296	9721.492	0.057994205		9721.492	0.355971631	55% 400m	0.06	0.00		0.19	0.14
35% no c													
35% 100mg	5.908629442	45.43485618	2984.868	0.197952777		2984.868	1.522172958	35% 100m	0.20	0.01		0.89	0.63
35% 400mg	5.705583756	49.2927242	10477.02	0.054458062		10477.02	0.470484067	35% 400m	0.06	0.00		0.21	0.22
LM no c													
LM 100mg	5.705583756	15.11336717	2158.8	0.264294266		2158.8	0.700081964	LM 100mg	0.28	0.03		0.50	0.18
LM 400mg	7.329949239	23.84433164	7819.293	0.093741842		7819.293	0.304942299	LM 400mg	0.08	0.01		0.21	0.08
TRIAL 2	DF factored in	DF factored in											
55% no c													
55% 100mg	5.637901861	48.41285956	2935.539	0.192056814		2892.764	1.673585058						
55% 400mg	5.840947547	14.36886633	9750.257	0.059905578		9741.729	0.147498116						
35% no c													
35% 100mg	6.043993232	26.61928934	3024.259	0.199850382		3003.684	0.886221441						
35% 400mg	5.570219966	9.969543147	10520.75	0.052945107		10516.35	0.094800446						
LM no c													
LM 100mg	5.840947547	7.803722504	2168.072	0.269407441		2166.109	0.360264481						
LM 400mg	5.908629442	11.9323181	7837.229	0.075391822		7831.205	0.152368865						
TRIAL 3	DF factored in	DF factored in											
55% no c													
55% 100mg	5.908629442	11.66159052	2935.268	0.201297795		2929.515	0.398072411						
55% 400mg	5.773265651	7.668358714	9750.324	0.059211012		9748.429	0.078662506						
35% no c													
35% 100mg	6.382402707	8.14213198	3023.921	0.21106383		3022.161	0.269414245						
35% 400mg	6.314720812	7.465313029	10520	0.060025857		10518.85	0.070970807						
LM no c													
LM 100mg	6.720812183	9.225042301	2167.192	0.310116107		2164.688	0.426160366						
LM 400mg	6.179357022	13.3536379	7836.958	0.078848925		7829.784	0.170549259						

	OD CaCl2 soln	ug/mL cur.	mg/ml cur	g FD beads	mg FD bead	theoretical cur (mg)	TE		mL CaCl2	mg cur in total CaCl2 solr	mg cur in CaCl2 soln minus c	entrapped cur in bea	EE (%)
72% no c	0.006	1.460236887	0.001460237	1.1	1100	0			110	0.160626058			
72% 100m	0.187	4.52284264	0.004522843	1.1	1100	100	0.090457		110	0.49751269	0.336886633	99.66311	99.66311
72% 400m	0.566	10.9357022	0.010935702	1.4	1400	400	0.284839		112	1.224798646	1.064172589	398.9358	99.73396
55% no c	0	1.358714044	0.001358714	3.2	3200	0			400	0.543485618			
55% 100m	0.02	1.697123519	0.001697124	3.4	3400	100	0.029213		398	0.675455161	0.131969543	99.86803	
55% 400m	0.025	1.781725888	0.001781726	4.1	4100	400	0.097391		390	0.694873096	0.151387479	399.8486	
35% no c	0.008	1.494077834	0.001494078	3.1	3100	0			398	0.594642978			
35% 100m	0.018	1.663282572	0.001663283	3.3	3300	100	0.030101		400	0.665313029	0.070670051	99.92933	
35% 400m	0.032	1.900169205	0.001900169	3.8	3800	400	0.105063		400	0.760067682	0.094754653	399.9052	
LM no c	0.006	1.460236887	0.001460237	4.6	4600	0			350	0.51108291			
LM 100mg	0.045	2.120135364	0.002120135	4.6	4600	100	0.021572		363	0.769609137	0.258526227	99.74147	
LM 400mg	0.062	2.407783418	0.002407783	5.1	5100	400	0.078257		370	0.890879865	0.379796954	399.6202	
									EE(%)= 100- (amount of precipitated curcumin / total amount of curcumin) *100				
										Teng, 2014			

Pectin san	% EE	% released in HC	% released in Na4PO3 (pH 6.5)
55P 100mg	99.87	0.20	1.01
55P 400mg	99.96	0.06	0.19
35P 100mg	99.93	0.20	0.89
35P 400mg	99.98	0.06	0.21
LM 100mg	99.74	0.28	0.50
LM 400mg	99.91	0.08	0.21