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Mechanisms of Calcium-induced Firmness in Fruits, Vegetables, and Mushrooms

Xiaoyun Deng
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Xiaoyun Deng entitled "Mechanisms of Calcium-induced Firmness in Fruits, Vegetables, and Mushrooms." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

We have read this thesis and recommend its acceptance:

John R. Mount, Carl E. Sams

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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MECHANISMS OF CALCIUM-INDUCED FIRMNESS IN FRUITS, VEGETABLES, AND MUSHROOMS

A Thesis Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Xiaoyun Deng

August 2008

Dedication

I would like to dedicate this thesis to my parents and sisters who have been supporting me throughout all the points of my life, my husband Tao Wu whose love and encouragement fuel the fulfillment of my goals, and to my upcoming lovely baby.

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I would like to express my deepest thanks to those who made my masters education in Food Science and Technology fulfilled.

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Abstract

Six fractions from mushrooms (MFs), as cytoplasmic proteins (F1), cell membrane proteins (F2), alkali-soluble cell wall glucans (F3a), cell wall proteins (F3b), cell wall beta-glucans (F4a), and chitin (F4b) and five fractions from both apples (AFs) and cucumbers (CFs), as cytoplasmic proteins (F1), cell membrane proteins (F2), cell wall proteins (F3), pectic substances (F4), and cellulose (F5) were extracted and analyzed for their neutral carbohydrates, proteins, aminosugar material, soluble phenolics, and pectic substances. The dominant compounds consisted of the following: proteins in MF1, proteins and neutral polysaccharides in MF2, neutral polysaccharides in MF3a, neutral polysaccharides in MF3b, neutral polysaccharides and chitinous material in MF4a, neutral polysaccharides and chitinous material in MF4b; pectic substances in AF1, AF2 and AF4, proteins in AF3, neutral polysaccharides in AF5; proteins in CF1, CF2, and CF3; pectic substances in CF4, and neutral polysaccharides in CF5.

Experiments for binding calcium ($CaCl_2$) to fraction solids were performed in 10 mM Tris-HCl buffer (pH 7.0). The calcium-binding capacity was obtained by both Ion Selective Electrode (ISE) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements. The results indicated that calcium-binding capacity of fractions differed significantly in the order of MF1>MF2>MF3a ≥ MF3b≥MF4b~MF4a for mushroom fractions. AF2 and AF4 showed higher binding capacity than AF1 and AF5 for apple fractions. For cucumber, CF1, CF2, and CF4 showed higher binding capacity than CF3 and CF5.

The results revealed that calcium was bound by all fractions, but those fractions dominantly composed of cytoplasmic proteins (MF1) and cell membrane proteins (MF2)

in mushroom fractions, pectic substances (AF4) and cell membrane proteins (AF2) in apple fractions, cytoplasmic proteins (CF1), cell membrane proteins (CF2), and pectic substances (CF4) in cucumber fractions possessed higher capacities. The binding of calcium to the biopolymers was further investigated by Isothermal Titration Calorimetry (ITC) using pectin and bovine serum albumin (BSA). The results confirmed that interaction of calcium with pectic substances plays a primary role in firmness improvement. However, data also indicated that interactions between calcium and cytoplasmic and/ or membrane proteins may contribute to the firmness improvement in calcium-treated mushrooms, apples, and cucumbers.

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Chapter 1 Literature Review

1.1 Introduction

Botanically, fruits are defined as the ripened ovaries containing seeds; mushrooms are microorganisms belonging to the fungal kingdom; and there is no botanical definition for vegetables. However, in discipline of food science, fruits are generally defined as those botanical fruits that are edible and sweet; vegetables are referred to as the edible parts of plants which can be fruits, leaves, seeds etc; and mushrooms are considered to be vegetables. In this text, food science descriptions are employed, but mushrooms are considered to be a separate group from vegetables.

Fruits, vegetables and mushrooms are vital components of the human diet and are indispensable sources of fibers, antioxidants, vitamins, proteins, and minerals (Chang and Miles 1999; Prasanna and others 2007). Consumption of sufficient amounts of fruits, vegetables, and mushrooms can prevent major diseases such as heart disease, high blood pressure, cancers, vision loss and diabetes (Brownleader and others 1999; Prasanna and others 2007). Moreover, mushrooms can also provide great nutraceutical values, e.g. antitumor and enhanced immune response (Chang and Miles 1999). Unlike other food products, fruits, vegetables, and mushrooms are typically consumed as parts of living organisms. During and after harvest, they are susceptible to physical, chemical, and microbiological spoilage, which significantly impacts the quality and shelf-life, with softening being one of the most undesirable types of quality degradation. Thus, maintaining firmness is one of the major issues for fresh and processed products such as

white button mushrooms and pickled cucumbers (Burton 1986; Brady 1987; Beelman and others 1987(a); Watada and Qi 1999).

Many studies have been conducted to improve the understanding of the mechanism of softening and to develop effective ways to retard it. It is believed that texture of fruits, vegetables and mushrooms is dominantly determined by cellular morphology, composition, and cell turgor pressure (Poovaiah and others 1988; McGarry and Burton 1994). Large amounts of pectin in plant cell walls have been shown to play a critical role in maintaining the integrity of plant cell anatomy. The primary cause of texture loss in plant-based products is the loss of cell morphology resulting from depolymerization and solubilization of pectin (Waldron and others 1997; Willats and others 2001; Prasanna and others 2007). Previous research has also indicated that degradation of other polymers such as starch, hemicellulose and cellulose may also be involved in texture loss of fruits such as citrus and bananas (Sanchez-Romero and others 1998; Prasanna and others 2007). In mushrooms, the primary polymers involved with softening may be the glucans, chitin, and/or proteins which has dominant role is still being debated (Zivanovic and others 2000; Zivanovic and Buescher 2004). In addition to cell walls, cell membranes in many tissue-based foods are also believed to play an important role in preventing texture loss by preserving cell turgor loss (Poovaiah and others 1988; Picchioni and others 1995; 1998; Hepler 2005).

A large number of factors affect the texture of fruits and vegetables including environmental condition during growth; practical, physiological, and genetic factors; post-harvest treatments and storage conditions (Sams 1999). Various approaches have been researched and applied to prevent texture loss in fruits, vegetables and mushrooms,

such as controlled atmosphere (CA), low temperature, and calcium salt treatment (Smock 1979; Poovaiah 1986; Okereke and others 1987). Among them, calcium treatment has been a successful practice adopted by the food industry due to low cost and multiple extra benefits such as reduced physiological disorders, browning, rate of ethylene production, and enhanced resistance to pathogen infection (Kukura and Beelman 1998; Martin-Diana and others 2007). The importance of preserving the quality of these products by calcium treatment is important not only to fresh produce, but also to processed products (Gu and others 1999; Zivanovic and Buescher 2004; Martin-Diana and others 2007). It is significant that quality improvement of calcium-treated products has been demonstrated by both instrumental analyses and sensory evaluation (Rico and others 2007).

It is generally accepted that calcium ions form cross-linkages between pectin molecules via an "egg-box" model protecting pectin from hydrolysis thus preserving cell structure (Poovaiah and others 1988). Nevertheless, many researchers have shown that calcium ions can also effectively improve the firmness of mushrooms in which pectic substances do not exist (Kukura and Beelman 1998; Zivanovic and others 2000; Philippoussis and others 2001). As a result, the hypothesis of this research is that the possible sites for calcium binding in mushrooms include cell wall polymers, membrane proteins and phospholipids, as well as cytoplasmic soluble and/or insoluble proteins. The same mechanism may be involved in plant cells as side effects to the "egg-box" model. This research is designed to determine potential polymers and sites of calcium binding in plant and mushroom cells through an *in vitro* calcium binding to apple, cucumber, and mushroom fractions which represent macromolecules from different locations in cells. A full understanding of mechanisms by which calcium affects firmness in these food

products would ultimately contribute to the application of calcium in texture improvement.

1.2 Role of texture in quality of fruits, vegetables and mushrooms

Bourne defines that "the texture properties of a food are that group of physical characteristics that are sensed by the feeling of touch, are related to the deformation, disintegration and flow of the food under the application of a force, and are measured objectively by functions of force, time and distance". He states that the texture is composed of several parameters including mechanical, geometrical and chemical characteristics (Bourne 1980). Texture of food is important in evaluating quality and determining consumer acceptability. Tests reveal that consumers are more sensitive to differences in texture than to those in taste, and the texture positively contributes to the enjoyment of eating (Shewfelt 1999; Szczesniak-Surmacka 2002). Due to increased awareness of their health benefits, the consumption of fruits, vegetables, and mushrooms, especially as minimally processed products has grown rapidly in recent years (Martin-Diana and others 2007). As expected, texture perception is an important factor in determining consumer acceptability to these products (Konopacka and Plocharski 2004). However, the consumption of these products is limited by their short shelf-life and rapid deterioration, in which texture loss is one of the primary causes of reduced quality (Burton 1986; Brady 1987; Beelman and others 1987(a); Willats and others 2001).

1.3 Cell components and the texture in fruits, vegetables, and mushrooms

Texture of fruits, vegetables and mushrooms is believed to be primarily determined by cellular morphology, chemical composition, and cell turgor pressure (Poovaiah and others 1988; McGarry and Burton 1994; Jarvis and others 2003). Plant and fungal cells are composed of cell wall, cell membrane, and cytoplasm. Inside the cytoplasm, there are large vacuoles, nucleoli, nucleus, and many other organelles. The large vacuole and organelles are also surrounded by a double layer membrane (Crang and Vasseilyev 2002). The cell wall and cell membrane have been recognized as crucial components for maintaining desirable textural characteristics of fruits, vegetables, and mushrooms (Poovaiah and others 1988; McGarry and Burton 1994), while organelle membranes may also play a role in quality maintenance (Kukura and Beelman 1998). Chemical composition of apple, cucumber, and mushroom tissue is given in **Table 1.1**.

1.3.1 Cell wall

The cell wall serves a similar purpose in fruits, vegetables and mushrooms. Its main function is in maintaining size and shape of the cell, tissue texture, mechanical support, restriction of osmotic distention, protection, and cell-to-cell communication (Ainsworth and Sussman 1965 ; Farkas 1985; Heredia and others 1995; Brett and Waldron 1996). However, plants and fungi have distinct cell wall chemical compositions and architectures.

Table 1.1 - Composition of apples, cucumbers, and mushrooms.

Composition	Apple	Cucumber	Mushroom
Moisture content (% fresh tissue)	86.67[a]	96.73[a]	92.43[a]
Proteins (% fresh tissue)	0.27[a]	0.59[a]	3.09[a]
Carbohydrates (% fresh tissue)	12.76[a]	2.16[a]	3.28[a]
Cellulose (% fresh tissue)	0.62[b]	0.16[b]	N/A
Total sugar (% fresh tissue)	10.10[a]	1.38[a]	1.65[a]
Pectin (% fresh tissue)	0.89[b]	0.22[b]	N/A
Lipids (% fresh tissue)	0.13[a]	0.16[a]	0.34[a]
Calcium (mg/100g fresh tissue)	5[a]	14[a]	3[a]
Cell wall (mg/g fresh tissue)	10[c]	14.3[d]	13.5[e]
Protein (% DW cell wall)	0.91[c]	N/A	8.3[f]
Neutral polysaccharide (% DW cell wall)	77.1[c]	53.2 [d]	47.5[e]
Calcium (% DW cell wall)	0.1[c]	0.5[d]	N/A
Pectin (% DW cell wall)	22.9[c]	24.6[d]	N/A
Chitin (% DW cell wall)	N/A	N/A	35.3[f]

DW represents dry weight; N/A represents not available; Superscripted letters represent the references as follows: a, USDA nutritional database (USDA, National Agricultural Research Service); b, (Sherz and Senser 2000); c, (Tobias and others 1993); d, (McFeeters and Lovdal 1987); e, (Hammomd 1979); f, (Mendoza and others 1987)

1.3.1.1 Cell wall of plant cells

The plant cell wall is composed of cell middle lamella, primary cell wall, and in some cases secondary cell wall, from outer to inter cell, respectively. The cell middle lamella, the outermost layer, binds adjacent cells and maintains cell-to-cell adhesion and tissue coherence. The primary cell wall is composed of a skeleton of cellulose microfibrils embedded in a gel-like matrix with hemicelluloses, pectic substances, proteins, and in some cases, lignin. The secondary wall is made of cellulose, hemicellulose and lignin; however, it is not common in fruit and vegetable products (Heredia and others 1995; Brett and Waldron 1996).

Pectic substances are abundant in the primary cell wall and middle lamella. They are composed of α-1, 4-linked galacturonic acid units with various degrees of esterification (DE). The polygalacturonic backbone is occasionally interrupted with rhamnose and side chains consisting of xylose, galactose, and arabinose. Pectic substances serve a variety of functions including controlling wall porosity, providing cell-to-cell adhesion, deposition, slippage and extension of cellulosic-glycan network (Willats and others 2001). It has also been established for dicot plant cells that cross-linked low-ester pectic homogalacturonans are most abundant in the middle lamella, especially in the "reinforcing zone" located at the point of maximum cell stress in dicot plant cells (Jarvis and others 2003). Most pectic substances are water-soluble; the water insoluble pectin fractions can be extracted by chelating agents, acid or alkali (Van Burn 1979).

Other than pectic substances, cellulose, hemicellulose, lignin, and proteins are also present at high levels. Cellulose, the least soluble component, is a large linear β – 1,

4 glucan that forms a stiff net-work of crystalline microfibrils due to extensive hydrogen bonds. The microfibrils are responsible for cell wall strength and rigidity. Hemicellulose is a group of alkali soluble neutral sugars built of glucose, galactose, rhamnose, xylose, mannose and other sugar molecules in the cell wall. They are usually branched, short chain polymers with random amorphous structures. In addition to carbohydrates, cell walls contain a variety of proteins classified into structural and nonstructural proteins. One type of structural protein is a glycoprotein rich in hydroxyproline. Nonstructural proteins are mainly enzyme mediating reactions that may alter wall composition. Lignin is a polymer of phenolic compounds but is usually present extremely low levels in most consumable parts of fruits and vegetables (Van Burn 1979; Heredia and others 1995; Brett and Waldron 1996). In Poaceas plants, the phenolic cross-linking of arabinoxylans is dominant in “reinforce zones” (Jarvis and others 2003).

1.3.1.2 Fungal cell wall

Similar to plant cell walls, fungal cell walls also have layered ultrastructural features based on cell wall components, and it is composed of chitin microfibrils embedded in protein, principally protein, glycoprotein reticulum with glucans merging into protein, and mixed glucan layers from inner to outer cell (Farkas 1985). However, the chemical composition is distinct from that of plant cell wall. Fungal cell walls are composed primarily of neutral polysaccharides, chitin and proteins (Peberdy 1990). Different from plant cell walls, they usually do not contain pectic substances and cellulose (Ainsworth and Sussman 1965 ; Griffin 1994).

The polysaccharides in fungi may be classified into two groups: skeletal and matrix polysaccharides. The primary skeletal constituents of fungal cells wall are chitin and β-1, 3 glucan. Chitin is a β -1, 4 linked acetyl-glucosamine homopolymer with highly developed hydrogen bonds. Similarly to cellulose, it is water-insoluble and highly crystallized giving rigidity to the cell. Chitin and β-1, 3 glucan often occur as a complex possibly due to the covalent peptide links between non-acetylated amino groups in chitin and the reducing ends of glucan (Farkas 1985; Wessels and others 1990). The matrix polysaccharides of wall are usually amorphous, slightly crystalline, and mainly-soluble. They fill the space between skeletal polysaccharide microfibrils serving as "cementing" substances. Glucan mucilage, a highly branched β-1, 3/1, 6 glucan forms an extracellular amorphous gel holding hyphae together during mycelial growth but becomes less apparent during fruit body development (Angeli-Papa and Eyme 1978; Farkas 1985). Another amorphous matrix compound in fungal tissue is glycoprotein. The carbohydrate portion of glycoprotein may vary from a single sugar to a polysaccharide chain. They hold wall polysaccharides by linkages between the amino group of amino acids and the reducing ends of polysaccharides. Some of these glycoproteins may also act as enzymes modulating cell wall synthesis and hydrolysis (Farkas 1985; Ruia-Herrera 1992).

1.3.2 Softening associated with cell wall

1.3.2.1 Softening of fruits and vegetables associated with cell wall

Cell wall autolysis has been recognized as a main phenomenon in texture loss, which is followed by expanded intercellular space, loosening of cell microfibrils, and cell separation (Poovaiah and others 1988). Such texture loss is considered to be mainly due

to the degradation of biopolymers in the cell wall and middle lamella, and pectic substances are recognized as the most crucial components involved in softening of plant-based products (Picchioni and others 1998; Prasanna and others 2007). It has been found that the soluble pectic fraction increases upon tissue softening, and it is believed that depolymerization and solubilization of pectic substances in the cell wall play a key role in fruit ripening and the texture loss during storage and processing (McFeeters 1992; Waldron and others 1997; Chardonnet and others 2003).

Pectic substances can be depolymerized by a variety of chemical reactions, e.g., alkaline/acid/enzyme-catalyzed de-esterification, hydrolysis of glycosidic bonds by β-elimination, acid and enzyme catalysis. β-Elimination takes place under pH > 4.5 in pectin with higher DE, usually during the cooking or heating process (Sila and others 2006). However, under natural physiological pH, enzyme-catalyzed depolymerization takes a lead role in most fruits and vegetables during storage and processing (Van Burn 1979; McFeeters 1992). Therefore, pectin-degrading enzymes substantially play vital roles in tissue softening, and it is generally accepted that pectin methylesterase (PME) and polygalacturonases (PGs) are primarily responsible for pectin degradation. PME is a wall-bound enzyme functioning in de-esterification of polygalacturonic polymers. The de-esterified pectin appears to be digested easier by PGs. PGs hydrolyze the α-1, 4-linked glycosidic linkages in pectic substances. Two types of softening-related PGs have been identified: endo-PG digesting pectin chain randomly and exo-PG digesting pecin chain from the non-reducing end (Prasanna and others 2007).

Even though other components such as cellulose, hemicellulose, lignin, and proteins apparently remain unchanged in most plant-based products upon softening

(Poovaiah and others 1988; Waldron and others 1997; Willats and others 2001), there are reports suggesting that they are involved in post-harvest softening as well (Sanchez-Romero and others 1998; Prasanna and others 2007).

1.3.2.2 Softening of mushrooms associated with cell wall

The softening of mushrooms has not been studied as extensively as that of vegetables and fruits. Nevertheless, similar to plant texture loss, the softening of mushrooms is strongly correlated with cell morphology and increased solubility of cell wall polymers. Significantly decreased tissue stiffness has been determined during post-harvest of mushrooms (McGarry and Burton 1994). Changes in mushroom tissue morphology followed by cell collapse and autolysis led to the formation of large void spaces and losses of cellular content, resulting in texture degradation (Evered and Burton 1995). A positive relationship between solids content and firmness has also been found (Beelman and others 1987(b)). Additionally, mushrooms tend to become softer and tougher during storage. This softening may be caused by reduced-intercellular space and may be associated with degradation of certain polysaccharides and proteins, while the toughness may be related to increased amounts of chitin in cell walls (Hammomd 1979; Zivanovic and others 2000; Zivanovic and Buescher 2004).

1.3.3 Cell membrane

Plants and fungi have bilayered membranes composed of a typical continuous bilayer of lipid molecules interspersed with proteins forming a fluid mosaic. They are known to act as a semi-permeable barrier and regulate the internal chemical environment of cytoplasm and organelles. The lipid bilayer provides basic membrane structure, and

individual lipid molecules can diffuse within lipid bilayers. Protein molecules function as the transporting specific molecules, catalyzing membrane-associated reactions, signal transduction, and structural links (Alberts and others 2002).

The most abundant membrane lipids are phospholipids. Generally, membrane phospholipids consist of two hydrophobic fatty acyl side tails and a hydrophilic head group which consists of either phosphate alone or a phophorylated head. The hydrophobic chains are present in the interior, and the hydrophilic head is present in the exterior in the membrane arrangements (Briskin 1994; Alberts and others 2002). The composition of lipid and temperature of the environment affects the fluidity of lipid layers thus affecting some membrane-associated enzyme activities (Poovaiah and others 1988). In addition to phospholipids, membranes also contain cholesterol and glycolipids but at a lower level. Membrane proteins exist in compact globular arrangements immersed in lipid bilayers. They can be classified as peripheral or integral proteins based on their arrangements and locations. The peripheral proteins are associated with lipid head group or other proteins on the surface of membrane. The integral proteins are amphiphilic embedded into the membrane. Compared to lipids and proteins, carbohydrates represent just a minor portion of membranes. Membrane carbohydrates are mostly associated with proteins and lipids, known as glycoprotein and glycolipids, respectively (Briskin 1994). The composition of these contents in membrane varies depending on cellular membrane origin. Generally, fungal membranes tend to have higher carbohydrate content compared with plant cell membranes (Griffin 1994).

1.3.4 Softening associated with cell membrane

Although no direct association between cell membrane alteration and softening has been reported, evident correlation between loss of membrane integrity and senescence has been observed, which is the prerequisite of softening in fruits (Poovaiah and others 1988). The loss of cell membrane integrity may induce an increased membrane permeability contributing to softening due to the loss of cell turgor pressure, one of the factors determining the texture of fruits, vegetables, and mushrooms (Beelman and others 1987(a); Poovaiah and others 1988; McGarry and Burton 1994; Waldron and others 1997). Membrane integrity directly relates to its lipid and protein content, and decreased membrane protein and altered lipid content in senesced fruits and plants has been reviewed (Poovaiah and others 1988). On the other hand, activity of texture-related enzymes may be regulated by cell membrane microenvironment, e.g., increased microviscosity results in an increased membrane-bound enzyme activity (Poovaiah 1986). However, in mushrooms, Braaksma et al find that although membrane proteins decreased, the ratio of sterol to phospholipids and fluidity of liposomes was unaffected by storage, and they concluded that mushroom senescence is independent of membrane composition (Braaksma and others 1994).

1.4 Mechanism of calcium-induced firmness

Calcium has an essential role in plant structure and development. Before being absorbed by plants, calcium regulates the uptake of ions and adjusts the pH of soil (Quintero 1991). Once in the plant system, calcium participates in polarity growth, secretion, hormonal actions, cell division, cationic transport, photosynthesis, gene

expression, and many other processes. Most of these functions are achieved by interacting with calcium-related proteins such as Ca-calmodulin which is the best known calcium- binding proteins (White and Broadley 2003). The Ca-calmodulin complex is known to regulate enzymes such as adenosine triphosphate decomposition enzyme (ATPases), oxidoreductases, and protein kinases. Deficient or excessive calcium content causes more than thirty calcium-related physiological disorders in plants (White and Broadley 2003; Hepler 2005). Besides these functions, about 60% of calcium is located in the cell wall where pectic substances are abundant and calcium is known to perform a crucial role in determining cell structure and integrity, cell-to-cell adhesion, and tissue coherence (Poovaiah and others 1988).

Similar to its role in plants, calcium generally contributes to mushroom physiology by being involved in transporting amino acids, ions, and sugar; simulation of tip growth and fruiting; increasing mycelia productivity; protecting the cell from microbial contamination and damage, and improving color and texture. It is located within mostl cell organelles of fungi (Morales and Ruiz-Herrera 1989; Yan and others 2002).

1.4.1 Current protocols to research mechanisms of calcium-induced firmness

Several approaches have been applied to explore the mechanisms of calcium-induced firmness in fruits, vegetables and mushrooms. Generally, the improvement of firmness is examined by texture analysis, usually a penetration test in which puncture force reflects the firmness (Zivanovic and others 2000). The *in vivo* microscopy-based observation is the most direct and simple way to determined the cell anatomy

modification in calcium-treated tissues (Poovaiah and others 1988; Evered and Burton 1995; Quiles and others 2004). Free Ca^{2+} and/or bound calcium have also been tracked by fluorescent dye, X-ray analysis, atomic absorption spectrometry, and potassium antimonite precipitation (Betoret and others 2005). Also, localization and possible calcium-induced conformation change of pectin have been studied by enzyme-gold complex, immunolabeling, ingenious fluorescent probe, cationic colloidal gold probe (Roy and others 1994; Jarvis and others 2003).

Many *in vitro* experiments are performed to investigate the alteration of overall chemical composition, enzymatic activity, and calcium content (Geduspan and Peng 1986; Poovaiah and others 1988; Pagel and Heitefuss 1990; Chardonnet and others 2003). Cell wall fraction extraction and analysis is extensively employed in researching the association between calcium-induced chemical composition alteration and firmness. Most often, fresh tissue of apples, cucumbers, or mushrooms is extracted with alcohol and total alcohol insoluble solids (AIS) are considered to be mainly composed of cell wall macromolecules (Selvendran 1975; Howard and Buescher 1990; Zivanovic and Buescher 2004). The basis for this assumption is that alcohol insoluble cytoplasmic and cell membrane compounds are low in proportion to total AIS. However, depending on species, the contamination from cytoplasmic proteins and the loss of cell wall alcohol-soluble content can cause a deviation from true values (Selvendran 1975). The extracted cell wall content is analyzed for its chemical composition including polysaccharides, proteins, pectic substances, and calcium. Pectic substances are often divided into 3 to 4 fractions based on its solubility properties as explained earlier (Howard and Buescher 1990).

1.4.2 Calcium-induced firmness associated with cell wall

1.4.2.1 Calcium-induced firmness associated with plant cell wall

A reduced degradation of the cell wall following calcium treatment has been observed by microanalysis and calcium is mainly located in the cell wall and intercellular spaces (Glenn and Poovaiah 1990; Roy and others 1994; Betoret and others 2005; Alandes and others 2006). Further investigations of the chemical composition of calcium-treated fruits and vegetables revealed that calcium was mainly involved in the reduction of soluble pectin substances in cell walls (Howard and Buescher 1990; Chardonnet and others 2003; Manganaris and others 2007).

It is generally accepted that calcium acts as a bivalent cation bridge cross-linking carboxyl groups of pectic substances via an "egg-box" model preventing solublization and hydrolysis of pectic polymers and strengthening the plant cell wall resulting in fimer texture in fresh and thermally processed fruits and vegetables (Poovaiah and others 1988). Pectic substances tend to cross link in the presence of calcium due to their non-esterified carboxyl groups. This cross-linking results in a lower pectin solubility and firmer texture of plant cells and is favored by a low percentage of DE. Therefore, activating endogenous or adding exogenous PME can effectively enhance firmness. Thus, pre-cooked french fries and fungal PME-infused eggplants showed a firmer texture (Bartolome and Hoff 1972; Banjongsinsiri and others 2004). However, the "egg-box" complex may also hinder the hydrolysis of pectic substances. Excessive de-esterifiaction of pectic substances is inhibited by calcium addition which may lower the accessibility of PME to methoxyl residues and this is proposed as the major effect in the retention of firmness of thermally

processed plant-based products (Hudson and Buescher 1986; Howard and Buescher 1990). In calcium-treated pickles, the delay of calcium addition does not result in the same texture improvement as that of addition before thermal processing. The possible explanation is that elevated temperature also activates PGs causing extensive depolymeration, and delay of calcium addition may not be capable of aiding firmness (Buescher and Hudson 1986). Furthermore, interactions between calcium ions and macromolecules other than pectin could also contribute to cell wall firmness (Hepler 2005). Ferreira et al found that calcium and magnesium ions are electrostatically involved in the macromolecular aggregation of conglutins, legume seed storage proteins (Ferreira and others 2003).

It has also been suggested that maintenance of calcium-associated firmness may be possible through modulating the activity of cell wall hydrolysis-related enzymes (Poovaiah and others 1988). Calcium increases PME activity, and increased PME activity results in decreased pectin solubility due to enhanced calcium-binding to de-esterified galacturonic acid units (Anthon and others 2005). Consequently, the β-elimination, which is highly dependent on the higher DE of pectin, is inhibited as well (Sila and others 2006). However, other reports indicated that the PME activity was negatively related to tissue calcium concentration (Sams and Conway 1993). It is reported that Exo-PG does not show much effect on pectin solubility and can be activated by calcium ions, whereas, Endo-PG has a pronounced influence on softening and is inhibited by calcium ions (Pagel and Heitefuss 1990; Prasanna and others 2007). In addition to PME and PGs, β-D-galactosidase which degrades the pectic substances and shows lower activity in calcium-treated golden delicious apples (Siddiqui and Bangerth 1995).

1.4.2.2 Calcium-induced firmness associated with mushroom cell wall

Compared to plants, fewer studies have been reported regarding the mechanisms of calcium-induced firmness in mushrooms. The reduction of water-soluble proteins and polysaccharides has been observed in thermally processed mushrooms after calcium treatment (Zivanovic and Buescher 2004). Cell walls of *Botrytis cinerea* after calcium treatments become thicker, and calcium content increased (Chardonnet and others 1999). As mentioned previously, the mushroom cell wall is composed mainly of neutral polysaccharides, chitin, and proteins in which no pectin exists (Peberdy 1990). Therefore, the role of calcium in improving mushroom firmness is possibly in formation of cross-links between proteins and/ or polysaccharides other than pectin that results in reduction of solubility of cell wall components (Beelman and others 1987(b); Zivanovic and Buescher 2004).

1.4.3 Calcium-induced firmness associated with cell membranes

Calcium is not only important for cell wall structure but has pronounced effects on membrane integrity which consequently impacts membrane permeability and cell turgor pressure. Calcium can bind to negatively charged heads of phospholipids, and stabilize lipid bilayers, thus providing structural integrity to cellular membranes (Poovaiah and others 1988; Hepler 2005). Poovaiah and others have reviewed the effects of calcium on reduction of membrane fluidity, microviscosity, and permeability, and believe that reduced permeability was caused by calcium-induced shrinkage of the membrane surface of plant cells (Poovaiah and others 1988). A retarded firmness loss in calcium-infiltrated apples fruits has been observed, and this effect is related to delayed

galactolipid degradation in cell membranes which may increase the rate of sterol conjugation contributing to cell membrane integrity (Picchioni and others 1995; 1998). Another report also indicates that calcium treatment reduces lipase activity in fresh cut cantaloupe melon, which can prevent the degradation of membrane lipids (Lamikanra and Watson 2004). On the other hand, it is believed that the interaction between calcium and membrane proteins may also be associated with maintaining cell texture by modulating cell wall synthase or calcium-calmodulin-regulated processes (Poovaiah and others 1988). In mushrooms, there is not direct evidence regarding calcium-induced firmness associated with mushroom membranes. However, Kukura and others indicate that reduction in browning of calcium-treated mushrooms is due to increased stability of vacular membranes preventing tyrosinase from mixing with its phenolic substrates which are normally isolated in the vacuoles (Kukura and Beelman 1998).

1.5 Current application of calcium in maintaining firmness

1.5.1 General methods in maintaining firmness

Favorable texture can be achieved by many production practices, e.g., proper sunlight, low growing temperature, calcium and N-dimethylaminosuccinamic acid treatment, harvest at proper stage of maturity, and genetic breeding (Sams 1999). Texture of peaches, apples and strawberries differ significantly among cultivars and many plant breeding programs are carried out to modify the plant texture (Kader and others 1982; Smith and Stow 1985; Shaw and others 1987). As for post-harvest practices, storage of fruits, vegetables, and mushrooms at refrigerated temperature is the first step and the most common procedure to extend shelf life. Under cold condition, ripening, softening,

color changes, respiration, moisture loss, undesirable metabolic process, and spoilage are effectively retarded (Hardenburg and others 1986). However, for long term storage, the growth of insects and pathogens cannot be efficiently prevented (Wills and others 1989). Another option for maintaining fruit quality is controlled atmosphere (CA) storage by modifying the gas composition of the storage atmosphere. CA storage delays softening due to the biochemical and physiological changes such as reduced-aspiration, ethylene production, and pathogen-induced decay (Sams and Conway 1985; Kader 1992). However, CA storage is expensive and not always available (Thompson 1992). Heat treatment at 38 °C before storage effectively maintained the firmness of apple fruits by modulating firmness-related enzyme activity (Klein and others 1990). Another approach is calcium treatment, and its role in decreasing softening is well documented and adopted by industry (Guthrie 1984; Sams and Conway 1993; Martin-Diana and others 2007). The combination of calcium and heating treatment has also been researched in apple fruits and pickles (Buescher and Hudson 1986; Whitaker and others 1997).

Additionally, it appears that other cations such as magnesium, aluminum, and strontium are less effective than calcium in apple fruits (Conway and Sams 1987; Sams 1999). Investigations of inhibition of tissue softening by various cations showed that calcium poses the highest efficiency in improving cucumber and apple texture (Conway and Sams 1987; McFeeters and Fleming 1989). However, pectin does not show the highest binding affinity to calcium among various cations by *in vitro* binding examination (Kohn 1987). Compared to other cations, the effect of calcium in improving firmness may be due to unique electronic or size characteristics of the calcium ion, or there are

other specific calcium ion binding sites related to texture other than carboxyl groups in pectin (McFeeters and Fleming 1989).

1.5.2 Calcium application

1.5.2.1 Calcium application in fruits and vegetables

Calcium salts have been applied widely to improve firmness of various fruits and vegetables both in fresh and processed products. Applications of calcium salts in whole apple fruits demonstrate that pre and post-harvest treatments with calcium salts can effectively increase the calcium content and delay the senescence, resulting in firmer, higher quality fruits (Sams and Conway 1993). It also contributes to reduced respiration, ethylene production, physiological disorder, and risk of salt-related injuries (Sams and Conway 1984). After calcium treatment the apple fruits are less susceptible to pathogen grown during storage (Conway and others 1988; Tobias and others 1993). Increased firmness after calcium treatment has also been observed in numerous fresh fruits such as honeydew, strawberry, grapefruit and peaches (Martin-Diana and others 2007). The processed products treated with calcium also appear more acceptable in color and firmness, e.g., applesauce produced from calcium-treated apples shows higher viscosity and lighter color (Quintero 1991; Manganaris and others 2005). In vegetables, calcium has been applied to fresh eggplant, carrots, iceberg lettuce, hot pepper, and diced tomatoes with significantly increased firmness (Martin-Diana and others 2007). Additionally, calcium has been successfully used to improve firmness of pickles. A direct effect has been observed when cucumber pickles were treated with calcium solution before thermal processing (Buescher and Hudson 1979).

1.5.2.2 Calcium application in mushrooms

Although the chemical composition of mushrooms differs from fruits and vegetables, mushroom firmness also significantly increases with calcium treatment. Calcium-induced firmness along with retarded the opening and browning, extended shelf-life, and fortified calcium content has been achieved by adding calcium to the casing layer, irrigation water, and washing solution (Guthrie 1984; Okereke and others 1987; Beelman and others 1987(b)). It has been shown that irrigation with calcium chloride solution does not affect the protein and total nitrogen content, but calcium content can be doubled in mushroom tissue (Simons and Beelmam 1995; Beelmam and Simons 1996; Miklus and Beelman 1996). Firmness of blanched and retorted mushroom increases significantly with the addition of calcium chloride in irrigation water (Simons and Beelmam 1995). Additionally, canned mushrooms become firmer and tougher with increased product yield by adding 5 and 25 mM calcium chloride in the brine (Zivanovic and Buescher 2004).

1.5.2.3 Type of calcium salts

Different forms of calcium such as calcium chloride, calcium lactate, calcium phosphate, calcium propionate, and calcium gluconate have been examined to improve the firmness of fruits, vegetables, and mushrooms (Martin-Diana and others 2007). The use of calcium chloride is dominant in industry, but it is associated with bitterness and off-flavors due to residual chlorine remaining in the products. Sensory evaluation indicates that a calcium chloride concentration above 800 µg/ g dry weight apple tissue may affect sensory acceptability of applesauce (Sams and Conway 1993). Dipping of

fresh-cut cantaloupe and immersion of apple slices in more than 1% calcium chloride solution resulted in apparent bitterness of flavor (Luna-Guzmán and others 1999; Sham and others 2001). Instead, the use of calcium lactate and calcium gluconate can avoid bitterness and residual flavor (Conway and Sams 1987; Quintero 1991; Manganaris and others 2005; Martin-Diana and others 2007).

1.5.2.4 Approaches in calcium application

Methods including pre- and post-harvest calcium treatments have been developed to improve firmness in food products. Generally effective pre-harvest treatments include adding calcium salt in growth media and sprays (Beelman and others 1987(b); Quintero 1991; Simons and Beelmam 1995). The concentration of calcium salt solution for pre-harvest treatment generally is around 0.1-1% (Beelman and others 1987(b); Siddiqui and Bangerth 1995; Philippoussis and others 2001). Post-harvest treatments include adding calcium in water for dipping, washing, vacuum/ pressure/ hydrocooling infiltration, coating or a combination of these techniques (Martin-Diana and others 2007). Fruits dipped in approximately 1-5% calcium chloride have shown firmer texture (Luna-Guzmán and others 1999), but salt-related injury may be caused if high concentrations of calcium salt (greater than 1000 µg/g dry tissue) remains on the surface (Sams and Conway 1993; Forcherio 1994). The concentration of calcium salts used in a washing solution generally ranges from 0.5-3.0% (Martin-Diana and others 2007). Pressure infiltration is the most effective method currently used in fruits and vegetables, and the concentration of calcium solution varies from 1 to 8% (Forcherio 1994; Martin-Diana and others 2007). Increases of calcium content in fruits by pressure infiltration are two to

three times greater than that by spray and dips (Sams and Conway 1993). A lower concentration of calcium salts (about 0.1-0.5%) is used in the brine of pickles and canned mushrooms (Guillou and others 1992; Zivanovic and Buescher 2004). The pH of calcium solution generally ranges from 5.5 to 6.5 (Martin-Diana and others 2007). However, McFeeters and Fleming found that in the inhibition of cucumber mesocarp tissue softening, effectiveness increases with decreased pH under pH < 5, which may be due to the β-elimination at higher pH (McFeeters and Fleming 1991). Adding calcium during or before warm temperature treatment strengthens the firming effects, but it can not be fulfilled if the calcium was added after temperature treating (Buescher and Hudson 1986; Martin-Diana and others 2007).

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Chapter 2 Mechanism of Calcium-Induced Firmness in Mushrooms (*Agaricus bisporus*)

2.1 Abstract

Calcium treatment has been widely applied in the food industry to improve the texture of fruits and vegetables. This practice is based on the well established pectin-calcium cross-linking mechanism. It has also been used to improve the texture of mushroom products in which no pectic material exists. The objective of this study was to investigate the mechanism of texture improvement in mushrooms by calcium treatments through an *in vitro* calcium binding.

Six fractions (MFs), as cytoplasmic proteins (F1), cell membrane proteins (F2), alkali-soluble cell wall glucans (F3a), cell wall proteins (F3b), cell wall β-glucans (F4a), and chitin (F4b) were extracted by a stepwise procedure from *Agaricus bisporus* cap tissue and analyzed for total carbohydrates, proteins, chitinous material, soluble phenolics, and pectic substances. The determined dominant compounds in each fraction were as follows: proteins in MF1, proteins and neutral polysaccharides in MF2, neutral polysaccharides in MF3a, neutral polysaccharides in MF3b, neutral polysaccharides and chitinous material in MF4a, neutral polysaccharides and chitinous material in MF4b. *In vitro* binding of calcium ($CaCl_2$) to fraction solids was performed in 10 mM Tris-HCl buffer (pH 7.0). The concentration of free Ca^{2+} in the binding solution was monitored with an Ion Selective Electrode (ISE) and total calcium bound by fractions was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ISE measurements showed the binding of calcium by solids in each fraction solid reached plateau within 30 mins and stayed at that level during the following 20 hrs. Both ISE and ICP-MS measurements indicated that average binding capacity of fractions to calcium

differed significantly and were in the order of MF1>MF2>MF3a ≥ MF3b≥MF4b~MF4a. The results revealed that calcium was bound by all fractions, but cytoplasmic and cell membrane proteins possessed higher capacities, indicating that interactions between calcium and protein molecules might result in an improved texture in calcium-treated mushrooms.

2.2 Introduction

Mushrooms are important agricultural products world-wide with numerous valuable nutritional and medical compounds (Chang and Miles 1999). In the United States, the white button mushroom (*A. bisporus*) is the most popular with total consumption of 827 million pounds in 2006-07 (USDA, National Agricultural Statistics Service, 2006-2007). However, the production and consumption of mushrooms have been limited by their short shelf-life and low post-harvest quality, which are primarily caused by a number of deteriorative events, generally referred to as senescence and characterized by discoloration, tissue softening, loss of flavor, increase in toughness, and advancing maturing (Burton and others 1995). Among them, discoloration and tissue softening are the most detrimental damage to both fresh and processed mushrooms (De la Plaza and others 1995).

Gene differential expression, changes of enzyme activity and cellular morphology have been examined also they relate to senescence processes (Burton and others 1995; Screenivasaprasad and Burton 1995; Zivanovic and others 2000). It is believed that tissue discoloration is caused by increased activity of polyphenol oxidase (PPOs: laccases and tyrosinases), especially tyrosinases, or increased exposure of enzymes to phenolic

substrates resulting from the damage of membrane structure (Smith and others 1993; Jolivet and others 1998). Compared to discoloration, insufficient knowledge has been accumulated regarding the internal/ external factors that influence texture and how these factors can be controlled. It was determined that tissue stiffness rapidly decreased during post-harvest storage at 5 or 18 °C (McGarry and Burton 1994). Changes of tissue morphology, cell collapse and autolysis led to the formation of large void spaces between cells and loss of cellular content, resulting in degradation of texture (Evered and Burton 1995). Additionally, mushroom softness was positively associated with protein and polysaccharide degradation, and tissue toughness was related to chitin synthesis after harvest (Zivanovic and others 2000). Although protein degradation occurred in cell membranes, the ratio of sterol to phospholipids and fluidity of liposomes were unaffected by the storage; and therefore, composition of membrane lipids may be independent of senescence (Braaksma and others 1994). In contrast, degradation of both proteins and lipids in cell membranes was observed in plant senescence (Poovaiah and others 1988).

Many studies have demonstrated that quality attributes of mushrooms can be ameliorated by calcium salt treatments. Addition of calcium salt to the casing layer or irrigation water can significantly improve the post-harvest quality and processing characteristics of mushrooms, especially their color and texture (Okereke and others 1987; Beelman and others 1987(b); Simons and Beelman 1995; Kukura and others 1998; Philippoussis and others 2001). However, there is no correlation between calcium content and initial firmness at harvest (Beelman and others 1987(b); Kukura and others 1998). Mushroom softening and cap opening can also be retarded by washing mushrooms with 0.1% calcium chloride (Beelman and others 1987 (a)). Canned mushrooms become

firmer and tougher with addition of 5 and 25 mM calcium chloride in the brine (Zivanovic and Buescher 2004). Contradictory effects of calcium on mushroom yield have been reported. Beelman et al showed that yield was negatively influenced by calcium chloride treatment during growth, while solid content significantly increased (Beelman and others 1987(b)). However, another study indicates that the yield and average weight were not significantly affected by addition of calcium chloride (Diamantopoulou and Philippoussis 2001). Moreover, it has been shown that irrigation with 0.3% calcium chloride solution had no impact on proteins and total nitrogen content but increased calcium content of fresh mushrooms (Simons and Beelman 1995; Miklus and Beelman 1996). Nevertheless, the effects of calcium treatments on mushroom texture improvement have been well documented (Guthrie 1984; Beelman and others 1987(b); Philippoussis and others 2001).

Similar to plants, calcium generally functions in promoting spore germination, sexual reproduction, nutrient uptake and biopolymer synthetase activity, simulating tip and polarization growth and fruiting, protecting from microbial contamination and damaging, and maintaining wall rigidification, tissue color and texture (Pitt and Ugalde 1984; Ruiz-Herrera 1992; Chiu and others 1998). It is located within nearly all cell organelles of fungi (Morales and Ruiz-Herrera 1989). Kukura and others indicated that reduction of browning by calcium treatments was caused by the increased stability of vacuole membranes in preventing tyrosinase from mixing with its phenolic substrates which is normally isolated in the vacuoles (Kukura and others 1998). This is probably a result of calcium interacting with negatively charged phospholipids and/ or proteins in the cell membrane matrix. Studies have documented a calcium-induced shrinkage of

membrane surface, decrease of fluidity and permeability, delayed galactolipid degradation (Poovaiah and others 1988; Picchioni and others 1995).

The softening of plant tissue is believed to relate to depolymerization and solubilization of pectic substances in the cell wall and middle lamella (Waldron and others 1997). Calcium is well known to significantly improve firmness in fruits and vegetables by cross-linking pectic substances through an "egg-box" model, consequently reducing solubility of pectic substances in the cell wall (Poovaiah and others 1988). However, mushroom cell walls are mainly composed of neutral polysaccharides, chitin and proteins with minor amount of chitosan, galactosamine polymers, melanin and lipids in which no pectin exists (Peberdy 1990). Therefore, the role of calcium in mushroom firmness is possibly due to its interaction with proteins and/ or polysaccharides other than pectin that consequently reduces solubility of cell macromolecules and elevates firmness (Beelman and others 1987 (a); Zivanovic and Buescher 2004). The objective of this study was to investigate the mechanism of texture improvement in mushrooms with calcium treatments. Fractions representing different cellular parts in cap tissue were extracted by a stepwise approach, and calcium-binding capacity of each fraction was determined *in vitro*. This data may help in the optimization of calcium applications to improve mushroom quality.

2.3 Experimental Details

2.3.1 Source of mushrooms

Whole fresh white common mushrooms were grown at the Mushroom Test Demonstration Facility at the Pennsylvania State University Campus (University Park,

PA). The mushrooms were harvested at "close cap" stage and shipped in cool state to the experimental laboratory. Cap tissue, free of gills and skins, was immediately cut into cubes upon arrival and kept in the freezer at -40 °C until extraction and lyophilization. The dry weight of freshly cut cubes was determined gravimetrically with triplication.

2.3.2 Stepwise extraction of mushroom cap tissue

Six fractions were extracted with a stepwise procedure as shown in **Figure 2.1**. Semi-thawed cap tissue was first homogenized in 40 mM Tris-HCl buffer (pH 7.0, 1:1 w/v) with a Waring commercial blender (Dynamics Corp., New Hartfor, CT) at high level for 5 mins and centrifuged at 1000 g, 4 °C for 30 mins (Sorval RC 5B Plus, Kendro Inc. Newtown, CT). The pellet was re-extracted twice with the same buffer and all the supernatants were combined, precipitated with ethanol (final concentration of 80%) at 4 °C overnight, and centrifuged at 13000g, 4 °C for 20 mins. Fraction precipitate (**F1**) was extensively washed with acetone and vacuum dried at room temperature.

The pellet remained after F1 extraction was re-suspended in 1.0 M NaCl containing 0.1% TritonX-100 (1:2 w/v) and sonicated at power 3 for 3 mins in an ice bath (1s on/1s off, Sonicator 3000, Misonix, NY). The sonicated sample was homogenized by a polytron (Polytron PT 10/35 Brinkman Instruments, Inc. Westbury, NY) at power level 8 for 3 mins, stirred at 4 °C for 1 hr at power level 8 (Model PC-420, Corning Inc. Lowell, MA), and centrifuged at 2000 g, 4 °C for 30 mins. The residual pellet was re-extracted twice with the same buffer, and all supernatants were combined and precipitated with ethanol. Fraction precipitate (**F2**) was obtained by the same procedure as F1.

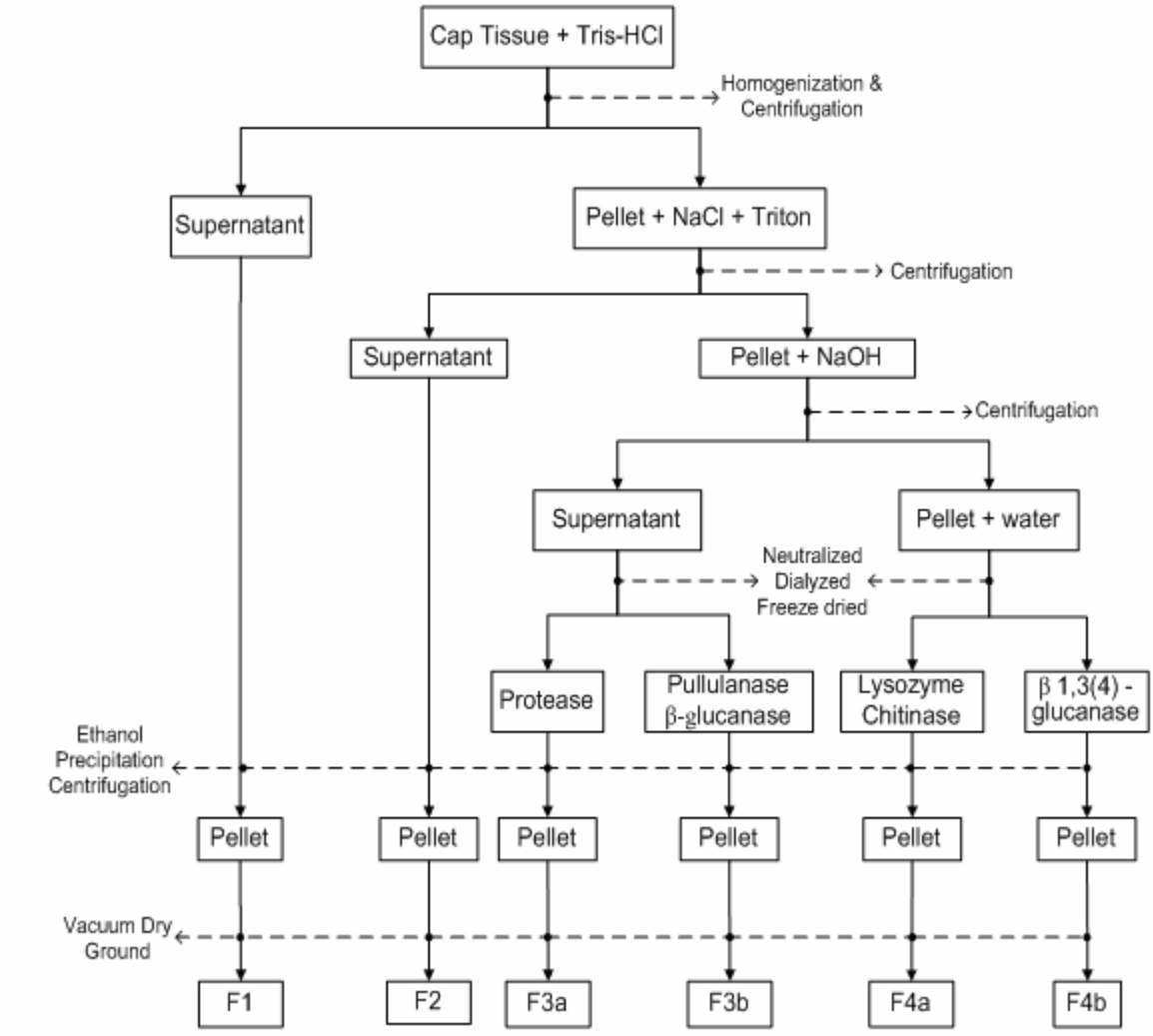

Figure 2.1 - Flow chart of fractions stepwise extraction from *A. bisporus* cap tissues**.**

The residual pellet after F2 extraction was re-suspended in 1.0 M NaOH (1:5 w/v), homogenized by Polytron at power level 8 for 3 mins, and shaken in a reciprocal shaking water bath (Precision Scientific, Cambridge, MA) at 120 rpm, 60 °C for 1 hr. After centrifugation at 2,000 g, 4 °C for 30 mins, the pellet was twice washed with d.i. water (1:1 w/v), and all supernatants were combined. Combined supernatants and residual pellets re-suspended in d.i. water were both neutralized and dialyzed through molecular porous membrane tubing (cutoff 6,000-8,000) against d.i. water at 4 °C for 24 hrs. Dialyzed samples were lyophilized and divided into two equal parts.

Two parts from the supernatant were separately re-suspended in 20 mM acetate buffer (pH 5.0 1:25 w/v). One part was digested by fungal protease from *Aspergillus oryzae* (200 U per gram freeze-dried sample, Sigma, St. Louis, MO), and the other part was digested by pullulanase from *Bacillus acidopullulyticus* (270 U per gram freeze-dried sample, Sigma, St. Louis, MO) and β-glucanase from *Aspergillus niger* (45 U per gram freeze-dried sample, Sigma, St. Louis, MO). Digested samples were precipitated by ethanol, and fraction precipitates (**F3a and F3b**) were obtained by the same procedure described earlier.

The pellet was divided into two portions, and portions were separately re-suspended in 20 mM Tris-HCl buffer (pH 7.2, 1:40 w/v. One part was digested by lysozyme from chicken egg white (5% in freeze-dried sample, Sigma, St. Louis, MO) and chitinase from *Streptomyces griseus* (8 U per gram freeze-dried sample, Sigma, St. Louis, MO). Another part was re-suspended in 20 mM acetate buffer (pH 5.0, 1:40 w/v) and digested by endo-β-1, 3 (4)-glucanse from *Penicillium* (6 U per gram freeze dried sample,

Sigma, St. Louis, MO). Digested samples were precipitated, and fraction precipitates (**F4a and F4b**) were obtained by the previously described procedure.

2.3.3 Analysis of fractions

Total polysaccharides were determined by Anthrone reagent (Fairbairn 1953) after 5 mg fraction solids were suspended in 0.5 mL d.i. water and sonicated for 10 mins, using glucose as standards. Proteins were determined with Bradford reagent following the product protocol (Sigma, St. Louis, MO). The 6 mg fraction solids were soaked with 2 mL 1 M NaOH and shaken in water bath at 60 °C for 30 mins; bovine serum albumin (BSA) was used as standard. The chitinous material in 6 mg fractions was released with 6 M HCl in a vacuum hydrolysis tube at 110 °C for 3 hrs and determined by 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) colorimetric assay with glucosamine chloride as standard (Wu and others 2004). Total soluble phenolic compounds in 10 mg fractions were extracted by 2 mL 80% methanol at 80 °C for 2.5 hrs and determined with Folin-Ciocalteau reagent (Sigma, St. Louis, MO) using gallic acid as standard (Singleton and Rossi 1965). A 7.5 mg sample was digested with concentrated H_2SO_4, and pectic substances were determined with m-hydroxydiphenyl using galacturonic acid as standard (Kintner and Buren 1982).

2.3.4 Calcium binding experiment

Lyophilized cap tissue was ground with a Thomas Wiley mill (Thomas Co., Philadelphia, PA), and the dried fraction powders were ground with a tissue grinder (Kontes Duall ®, Kontes Glass Co. Vineland, NJ). Finely ground samples, passed through

a 250µm sieve, were used for *in vitro* binding. Calcium chloride (0.1 M standard solution, Fisher Scientific Inc. Atlanta, GA) was used as calcium source.

To monitor kinetics of calcium binding to mushroom tissue powder, powder (0.1–1g) was thoroughly mixed with 50 mL 10 mM Tris-HCl buffer (pH 7.0) and a predetermined amount of 0.1 M calcium chloride (0.5 - 2.5 mL) was added and shaken on a Labquake shaker (Barnstead/Thermolyne, Dubuque, Iowa) at 23 or 35 °C. The same tissue powder suspensions (without adding calcium chloride) were used as blanks. At a designated time, 4 mL slurry was taken out and filtered with Whatman No. 4 filter paper. The 2.5 mL supernatant was diluted with HPLC water up to 25 mL, and the concentration of free ionic calcium in supernatant was determined with calcium ISE (Accumet, model 13-620-536, Hudson, MA). The electrode was connected with an Excel XL 50 meter (Accumet, Hudson, MA). To further determine bound calcium by tissue powder, a similar set of tissue powder suspensions was prepared as before. The 15 mL slurry was filtered, and solids were washed with HPLC water three time, and freeze dried after binding for 0.5, 5, and 20 hrs, respectively. Approximately 25 mg powders were digested with 5 mL concentrated HNO_3 (ACS reagent, Sigma, St. Louis, MO) by a Microwave System (Advanced Microwave Labstation, Milestone. Inc., Monroe, CT) and diluted with acid solution (2% HNO_3 + 0.5% HCl) before ICP-MS analysis. Bound calcium was then determined by ICP-MS (Agilent 7500 series, Agilent Technologies, Santa Clara, CA).

For fraction binding experiments, 80 mg fraction powder was mixed with 20 mL 10 mM Tris-HCl buffer (pH 7.0) and shaken on a Labquake shaker for 1 hr at room temperature. 400 µL 0.1 M calcium chloride was subsequently added and continuously shaken at room temperature. The same fraction powder suspensions in buffer without

addition of calcium chloride were prepared for blanks. Total free ionic calcium in solutions was directly monitored with calcium ISE at predetermined times by immersing an electrode in the binding solution. After 20 hrs, slurry was precipitated in 80% ethanol and centrifuged at 13,000 g for 20 mins. The pellet was washed with 80% ethanol 3 times and freeze dried. The total bound calcium in the pellet was determined by ICP-MS.

2.3.5 Calculation and statistical analysis

The calcium bound by sample solids was calculated by equations (1), (2) in ISE and (3) in ICP-MS.

$$\text{Decrease of free}[Ca^{2+}] = 1 - \frac{\text{free}[Ca^{2+}]\text{ in binding solution - free}[Ca^{2+}]\text{ in control}}{\text{initial exogenous}[Ca^{2+}]\text{ in binding solution}} \quad (1)$$

$$\text{Bound calcium (mg Ca/g sample)} = \frac{\text{initial exogenous Ca mass} \times \text{decrease of free } Ca^{2+}}{\text{sample mass}} \quad (2)$$

$$\text{Bound calcium (mg Ca/g sample)} = [Ca^{2+}]\text{ in sample - }[Ca^{2+}]\text{ in control} \quad (3)$$

Results were analyzed using SAS program 9.13 (SAS institute Inc, 2003). Experiments were repeated twice and significant differences among treatments was determined by Duncan's multiple range test ($P<0.05$).

2.4 Results and Discussion

2.4.1 Extraction yield of fractions

As shown in **Table 2.1**, the total mass of extracted fractions was 37.37% of cap tissue on dry weight (DW) basis, 2.33% on fresh weight. This is slightly lower than

Table 2.1 - Characteristic and composition of fractions from *A. bisporus* cap tissue

Fraction	Color	Yield mg/g DW tissue	Chemical compositions									
			% DW fraction					mg/g DW tissue				
			PR	PO	CH	TSP	PS	PR	PO	CH	TSP	PS
MF1	light grey	99.05±4.85	39.63±3.82	7.54±1.61	3.35±0.35	0.33±0.11	2.14±0.14	39.15±2.61	7.50±1.80	3.33±0.44	0.33±0.10	2.12±0.05
MF2	black	11.16±1.77	15.87±1.85	14.36±2.13	3.81±0.91	0.44±0.12	N/A	1.76±0.22	1.58±0.21	0.43±0.15	0.05±0.02	N/A
MF3a	light yellow	25.17±1.52	1.25±0.17	44.10±4.72	6.11±0.62	0.61±0.20	N/A	0.32±0.06	11.08±1.08	1.54±0.17	0.16±0.06	N/A
MF3b	light yellow	24.45±0.10	1.98±0.28	40.86±1.03	6.46±0.44	0.53±0.20	N/A	0.48±0.07	9.99±0.25	1.58±0.10	0.13±0.05	N/A
MF4a	light yellow	92.00±9.59	1.50±0.16	40.46±4.71	25.82±3.11	0.31±0.17	9.45±0.12	1.27±0.12	34.37±5.06	21.80±1.98	0.26±0.15	8.69±0.01
MF4b	ivory	121.89±6.11	0.68±0.12	33.59±1.45	35.31±3.34	0.22±0.10	N/A	0.80±0.23	38.79±6.57	40.81±7.98	0.26±0.14	N/A
Total		373.74±9.71						43.78±2.57	103.32±9.81	69.49±6.99	1.18±0.45	
% total yield								11.72±0.85	27.65±2.93	18.60±2.08	0.32±0.12	

Values are average ± standard deviation (n=3); DW represents dry cap tissue, DW factor was 6.25±0.33 % fresh weight of cap tissue; PR – Protein; PO – Neutral polysaccharides; CH - Chitinous material; TSP – Total soluble phenolic compounds; PS – Pectic substances; N/A represents samples were not determined.

3.01%, reported by Zivanovic et al. (Zivanovic and Buescher 2004) and may be due to the variation of mushroom source and/ or difference in extraction procedures. In the eport, the authors blended mushroom tissue with alcohol and the pellet was collected as AIS. The extraction yields of each fraction ranged from 11.16 to 121.89 mg/g DW cap tissue and were in the order of MF4b>MF1>MF4a>MF3a>MF3b>MF2. Total extracts as the alcohol insoluble substance (AIS) in cap tissue actually consisted of 26.50% cytoplasmic content (MF1), 2.99% cell membrane content (MF2), and 70.51% cell wall compounds (CW) (MF3a+ MF3b+ MF4a + MF4b).

2.4.2 Chemical composition of fractions

According to the experimental design, expected prevailing compositions in each extracted fraction would be cytoplasmic proteins in MF1, membrane proteins in MF2, alkali-soluble cell wall glucans in MF3a, cell wall proteins in MF3b, cell wall β-glucans in MF4a, and chitinous material in MF4b respectively. The actual prevailing composition from chemical composition analysis were as follows: proteins (39.63%) in MF1, proteins (15.87%) and neutral polysaccharides (14.36%) in MF2, neutral polysaccharides (44.10%) in MF3a, neutral polysaccharides (40.86%) in MF3b, neutral polysaccharides (40.46%) and chitin (25.82%) in MF4a, neutral polysaccharides (33.59%) and chitin (35.31%) in MF4b (**Table 2.1**). The results showed that the extraction procedure was quite effective for MF1 and MF3a in which the analysis results matched well with the expected values. However, in the remaining fractions, the prevailing components did not match exactly with the expectation, e.g., in membrane protein MF2, almost equal amount of proteins and neutral polysaccharides were obtained. It may be caused by the fact that

membrane proteins are mostly present in the form of glycoproteins in mushroom cells (Farkas 1985; Ruiz-Herrera 1992). In the case of MF3b fraction, supposedly cell wall proteins, large amount of neutral polysaccharides were obtained, that may be due to the fact the level of proteins in mushroom cell walls is low and these proteins are tightly bound to cell wall glucan (Ruiz-Herrera 1992). The pullulanase and β-glucanase used in the extraction were not effective in hydrolysis of polysaccharides and resulted in essentially the same composition of fractions MF3a and MF3b (**Table 2.1**). High levels of chitin in MF4a and equal amounts of neutral polysaccharides and chitin in MF4b suggested incomplete enzymatic hydrolysis in the fractions even after increased enzyme amount and extended hydrolysis time were applied in the experiments. This may be due to the fact that glucan and chitin are covalently linked in the cell wall matrix which prevented the attack by enzymes (Sietsma and Wessels 1981). Since there were other undetermined contents such as lipids and minerals, 100% recovery was not obtained for all fractions.

2.4.3 Chemical composition in cap tissue

As shown in **Table 2.1**, the total AIS extracts consisted of 11.72% proteins, 27.65% neutral polysaccharides and 18.60% chitin, which was similar to previously reported values of 12.0%, 33.0% and 16.7% (Zivanovic and Buescher 2004). By calculating the composition based on 1 g DW cap tissue, it contained 43.74 mg proteins, 103.32 mg neutral polysaccharides, 69.49 mg chitinous materials and 1.18 mg total soluble phenolics, which were lower than overall chemical composition reported: 24-35% crude protein, 44-54% of nitrogen free carbohydrates, and 51-63% total carbohydrates

based on DW tissue(Chang and Miles 1999; Mattila and others 2002). This was because the extraction procedure only extracted the AIS content, in which alcohol soluble compounds were not included and thus not determined. Proteins were the primary compounds in cytoplasm (MF1) which consisted of most of the protein in AIS extracts. Proteins and neutral polysaccharides were the dominant components in the cell membrane (MF2). In cell wall (CW) extracts, proteins (PR), neutral polysaccharides (PO) and chitinous material (CH) were 1.01% (PR/CW), 35.76% (PO/CW) and 24.94% (CH/CW), respectively. These values for neutral polysaccharides and chitin content were close to 43% and 34% calculated from previously reports, but protein content was much lower than 9.9% (Hammomd 1979). The differing protein content might be caused by a difference in protein extraction and determination methods. Hammomd extracted cell wall content by collecting the residuals after homogenizing tissue with Tirs-HCl and NaCl solutions, and the proteins were determined by Lowry's method. The results also showed that the amount of the alkali-insoluble substances (MF4a +MF4b) were higher than that of alkali-soluble one (MF3a +MF3b) in cell wall. The alkali-insoluble wall material consists mainly of (1-3)-β-D/ (1-6) - β-D-glucan and chitin. The alkali-soluble wall materials consist mainly of proteins and glucans containing α-1, 3 /1,4 linkage (Farkas 1985).

2.4.4 Fraction discoloration

All freeze-dried fractions had various levels of discolorations as shown in **Table 2.1**. MF1 and MF2 were light gray and black; MF3a, MF3b and MF4a were light yellow; MF4b was ivory. The chemical composition analysis could be used to explain the fraction

discoloration. The discoloration in fresh mushroom tissue is mainly due to the oxidation of phenolic constitutes; e.g., L-tyrosine, L-3,4-dihydroxyphenylalanine (L-Dopa), glutaminyl-4-hydroxybenzene (GHB) and c-glutaminyl-3,4-dihydroxybenzene (GDHB) into quinones catalyzed by PPOs, especially tyrosinase. The quinones then polymerize into brown melanin pigments (Jolivet and others 1995). Previous reports state that PPOs are mainly bound to membranes in plants even though the location of mushroom PPOs still remains unclear (Jolivet and others 1998). Other researchers also observed the degradation of membrane proteins during mushroom senescence (Braaksma and others 1994). Loss of membrane integrity during senescence thus may result in the mixing of tyrosinases with their substrates. MF2, being the darkest fraction, contains high levels of proteins and relatively high levels of phenolic contents in which high levels of proteins may be an indication of higher PPOs content and relative high phenolics, which could promote enzymatic discoloration reaction. The MF1 fraction containing high protein content exhibited darker color than the rest of fractions dominantly containing glucans and chitin.

2.4.5 Experimental parameters affecting calcium binding to tissue powder

Pacheco *et al*. revealed that pH, ratio of Ca^{2+}-to-samples, temperature and type of binding buffers, may impact the binding capacity of samples to calcium ions (Pacheco and others 1999). Many acidic groups are un-dissociated at lower pH with fewer free binding sites available for calcium ions. Further, endogenously bound calcium may be released with pH changes (Luccia and Kunkel 2002). Approximate pH of fresh mushrooms is 6.0 - 6.7 (US FDA/CFSAN 2008: Approximate pH of foods and food

products). In this experiment, a consistent pH was achieved by using Tris-HCl buffer (pH 7.0). The influence of potential endogenous free calcium ions was subtracted using a blank binding solution without adding exogenous calcium ions.

Figure 2.2 shows the calcium binding kinetics at different Ca^{2+}-to-powder ratios based on ISE measurements. All four ratios demonstrated similar binding kinetics trends. The binding occurred immediately after adding calcium chloride. A sharp increase in binding was observed during the first 30 mins. The saturated binding level then stayed consistent during the next 5 hrs and slightly fluctuated in the following 15 hrs. The amounts of bound calcium by mushroom powder determined by ICP-MS and ISE at 0.5, 5 and 20 hrs are shown in **Figure 2.3**. The ISE measurements after 20 hrs showed that the level of bound calcium was the highest at a ratio of 1:10, but the values at this ratio had the largest standard deviations. Such significant difference was not observed in the ICP-MS data. Among these three ratios, 1:50 gave the most consistent results in both ISE and ICP-MS determinations and was selected as a standard ratio for all future experiments.

The effect of ambient temperature on binding capacity was tested at 23 and 35 °C. The ISE results indicated that binding capacity at 23 ˚C was higher that at 35 ˚C, whereas ICP-MS measurements showed no difference (**Figure 2.4)**. A previous study of calcium binding to food proteins showed that binding capacity increased only when the temperature was above 35 °C (Wallace and Sattelee 1977). The effect of calcium on retarding the loss of firmness in thermal processed mushrooms also suggested that high temperature does not hinder the binding of calcium to compounds in mushroom (Zivanovic and Buescher 2004). Based on our results, the following experiments were

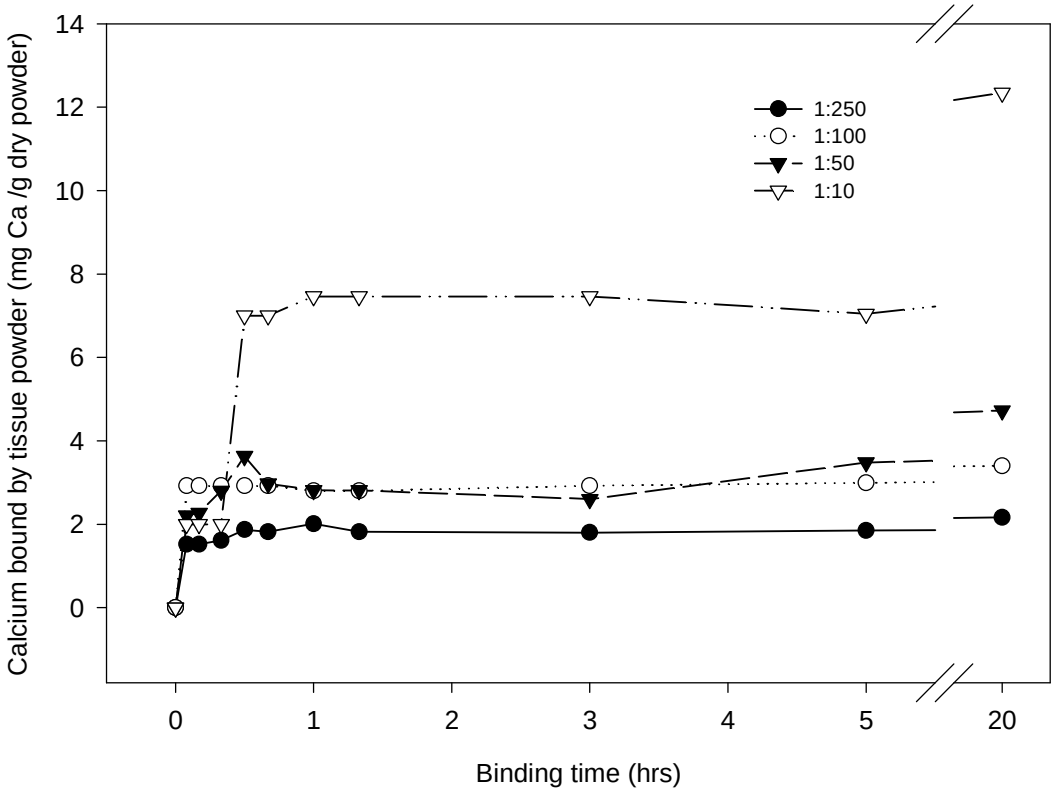

Figure 2.2 - Calcium binding kinetics to *A. bisporus* cap tissue powder with different Ca^{2+}-to-powder (w/w) ratios (by ISE). Experiments were performed in 50 mL 10 mM Tris-HCl buffer at 23 °C; 1: 250 = 1% powder + 0.004% Ca^{2+}; 1:100 = 2% powder + 0.02% Ca^{2+}; 1:50 = 1% powder + 0.02% Ca^{2+}; 1:10 = 0.2% powder + 0.02% Ca^{2+}.

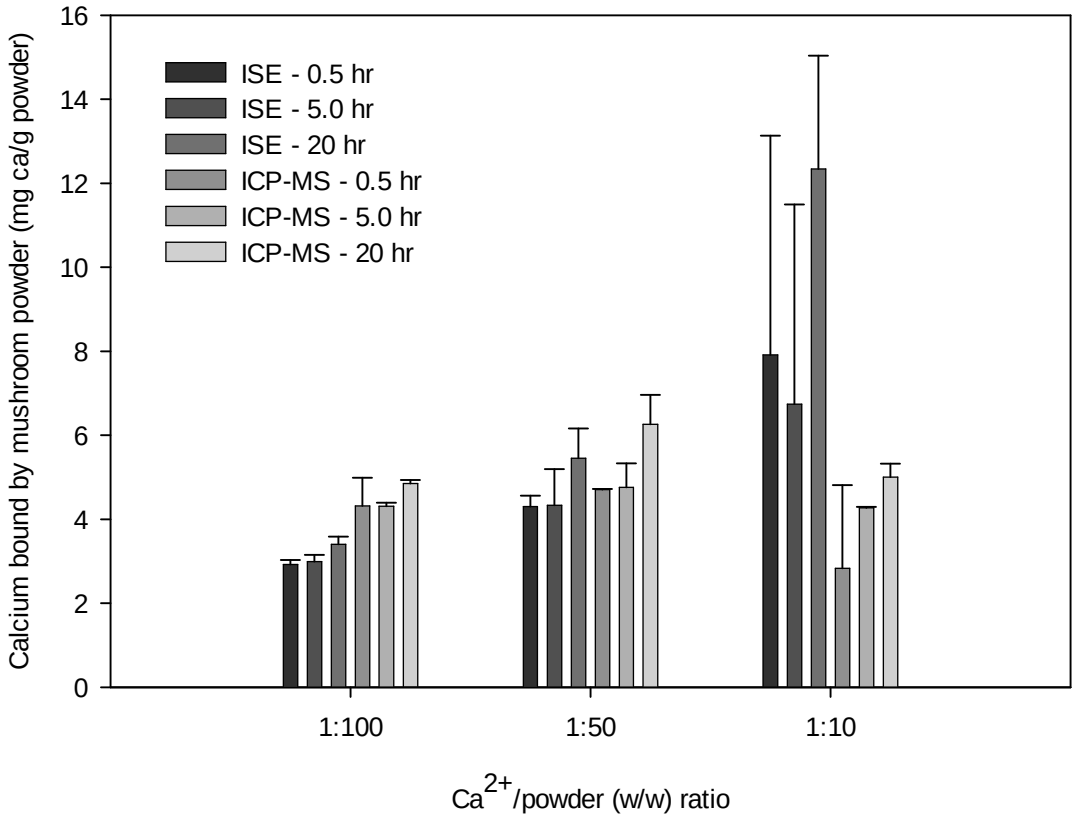

Figure 2.3 - Effects of Ca^{2+}-to-powder (w/w) ratio on calcium binding to *A. bisporus* cap tissue powder. Experiments were performed in 50 mL 10 mM Trish-HCl buffer at 23 °C; 1:100 = 2% powder + 0.02% Ca^{2+}; 1:50 = 1% powder + 0.02% Ca^{2+}; 1:10 = 0.2% powder + 0.02% Ca^{2+}; Bars represents standard deviation (n=2).

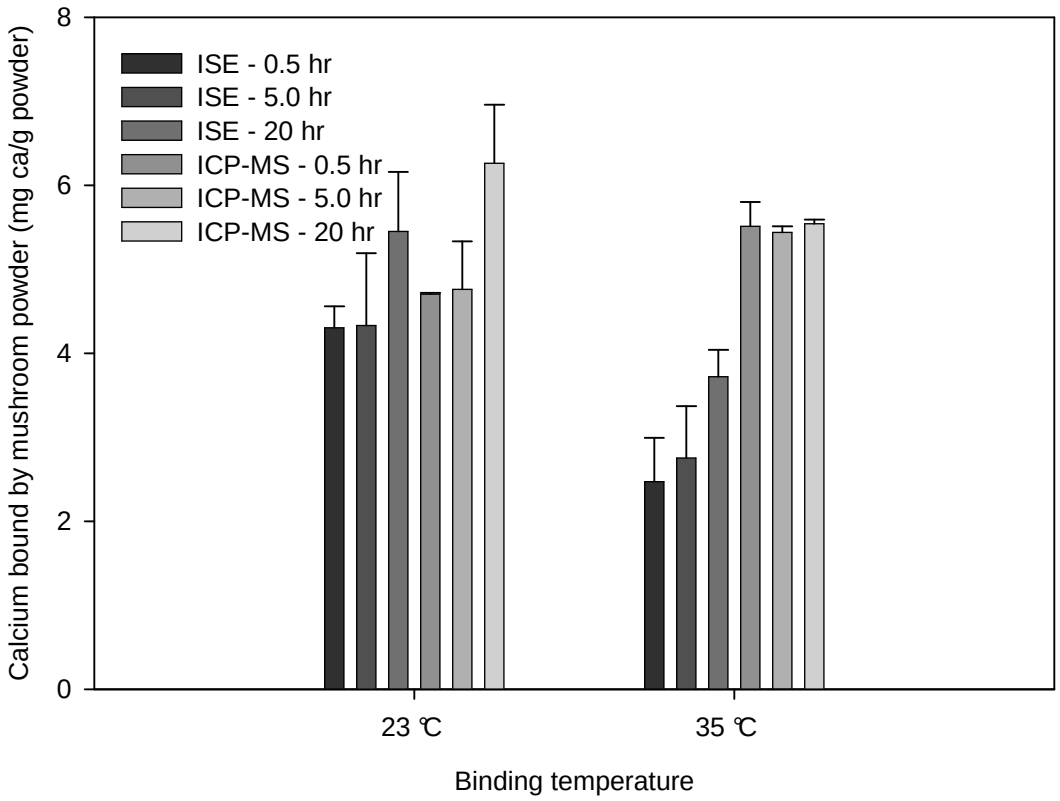

Figure 2.4 - Effect of temperature on calcium binding to *A. bisporus* cap tissue powder. Experiments were performed in 50 mL 10 mM Tish-HCl buffer with Ca^{2+}-to-powder ratio 1:50 (w/w); Bars represent standard deviation (n=2).

consequently carried out at Ca^{2+}-to-powder ratio 1: 50 during 20 hrs binding time at 23 °C.

2.4.6 Calcium binding to fractions

The binding kinetics and capacity of calcium to each fraction obtained by ISE measurements is shown in **Figure 2.5.** Six fractions showed similar binding trends. The binding occurred immediately after addition of calcium chloride to the suspension, and plateau was reached within 30 mins. After that time, the binding curves leveled off with some small fluctuations. The binding kinetics revealed that all six fractions bound calcium in a similar trend but with different binding capacity: MF1 was consistently the highest, followed by MF2, and MF4a was the lowest. Binding curves of MF3a, MF3b and MF4b overlapped occasionally and did not show significant difference. Comparisons at 20 hrs binding indicated that the binding capacity differed significantly in the order of MF1>MF2>MF3a ≥MF3b≥MF4b>MF4a and values ranged from 3.09 to 7.70 mg Ca/g DW fraction solids.

Data for endogenous calcium before the binding experiment, calcium level in blank control, and total calcium after binding determined by ICP-MS procedure are presented in **Table 2.2**. Total amount of calcium in mushroom tissue was consistent with reported results (Hartman and others 2000). The blank binding procedure caused a slight release of endogenous calcium in fractions F4a and F4b and a slight increase in tissue powder. Therefore, the ethanol precipitation step actually concentrated the calcium in tissue powder because macromolecules with bound calcium were collected and determined, whereas small molecules were lost, which also suggested that the most of

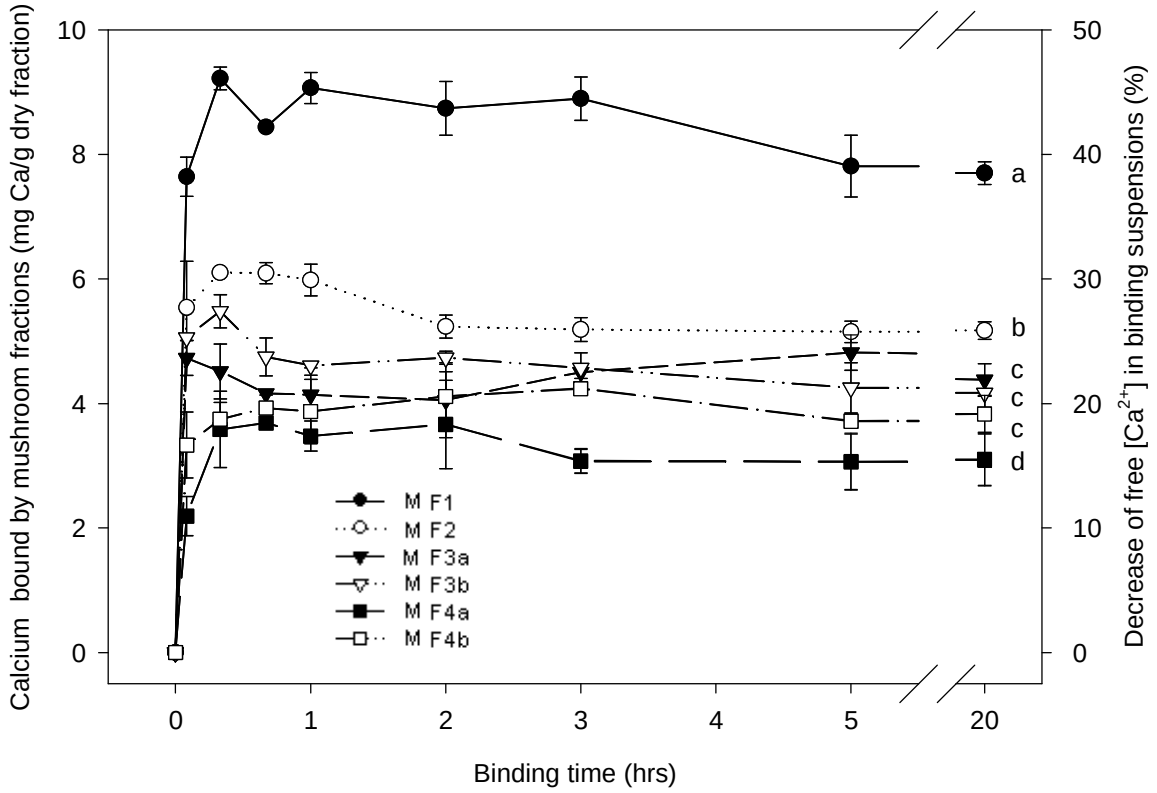

Figure 2.5 - Calcium binding kinetics to fractions from *A. bisporus* cap tissue (by ISE). Experiments were performed in 20 mL 10 mM Tris-HCl buffer at 23°C with Ca^{2+}-to-powder ratio (w/w) 1:50; Bars represent standard deviation (n=2); Values at binding time of 20 hrs with different letters were significant different (Duncan's comparison $P<0.05$).

Table 2.2 - Calcium in *A. bisporus* cap tissue powder and fractions (by ICP-MS).

Source	Calcium (mg/g DW sample)		
	Before binding	Blank	Calcium binding
Tissue powder	0.02	0.23±0.05	6.49±0.66
MF1	0.84	1.00±0.14	20.17±6.77
MF2	N/A	2.00±0.01	18.99±10.77
MF3a	N/A	0.43±0.04	5.76±0.14
MF3b	N/A	1.02±0.02	5.62±0.60
MF4a	0.72	0.17±0.01	2.59±0.00
MF4b	0.24	0.10±0.00	2.71±0.22

Values are average ±standard deviation (n=2) except values of before binding; N/A represents the samples were not tested

calcium was present as bound form to macromolecules. However, in the case of fractions which are already macromolecules with bound calcium, the blank binding reduced calcium level by washing it off over 20 hrs during the experiment. Nevertheless, total exogenous calcium was much higher than the original concentration in all samples. The amounts of exogenous calcium bound by fractions (**Figure 2.6)** were thus obtained by subtracting endogenous calcium in the blank from total calcium in the fraction. MF1 showed highest binding capacity with a value of 19.17 mg Ca/g fraction solids. MF2 was next to MF1 with value of 16.94 mg/g. MF4a showed lowest calcium binding capacity (2.42 mg/g). Calcium bound by fractions from both ISE and ICP-MS measurements are shown in **Figure 2.6** to compare two measurement methods. As it can be seen, the two methods gave similar binding capacity trends among six fractions. However, the ICP-MS measurements gave higher binding capacity values than that of ISE measurements. This might be caused by the interference of particles and some chemical compounds in ISE measurements. Free ionic calcium adsorbed at the surface of the particles during collection of the binding slurry might also result in higher calcium content in ICP-MS measurements.

In plants, pectic substances are the primary macromolecules that bind calcium that retards tissue softening. Previous reports have demonstrated that there are no pectic substances in mushroom tissue (Ainsworth and Sussman 1965). The MF1, showing highest calcium-binding capacity, and MF4a, showing lowest calcium-binding capacity, were analyzed for pectic substances. The results showed that very little amounts of pectic substances existed in each fraction, which could be the result of interference from other compounds, e.g., chitin. Around 1.5% of pectin was detected during calorimetric

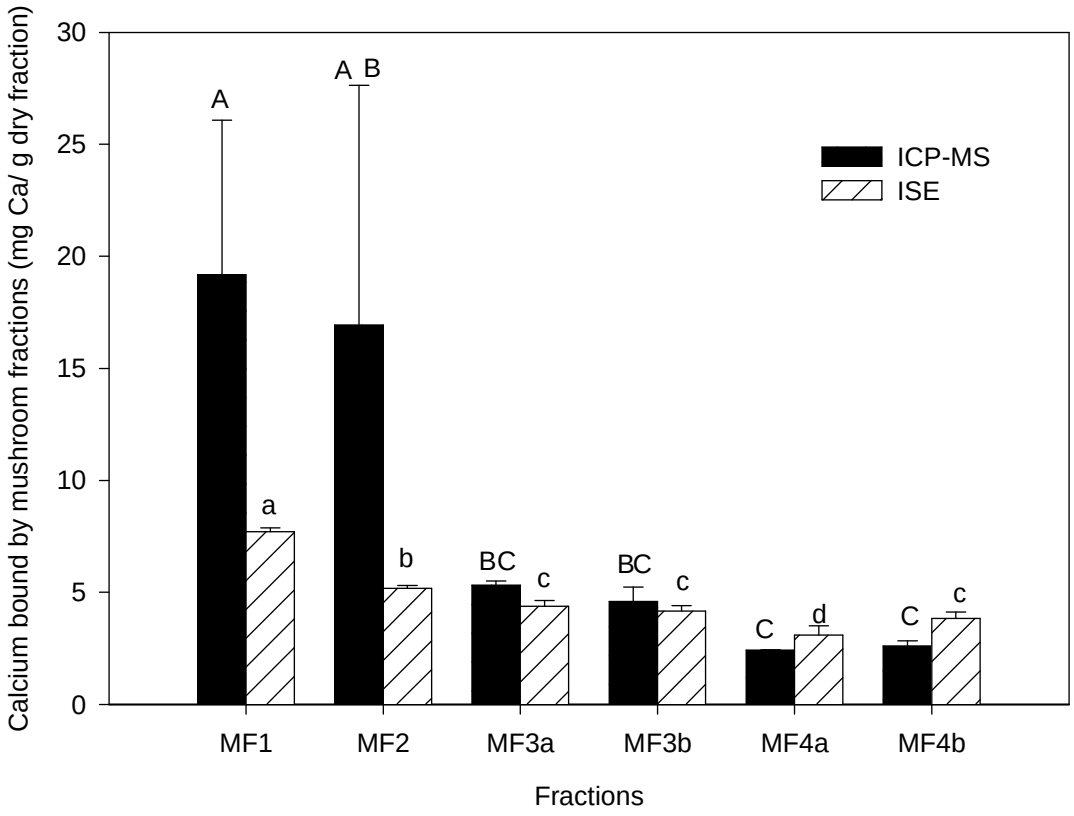

Figure 2.6 - Calcium bound to fractions from *A. bisporus* cap tissue. Values were obtained after binding for 20 hrs. Experiments were performed in 20 mL 10 mM Tris-HCl buffer with Ca^{2+} -to -fraction 1:50 (w/w) at 23°C; Bars represent standard deviation (n=2); Values with different letters within same type of measurement were significant different (Duncan's comparison $P<0.05$).

determination of pectic substances while using chitin as materials. Based on the chemical composition, MF1 and MF2 fractions, in which cytoplamic proteins and membrane proteins were dominant, respectively, showed significantly higher binding capacity to calcium than the remaining fractions. Protein generally can interact with oppositely charged ions through amino acid residues (Pacheco and others 1999). According to amino acid composition, negatively charged amino acids (aspartic acid and glutamic acid) were 29% of overall amino acids in mushroom tissue, and positively charged amino acid . (histidine, lysine, and arginine) only 13% (Mattila and others 2002). This may address the higher calcium binding potential of MF1 and MF2 in our study. A previous study suggested that the occurrence of cross-linking of proteins through Ca^{2+} bridges resulted in reduced total soluble content in mushroom cells, although protein contents was not affected by irrigation of calcium chloride (Simons and Beelman 1995). Wong and Cheung also showed that a high protein level may be the main factor for high calcium binding capacity to mushroom fibers (Wong and Cheung 2005). In addition to MF1 and MF2, the binding experiments also indicated that the rest of the four fractions, mainly consisting of glucans and chitin also showed calcium-binding capacity. Simple physical adsorption could be the case. Thus, previous studies regarding calcium binding to fibers have concluded that neutral compounds such as cellulose, methyl-cellulose hemicellulose and psyllium could bind calcium ions and this binding was simply a result of an adsorption phenomenon (Torre and others 1992; Luccia and Kunkel 2002). However, other compounds such as chitosan and lipids in the fractions may bind calcium to some extent. It has been reported that chitosan can interact with certain metal ions including calcium(Vold and others 2003).Thus, free aminogroups of deacylated units of

chitin are most probable sites for calcium binding in mushroom cell walls. The binding of calcium to compounds in mushrooms is complicated and remain unclear. Calcium may bind to calcium-binding proteins, negative residues of polysaccharides or proteins. Binding may take place by ion exchange, chelation, and adsorption (Vold and others 2003; Wong and Cheung 2005). However, considering the texture improvement, the most possible mechanisms would be calcium-induced cross-linking of polymers or activity alteration of softening related enzymes. Present studies have identified proteins as the most potential macromolecules involved in calcium binding and determined their location in mushroom cells. Possible polymer-calcium complexes need to be characterized to further confirm the potential function of calcium in retarding mushroom softening. The additional study on calcium-binding to BSA was performed by calorimetric analysis to determine binding energy between protein and calcium (**Chapter 4**).

2.5 Conclusion

To understand the mechanism of calcium-induced firmness in mushrooms, calcium binding was investigated *in vitro* with mushroom tissue powder and cell fractions. The dominant compounds in the extracted fractions were as follows: proteins in MF1, proteins and neutral polysaccharides in MF2, neutral polysaccharides in MF3a, neutral polysaccharides in MF3b, neutral polysaccharides and chitin in MF4a, neutral polysaccharides and chitin in MF4b. Calcium binding capacity of the fraction solids was saturated within 30 mins. It was temperature independent but was affected by the Ca^{2+}-to-solids ratio. Binding capacity of calcium to fractions varied significantly in the order of MF1>MF2>MF3a ≥ MF3b≥MF4b~MF4a. The results revealed that calcium was bound

by all fractions, but those dominantly composed of cytoplasmic and cell membrane proteins showed the highest capacity, which provided strong evidence that calcium possibly interacts with proteins in mushrooms to improve their firmness. This data may help in optimizing calcium applications in improvements of mushroom quality.

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Chapter 3 Interaction of Calcium with Apple and Cucumber Macromolecular Extracts

3.1 Abstract

Calcium can effectively enhance the firmness of fruits and vegetables by cross-linking pectin through the “egg-box” model. However, new studies suggest that pectin cross-linking may be insufficient to explain the texture improvement after calcium treatment. This study was conducted to investigate interactions of calcium with macromolecular extracts from apple and cucumber tissue in order to improve the understanding of calcium-induced firmness in fruits and vegetables.

Five macromolecular fractions: cytoplasmic protein fraction 1 (F1), cell membrane protein fraction 2 (F2), cell wall protein fraction 3 (F3), pectin fraction 4 (F4), and cellulose (F5) were stepwise-extracted from apple cortical (AFs) and cucumber mesocarp tissues (CFs), and analyzed for their chemical compositions. The actual dominant macromolecules in apple extracts were as follows: pectic substances in AF1, AF2 and AF4, proteins in AF3, and neutral polysaccharides in AF5. Dominant macromolecules in cucumber extracts were as follows: proteins in CF1, CF2, and CF3; pectic substances in CF4, and neutral polysaccharides in F5. *In vitro* binding of calcium ($CaCl_2$) by fraction solids was performed in 10 mM Tris-HCl buffer (pH 7.0). Bound calcium was determined by a calcium selective electrode (ISE) and an Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Calcium binding capacity of all tested fraction solids from apples reached plateau within 10 mins; AF2 and AF4 showed higher binding capacity than AF1 and AF5. For cucumber fractions, the plateau was reached in 1 hr; CF1, CF2, and CF4 showed higher binding capacity than CF3 and CF5. Therefore, calcium was bound by all tested fractions, but those fractions dominantly composed of

pectic substances, cytoplamic proteins and membrane proteins exhibited higher binding capacity. These results show that although interaction of calcium with pectic substances plays a primary role in enhancement of firmness plant tissues, interactions between calcium and cytoplasmic and membrane proteins also take place and possibly contribute to texture alteration.

3.2 Introduction

Calcium treatment has been widely practiced in the food industry to retard firmness loss in fresh and processed fruits and vegetables (Poovaiah 1986; Guillou and others 1992; Banjongsinsiri and others 2004; Alandes and others 2006; Martin-Diana and others 2007). The effects of temperature, type and concentration of calcium salt, pH and treatment methods were extensively studied and reviewed (Martin-Diana and others 2007). Considerable efforts have also been directed to elucidate the mechanisms of calcium in maintaining firmness. It is generally accepted that tissue firmness is mainly determined by the structure of the cell wall and cell middle lamella, in which pectic substances are abundant. Softening is induced primarily by degradation of pectic substances through enzymatic depolymerization, and consequently the dissolution of degraded pectic substances leads to loss of cell-to-cell adhesion and tissue coherence (Poovaiah and others 1988; Waldron and others 1997; Willats and others 2001). Pectin methylesterase (PME) and polygalacturonases (PGs) are the key enzymes involved. PME is a cell wall bound enzyme that causes pectin de-esterification, and PGs cleave α-(1-4) glycosidic bonds of pectin randomly or from the non-reducing end (Prasanna and others 2007). Degradation of other biopolymers in cell walls, such as hemicellulose and

cellulose, may be involved in excessive texture loss as well (Sanchez-Romero and others 1998; Prasanna and others 2007). Calcium ion protects plant-based food products from softening by cross-linking pectin through dissociated carboxyl groups or non-esterified galacturonic acid units, as described in the "egg-box" model. The cross-linked complex not only directly prevents the loss of cell and tissue coherence but also hinders the degradation of pectic substances by reducing accessibility by hydrolyses (Poovaiah and others 1988). Additionally, calcium may directly affect the activity of enzymes involved in pectin degradation, although contradictory results have been found in term of increasing or decreasing of enzyme activity (Poovaiah and others 1988; Pagel and Heitefuss 1990; Siddiqui and Bangerth 1995; Rico and others 2007). Recent studies showed that calcium treatment effectively improved the firmness of mushrooms which have no pectin, which suggests that other biopolymers may also interact with calcium ions and contribute to improvement of the firmness (Miklus and Beelman 1996; Zivanovic and Buescher 2004; Hepler 2005).

Calcium is important in maintaining plant cell wall structure and has pronounced effects on structure and function of cell membrane, which may be associated with firmness as well. A well preserved cell membrane structure prevents cell turgor loss and benefits firmness maintenance (Poovaiah and others 1988; Sajnin and others 2003). Calcium binds with negatively charged "head" of lipids and/or acidic amino acid residues of membrane proteins (Hepler 2005). As a result, fluidity, microviscosity, and permeability of membranes decrease with addition of calcium, and it is believed that reduced permeability was caused by calcium-induced shrinkage of the membrane surface of plant cells by interacting with membrane lipids (Poovaiah and others 1988). Retarded

softening in calcium-infiltrated apples has been observed, and this effect was related to delayed galactolipid degradation in cell membranes which could increase the sterol conjugation contributing to cell membrane integrity (Picchioni and others 1995; 1998). It is postulated that activity of cell membrane enzymes is affected either by calcium–induced alteration of membrane microenvironment or by direct interaction with calcium, which may consequently affect the texture (Kauss and others 1983; Poovaiah and others 1988). Lamikanra and Watson also found that lipase activity was inhibited by calcium addition in fresh-cut cantaloupe melon (Lamikanra and Watson 2004). In addition to the effects on the cell wall and cell membranes, calcium reduces the rate of ethylene and carbon dioxide evolution, increases ascorbic acid level, and retards the loss of chlorophyll (Glenn and others 1988).

Many studies have been carried out to investigate calcium-induced firmness in various fruit and vegetable products such as apples, strawberries, peaches, hot peppers, diced tomatoes, mushrooms (Miklus and Beelman 1996; Martin-Diana and others 2007). In order to explore the mechanism of calcium in improving firmness, researchers primarily focused on calcium content and distribution, enzyme activity, chemical composition, and microstructure of products (Glenn and Poovaiah 1990; Roy and others 1994; Chardonnet and others 2003; Banjongsinsiri and others 2004; Quiles and others 2004; Betoret and others 2005; Sila and others 2006). Little has been done to further identify macromolecules other than pectin with potential to interact with calcium to improve firmness. This is the first study conducted to *in vitro* identify these macromolecules in order to extend our understanding of effects of calcium on texture of plant-based products.

3.3 Experimental Details

3.3.1 Source of apples and cucumbers

'Golden Delicious' apples (*Malus domestica*) and cucumbers (*Cucumis sativus L*) were purchased from a local grocery store. The peel of the entire fruit was removed to a depth of around 2 mm with a peeler. The mesocorp of approximately 6 mm thickness was cut into cubes and kept in the freezer at - 40°C until extraction. The dry weight of freshly cut cubes was determined gravimetrically with triplication.

3.3.2 Stepwise extraction of apple and cucumber tissue

Five fractions were stepwise extracted following a procedure depicted in **Figure 3.1**. Semi-thawed tissue was homogenized in 40 mM Tris-HCl buffer (pH 7.0, 1:1 w/v) with a Waring commercial blender (Dynamics Corp. New Hartfor, CT) at high level for 5 mins and centrifuged at 3,000 g, 4°C for 30 mins (Sorval RC 5B Plus, Kendro Inc. Newtown, CT). The pellet was twice re-extracted with the same buffer and all supernatants were combined, precipitated with 80% ethanol at 4°C overnight and centrifuged at 13,000 g, 4°C for 20 mins. The precipitate, cytoplamic proteins fraction (**F1**) was extensively washed with 80% ethanol and freeze dried.

The pellet after F1 extraction was re-suspended in 1.0 M NaCl containing 0.1% TritonX-100 (1:2 w/v) and sonicated at power 3 for 3 mins in an ice bath (1s on/1s off, Sonicator 3000, Misonix, NY). The sonicated sample was homogenized by a polytron (Polytron PT 10/35 Brinkman Instruments Inc. Westbury, NY) at power level 8 for 3 mins, stirred at 4°C for 1 hr at power level 8 with a stir plate (Model PC-420, Corning Inc. Lowell, MA), and centrifuged at 3,000 g, 4°C for 30 mins. The residual pellet was twice re-extracted with the same buffer and all supernatants were combined and

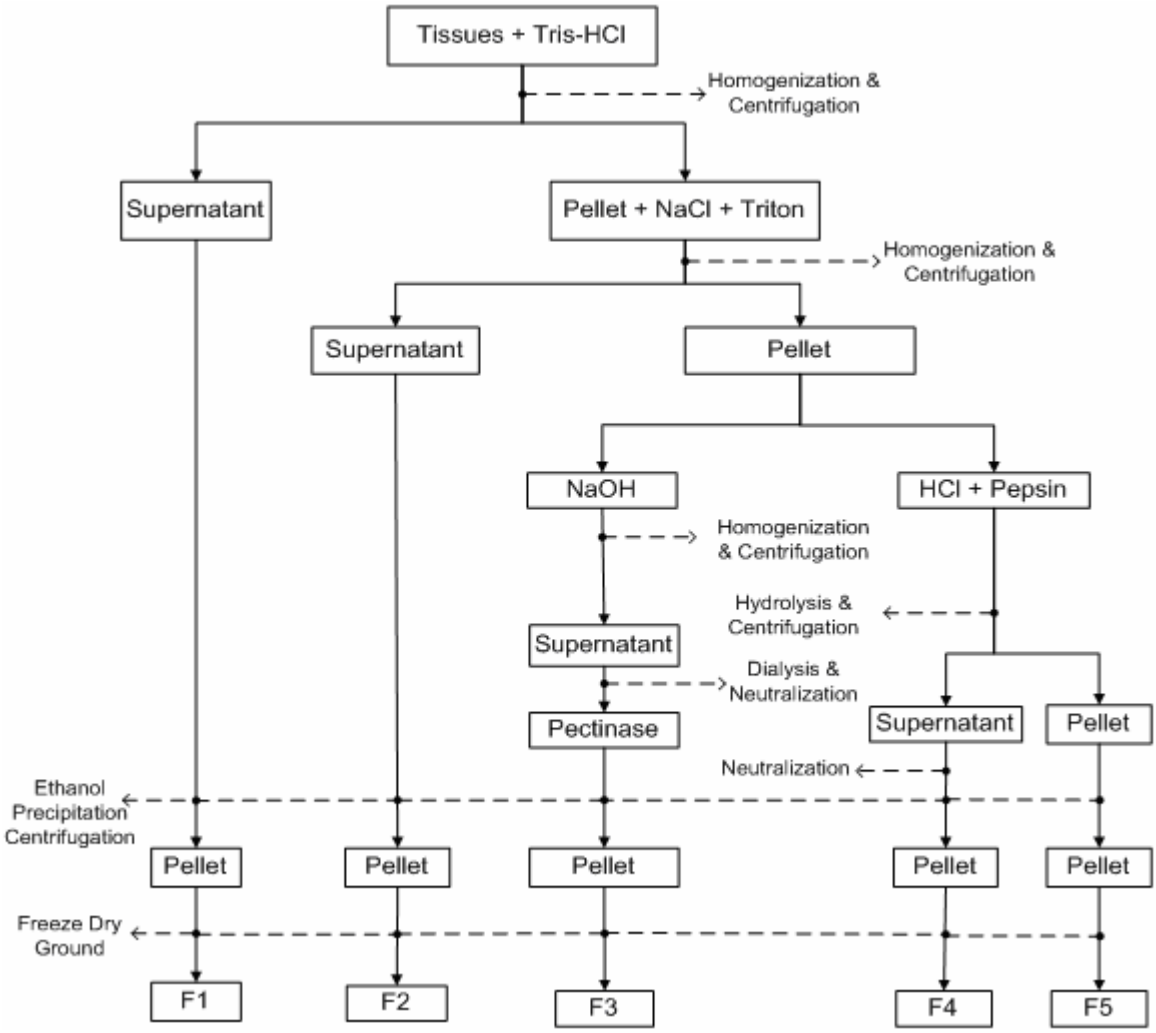

Figure 3.1 - Flow chart of fraction stepwise extraction from apple and cucumber tissue.

precipitated with 80% ethanol. The precipitate, cell membrane proteins fraction (**F2**), was obtained by the same procedure as for F1 preparation.

The remaining pellet after F2 extraction was then divided into two equal parts. One part was re-suspended in 1.0 M NaOH (1:5 w/v), homogenized by Polytron at power level 8 for 3 mins, shaken in water bath at 120 rpm, 60°C for 1hr, and centrifuged at 3000 g, 4°C for 30 mins. The residual pellet was washed with d.i. water (1:1 w/v) and centrifuged twice, and all supernatants were combined, dialyzed (molecular weight cutoff 6,000-8,000 Da) against d.i. water at 4°C for 24 hrs. The dialyzed solution was adjusted to a pH of 4.8 - 5.0 and digested by pectinase from *Aspergillus japonicus* (0.15 % pectinase in dialyzed solution, w/v, Sigma, St. Louis, MO). The digested solution was precipitated, and the precipitate, cell wall proteins fraction (**F3**), was obtained by the same procedure as for F1.

The other part of the pellet after F2 extraction was re-suspended in 10 mM HCl (1:5 w/v) and adjusted to pH 3. The solution was hydrolyzed with pepsin (0.01 mg pepsin/ml solution, Fisher Scientific Inc., Atlanta, GA), and the hydrolyzate was centrifuged at 3000 g, 4°C for 30 mins. The pellet was washed with d.i. water and centrifuged again. All supernatants were combined, neutralized with NaOH, and precipitated; and the precipitate, pectin substances fraction (**F4**), was obtained by the previously described procedure.

The residual pellet after F4 extraction was extensively washed with d.i. water, centrifuged at 3,000 g, 4°C for 20 mins and twice re-washed. The pellet was washed with 80% ethanol and twice centrifuged. The residual, cellulose fraction (**F5**), was freeze dried.

3.3.3 Analysis of fractions

Neutral polysaccharides were determined by Anthrone reagent (Fairbairn 1953) using glucose as standard. 5 mg fraction solids were suspended in 0.5 mL d.i. water, sonicated (1510R-MT, Branson, USA) for 10 mins prior 4.5 mL reagent was added. Proteins were determined with Bradford reagent following the product protocol (Sigma, St. Louis, MO). 6 mg fraction solids were extracted with 2 mL 1 M NaOH, shaken in water bath at 60°C for 30 mins, and bovine serum albumin (BSA) was used as standard. 6 mg fraction solids was hydrolyzed with 6 M HCl in vacuum hydrolysis tube at 110°C for 3 hrs and aminosugar was determined by 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) colorimetric assay using glucosamine chloride as standard (Wu and others 2004). Total soluble phenolics in 10 mg fractions were extracted by 80% methanol at 80°C for 2.5 hrs and determined with Folin-Ciocalteau reagent (Sigma, St. Louis, MO) using gallic acid as standard (Singleton and Rossi 1965). Pectic substances in 7.5 mg sample were hydrolyzed with concentrated H_2SO_4 and determined with m-hydroxydiphenyl using galacturonic acid as standard (Kintner and Van 1982).

3.3.4 Calcium binding experiment

Lyophilized apple cortical tissue and cucumber mesocarp tissue cubes were ground with a Thomas Wiley mill (Thomas Co., Philadelphia, PA) and lyophilized fractions were ground with a tissue grinder (Kontes Duall ®, Kontes Glass Co. Vineland, NJ). Finely ground samples of apple and cucumber lyophilized tissue and fractions were passed though a 250 µm sieve prior to *in vitro* binding with calcium chloride as calcium source (0.1 M standard solution, Fisher Scientific Inc., Atlanta, GA).

Calcium binding to fraction solids was determined as following: 100 mg fraction powder was mixed with 25 mL 10 mM Tris-HCl buffer (pH 7.0) and shaken on Labquake shaker (Barnstead/Thermolyne, Dubuque, Iowa) for 1 hr at room temperature. 500µL 0.1 M calcium chloride was then added and suspension was continuously shaken at room temperature for 20 hrs. Same fraction powder suspensions without addition of calcium chloride were prepared as blanks. Total free ionic calcium in solution was determined with an ionic selective electrode (ISE, model 13-620-536, Accumet, Hudson, MA) connected with Excel XL 50 meter (Accumet, Hudson, MA) at predetermined times by immersing the electrode in the binding solution. After 20 hrs, slurry was precipitated in 80% ethanol and centrifuged at 13,000 g for 20 mins. The pellet was washed three times with 80% ethanol and freeze dried. Approximately 45 mg dried powder was digested with 5 mL concentrated HNO_3 (ACS reagent, Sigma, St. Louis, MO) by a Microwave System (Advanced Microwave Labstation, Milestone. Inc., Monroe, CT) and diluted with acid solution (2% HNO_3 + 0.5% HCl, v/v). The diluted solution was subjected to an Inductively-Coupled Plasma Mass Spectrometer (ICP-MS; Agilent 7500 series, Agilent Technologies, Santa Clara, CA) to analyze the total calcium in the pellet using argon as carrier and plasma gas.

Calcium binding to apple and cucumber tissue powder was determined as follows: 500 mg tissue powder was thoroughly mixed with 50 mL 10 mM Tris-HCl buffer (pH 7.0), and 2.5 mL 0.1 M calcium chloride was added shaking on Labquake shaker at room temperature. The same tissue powder suspensions without adding calcium chloride were used as blanks. Free calcium ion in the binding solution was monitored with ISE meter at predetermined time as for fractions. After binding 20 hrs, the pellet was collected by the

previously described procedure and freeze dried. Total calcium in samples was determined by ICP-MS by the same procedure as for fractions.

3.3.5 Calculation and statistical analysis

The calcium bound by sample solids was calculated by equations (1), (2) in ISE measurement and (3) in ICP-MS, respectively.

$$\text{Decrease of free}[Ca^{2+}] = 1 - \frac{\text{free}[Ca^{2+}]\text{ in binding solution - free}[Ca^{2+}]\text{ in control}}{\text{initial exogenous}[Ca^{2+}]\text{ in binding solution}} \quad (1)$$

$$\text{Bound calcium (mg Ca/g sample)} = \frac{\text{initial exogenous Ca mass} \times \text{decrease of free } Ca^{2+}}{\text{sample mass}} \quad (2)$$

$$\text{Bound calcium (mg Ca/g sample)} = [Ca^{2+}]\text{ in sample - }[Ca^{2+}]\text{ in control} \quad (3)$$

Results were analyzed using SAS program 9.13 (SAS institute Inc, 2003). Experiments were repeated twice and significant differences among treatments was determined by Duncan's multiple range test ($P<0.05$).

3.4 Results and Discussion

3.4.1 Yield of apple fractions

The total yield of all extracted fractions representing alcohol insoluble solids (AIS) was 96.04 mg/g dry weight (DW) tissue. The total extracts were composed of 11.22% cytoplasmic content (AF1), 3.01% cell membrane content (AF2) and 85.76% cell wall contents (AF3+AF4+AF5) **(Table 3.1)**. The low-molecular-weight carbohydrates including sorbitol, glucose, sucrose, and fructose, which make up about 70% of dry weigh of apple tissue (Mikael and others 2000) were lost during extraction and might be the reason for the low yield of total extracts. Total cell wall content was 8.24% of DW cortical tissue, which was slightly lower than the previously reported value of around

Table 3.1 - Fraction characteristics from Golden Delicious apple cortical tissue.

Fraction	Color	Yield mg/g DW tissue	Chemical composition									
			% DW fraction					mg/g DW tissue				
			PS	PO	PR	AM	TSP	PS	PO	PR	AM	TSP
AF1	light brown	10.78±1.64	25.35±3.85	20.26±1.77	7.94±1.24	5.52±0.22	0.43±0.03	2.73±0.41	2.18±0.19	0.86±0.13	0.59±0.02	0.05±0.00
AF2	light brown	2.89±0.57	27.05±4.09	16.77±2.14	11.21±1.02	5.88±0.34	0.27±0.02	0.78±0.12	0.48±0.06	0.32±0.029	0.17±0.01	0.01±0.00
AF3	brown	0.24±0.07	18.67±1.87	15.02[a]	26.00±2.37	7.53[a]	1.08±0.28	0.04±0.00	0.04	0.06±0.01	0.02	<0.01
AF4	gray	4.50±0.82	54.27±4.35	18.82±0.53	3.66±0.17	6.20±0.72	0.68±0.13	2.44±0.20	0.85±0.02	0.16±0.01	0.28±0.03	0.03±0.01
AF5	white	77.62±4.54	24.24±1.38	54.76±2.72	1.64±0.10	3.92±0.42	0.16±0.01	18.82±1.07	42.50±2.11	1.27±0.08	3.05±0.33	0.12±0.01
Total		96.04±2.30						24.82±1.81	46.05±2.26	2.68±0.23	4.11±0.38	0.21±0.01
% total yield								25.84±1.41	47.97±3.09	2.79±0.25	4.27±0.47	0.22±0.02
Tissue								87.70±0.40	617.64±36.18[*]	15.27±1.62	19.70±4.82	4.06±0.30

Values are average ± standard deviation (n=3) except in F3 fraction ; [a] where no replication was conducted; DW represents dry weight; DW factor was 14.73 ± 0.35% of fresh weight for cortical tissue; PS – Pectic substances, PO – Neutral polysaccharides, PR – Protein, AM - Aminosugars, TSP – Total soluble phenolic compounds; [*] Total carbohydrates was determined.

10% (Siddiqui and Bangerth 1995; Chardonnet and others 2003). The extraction yields of each fraction were in the order of AF5>AF1>AF4>AF2>AF3 and ranged from 0.24 to 77.62 mg/g DW cortical tissue (**Table 3.1**). Negligible amounts of AF3 were obtained in this study, which was probably due to limited amount of protein in cell wall or the condensed cell wall structure prevented the release of proteins (Chardonnet and others 2003).

3.4.2 Chemical composition of apple fractions

The dominant macromolecules in apple fractions were as follows: pectic substances in AF1, AF2 and AF4, proteins in AF3, and neutral polysaccharides in AF5 (**Table 3.1**). In all fractions, aminosugars and total soluble phenolics existed at very low levels, while pectic substances and neutral polysaccharides were present in considerable amounts. Pectic substances can be extracted from cell wall and cell middle lamella with several solvents including water, chelating agents, alkali, and acid (Heredia and others 1995), although some fractions are not completely soluble even in HCl (Kalapathy and Proctor 2001). Therefore, it is no surprise pectic substances were present in all fractions. Nevertheless, the AF1, AF2, and AF3 fractions, aiming to extract proteins, had considerably higher protein content than fractions AF4 and AF5, which were designed to obtain pectin and cellulose, respectively.

3.4.3 Chemical composition of apple cortical tissue

In cytoplasmic (AF1) and cell membrane (AF2) portions, all abalyzed chemical compounds were presented in similar low levels based on DW cortical tissue (**Table 3.1**). In the cell wall portion (AF3+AF4+AF5), neutral polysaccharides, pectic substances, and proteins were 52.72%, 25.88%, and 1.81%, respectively, which was consistent with

previous reports (Siddiqui and Bangerth 1995; Chardonnet and others 2003). When composition of fractions is calculated based on 1 g DW cortical tissue, total extracts were composed of 46.05 mg polysaccharides, 24.82 mg pectic substances, 2.68 mg proteins, 4.11 mg chitinuous material, and 0.21 mg total soluble phenolics (**Table 3.1**). However, chemical analysis of un-extracted freeze-dried cortical tissue powder revealed that 1 g DW cortical tissue contained 617.64 mg total carbohydrates, 87.70 mg pectic substances, 15.27 mg proteins, 19.70 mg aminosugars, and 4.06 mg total soluble phenolics. The large difference between values calculated from fractions and directly determined from tissue powder was mainly due to the loss of substances soluble in alcohol such as low-molecular-weight carbohydrates, amino acids, and short peptides etc. (Mikael and others 2000). Our results indicated that alcohol soluble carbohydrates in cortical tissue were 573.59 mg/g DW cortical tissue, which was similar to 560 mg/g DW cortical tissue calculated from a previously report (Chardonnet and others 2003).

3.4.4 Discoloration of apple fractions

All fractions appeared discolored after extraction and freeze-drying: AF1 and AF2 were light brown, AF3 was brown, AF4 was gray, and AF5 was white (**Table 3.1**). Browning of apple tissue is mainly caused by oxidation of polyphenols catalyzed by polyphenol oxidase (PPO) (Toivonen and Brummell 2008). The brown color of the AF3 fraction was probably caused by the presence of the highest level of phenolic compounds. AF1 and AF2 fractions with relatively high protein content appeared light brown, while AF4 and AF5 fractions with low protein but high polysaccharide content had the lightest color. Although the discoloration was apparently associated with the protein content, it could not be prevented even when lyophilization was applied for drying.

3.4.5 Yield of cucumber fractions

Yield of cucumber fractions was in the order of CF5>CF1>CF2>CF4>CF3 and ranged from 14.18 to 111.12 mg/g DW mesocarp tissue (**Table 3.2**). The combined total extract yield was 220.36 mg is consisted of 25.72% cytoplasm (CF1), 12.77% membrane (CF2), and 63.71% cell wall contents (CF3+CF4+CF5). The cell wall content was 140.39 mg/g DW mesocarp tissue, which was lower than 246 mg/g DW pickled cucumbers tissue calculated from a previously report (McFeeters and Lovdal 1987). This could be caused by the difference of cucumber variety and extraction method. In the report, the mesocarp tissue was blended with 95% alcohol and overall AIS were considered as cell wall content. However, the cell wall content in our results only refers sum of fractions CF3, CF4, and CF5. The total weight of fraction extracts from cucumber tissue was much higher than that extracted from apple tissue. This was possibly because alcohol soluble substances are at lower levels in cucumber mesocarp tissue (350 mg/g DW, calculated from previous report) compared to that in apple cortical tissue (560 mg/g DW, calculated from previous report) (Chardonnet and others 2003; Zhang and others 2004).

3.4.6 Chemical composition of cucumber fractions

Similar to apple fractions, aminosugars and total soluble phenolics were detected at low levels in all fractions. As expected, pectic substances and neutral polysaccharides were present in all fractions in considerable amounts, while significantly higher levels of proteins were found in CF1, CF2, and CF3 than in CF4 and CF5. The results also indicated that much higher amounts of proteins were present in the cucumber extracts than in apple extracts. The dominant compounds in fractions were proteins in CF1, CF2, and CF3, pectic substances in CF4, cellulose in CF5.

Table 3.2 - Fraction characteristics from cucumber mesocarp tissue

Fraction	Color	Yield mg/g DW tissue	Chemical composition									
			% DW fraction					mg/g DW tissue				
			PS	PO	PR	AM	TSP	PS	PO	PR	AM	TSP
CF1	yellow-gray	56.55±1.25	9.01±1.64	17.52±3.21	37.04±2.91	2.67±0.51	0.25±0.27	5.1±0.93	9.92±1.82	20.96±1.65	1.51±0.29	0.14±0.15
CF2	light yellow	28.14±10.46	11.23±1.27	15.70±2.97	41.28±5.14	2.73±0.49	0.21±0.16	3.15±0.36	4.41±0.83	11.60±1.44	0.77±0.14	0.06±0.04
CF3	light gray	14.18±5.88	25.34±12.64	31.03±14.82	44.82±10.32	3.99±0.81	0.19±0.12	1.97±0.09	4.41±2.10	6.36±1.46	0.57±0.12	0.03±0.02
CF4	light gray	15.09±2.95	28.36±5.02	25.25±6.65	7.40±0.90	6.02±1.39	0.54±0.50	4.28±0.76	3.81±1.00	1.12±0.14	0.91±0.21	0.08±0.08
CF5	white	111.12±13.36	19.79±2.12	47.37±8.00	6.02±1.57	3.24±0.63	0.17±0.14	21.99±2.35	52.63±8.89	6.69±1.74	3.60±0.70	0.18±0.16
Total		220.36±23.83						36.49±2.58	75.18±8.5	46.74±3.40	7.35±1.09	0.49±0.22
%total yield								16.12±2.15	33.81±5.83	20.39±2.42	3.28±0.63	0.22±0.10

Values are average ± standard deviation (n=3); DW represents dry weight, DW factor was 4.05 ± 0.08% fresh weight for mesocarp tissue; PS – Pectic substances, PO – Neutral polysaccharides, PR – Proteins, AM - Aminosugars, TSP – Total soluble phenolic compounds.

3.4.7 Chemical composition of cucumber mesocarp tissue

Calculated based on 1 g of dry mesocarp tissue, the extraction yielded 36.49 mg pectic substances, 75.18 mg neutral polysaccharides, 46.74 mg proteins, 7.35 mg aminosugars and 0.49 mg total soluble phenolics (**Table 3.2**). Expressed on fresh weigh of mesocarp tissue, 1 g fresh tissue yielded 1.48 mg pectic substances, 3.04 mg neutral polysaccharides, and 1.89 mg proteins. The neutral polysaccharide and pectic substances contents were lower than 3.52 and 7.6 mg previously reported by McFeeters and Lovdal (McFeeters and Lovdal 1987). These variations were possibly caused by the differences in experimental materials, extraction and analysis methods. In the report, sugars and galacturonic acid in overall AIS which was mentioned earlier were determined by gas chromatographic analysis and calculated based on fresh tissue weight. Overall protein content in cucumbers was much higher than that extracted from apples, which was consistent with the values from USDA nutritional database (USDA, National Agricultural Research Service). Based on yield of cell wall DW portion (CF3+CF4+CF5), cell wall content contained 10.09% protein, 43.34% neutral polysaccharides and 20.11% pectic substances, in which the pectic substances were slightly lower than the reported value of 28.8% in pickled cucumbers (Howard and Buescher 1990).

3.4.8 Discoloration of cucumber fractions

Slight darkening of fraction solids was developed upon the extraction (**Table 3.2**). F1 appeared the darkest with yellow-gray color, while CF5 was the lightest white. Similar to the extracts from apples, the fractions with higher protein and/ or phenolics content showed darker color than the fractions containing high neutral polysaccharides

and pectic substances. Interestingly, even though the cucumber extract in total had higher combined protein and phenolics content, and higher protein content in CF1, CF2, and CF3 than that of apples, all fractions from cucumber tissue darkened to less extent than that of apple fractions. This indicates different composition and types of phenolics and proteins in apples and cucumbers.

3.4.9 Calcium binding to apple fractions

The binding kinetics and capacity of calcium to apple fractions from ISE measurements are depicted in **Figure 3.2**. AF3 was not used in binding experiments because limited amount of the sample was obtained. Four other fractions demonstrated similar binding trends but with different binding capacity: binding capacities of all fractions reached plateaus after addition of calcium chloride within 10 mins and stayed at similar levels thereafter. At 20 hrs' binding, the capacities of AF1, AF2 and AF4 slightly decreased but slightly increased in F5. Comparison at 20 hrs showed that binding capacity differed significantly in the order of AF4 ≥AF2>AF5>AF1 and the values ranged from 3.08 to 6.32 mg Ca/g fraction solids.

Endogenous calcium before the binding process, endogenous calcium in the blank and total calcium after binding determined by ICP-MS procedure are shown in **Table 3.3**. The total endogenous calcium concentration in apple tissue was 0.14 mg/g DW sample, which was slightly lower than the previously reported value of around 0.25 mg/g in the same variety of apple(Chardonnet and others 2003). This variation possibly results from the difference in apple source. Before binding, a high level of calcium existed in AF1 and AF4 fraction solids, which indicated that calcium was associated with macromolecules in

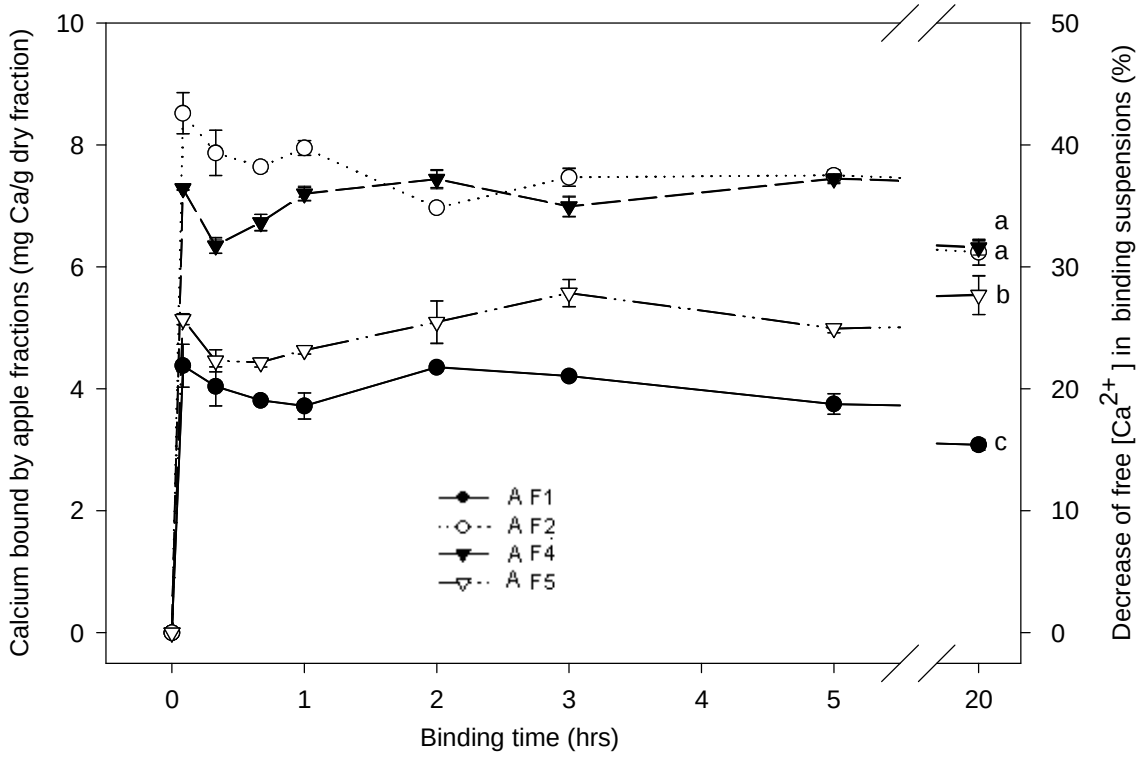

Figure 3.2 - Calcium binding kinetics to fractions from Golden Delicious apple cortical tissue (by ISE). Experiments were performed in 25 mL 10 mM Tris-HCl buffer at 23 °C with Ca^{2+}-to-powder ratio 1:50 (w/w); Bars represent standard deviation (n=2); Values at 20 hrs with different letters were statistically significant different (Duncan's comparison $P<0.05$).

Table 3.3 - Calcium in Golden Delicious apple cortical tissue powder and fractions (by ICP-MS).

Source	Calcium (mg/g sample)		
	Before binding	Blank	After binding
Apple powder	0.14	0.99±0.07	6.24±0.09
AF1	2.25	2.85±0.26	11.14±0.45
AF2	0.90	1.23±0.07	12.61±0.39
AF3	N/A	N/A	N/A
AF4	1.32	1.72±0.07	11.93±0.08
AF5	0.06	0.13±0.03	5.71±0.22

Values are average ± standard deviation (n=2) except the values before binding; N/A samples were not determined

cytoplasm and pectic substances. After *in vitro* binding with calcium chloride, the total bound calcium in tissue was 6.24 mg/g DW tissue. This was much higher than the value of 1-2 mg/g apple tissue treated by calcium infiltration in vivo with whole fruits during storage (Chardonnet and others 2003).

Amounts of calcium bound by fractions after 20 hrs binding obtained from ISE and ICP-MS measurements are displayed in **Figure 3.3.** Amounts of bound calcium by fractions obtained by ICP-MS varied significantly and were in the order of AF2>AF4>AF1>FA5 ranging from 5.58 to 11.38 mg Ca/g fraction solids. The values of AF1, AF2, and AF4 obtained by ICP-MS were much higher than that by ISE. Such variation may be caused by particle or compound interference with ISE electrode or free ionic calcium was trapped in pellets during precipitation of slurry for ICP-MS test. Even though the absolute values for bound calcium differ between the methods used, the trend is similar and indicated that AF2 and AF4 have higher binding potential than AF1 and AF5.

3.4.10 Calcium binding to cucumber fractions.

The binding kinetics and capacity of calcium by cucumber fractions obtained by ISE measurements are shown in **Figure 3.4**. Neither replication nor blank were run for CF3 and CF4 due to shortage of extracted samples. All five fractions demonstrated similar binding trends with different binding capacities. Binding capacity of all five fractions reached plateau one hour after addition of calcium chloride and stayed at similar levels thereafter; the binding capacities of CF1, CF2, CF3 and CF4 slightly decreased at 20 hrs, but slightly increased in CF2; comparison at 20 hrs showed that binding capacity

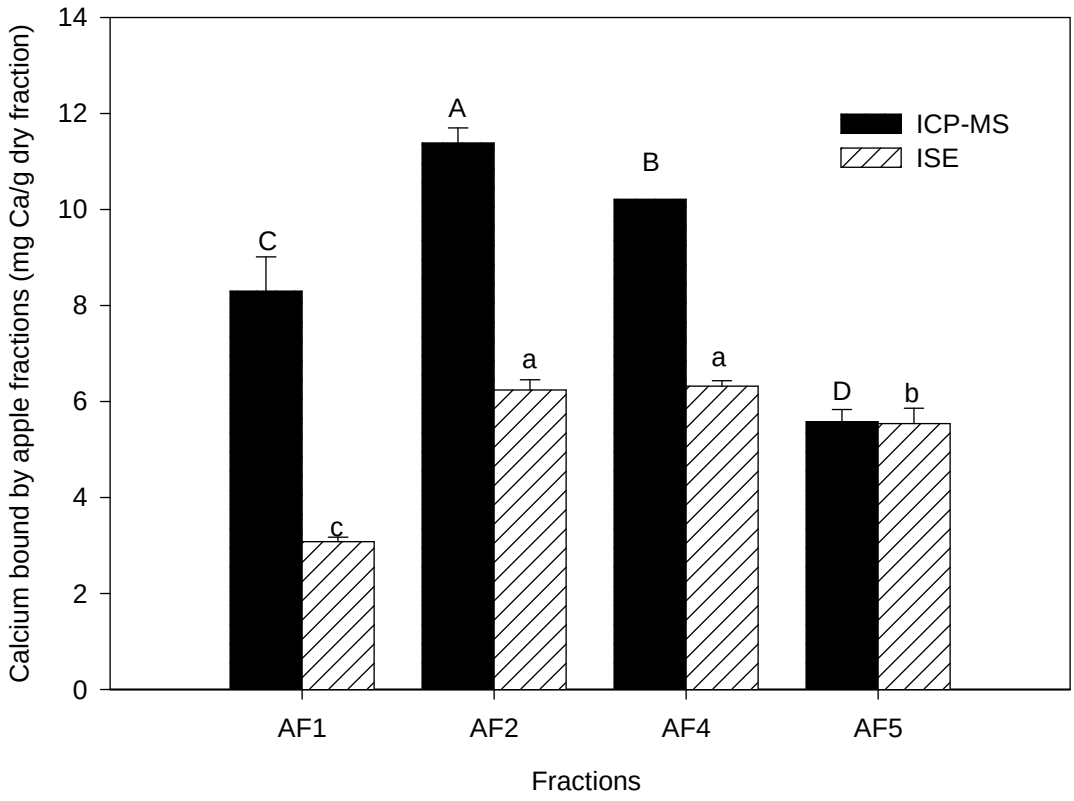

Figure 3.3 - Calcium binding to fractions from Golden Delicious apple cortical tissue. Values were obtained after binding for 20 hrs; Experiments were performed in 25 mL 10 mM Tris-HCl buffer with Ca^{2+}-to-fraction ratio 1:50 (w/w) at 23 °C; Bars represent standard deviation (n=2); Values with different letters within same type of measurement were significant different (Duncan's comparison $P<0.05$).

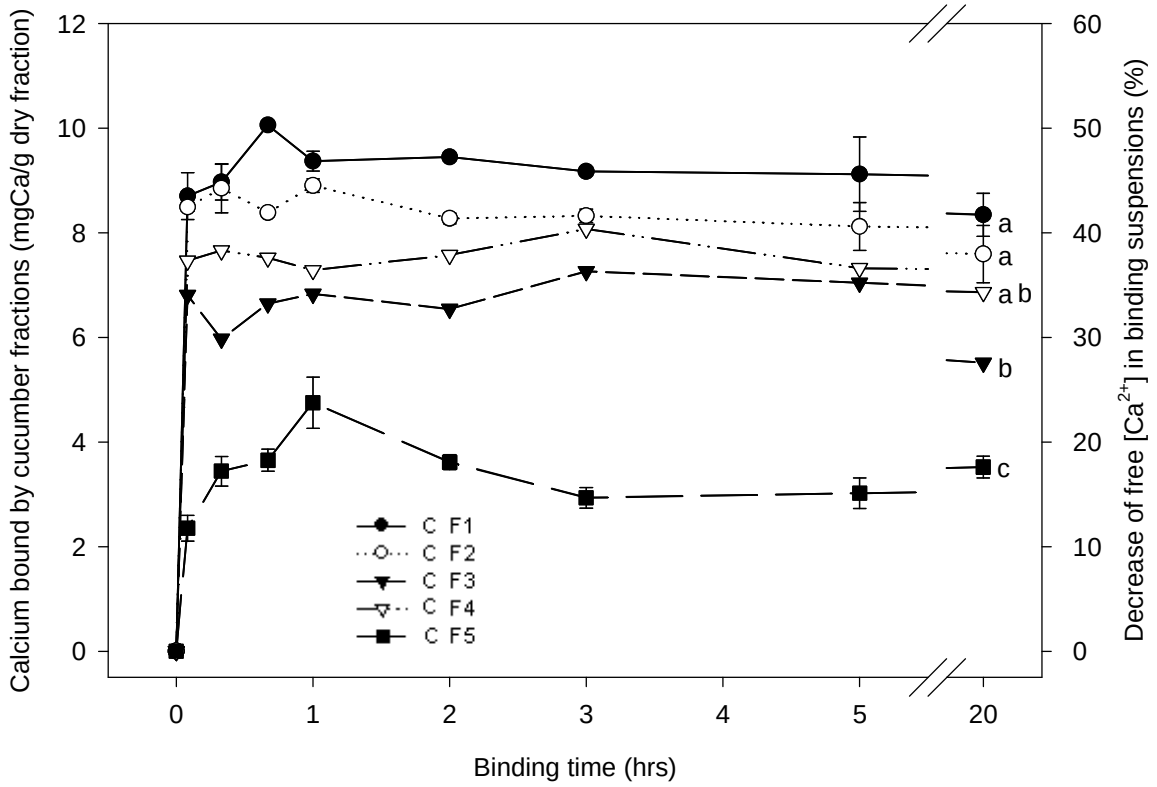

Figure 3.4 - Calcium binding kinetics to fractions from cucumber mesocarp tissue (by ISE). Experiments were performed in 25 mL 10 mM Tris-HCl buffer at 23 °C with Ca^{2+}-to-powder ratio 1:50 (w/w); Bars represent standard deviation (n=2); Values at 20 hrs with different letters were statistically significant different (Duncan's comparison $P<0.05$).

differed significantly in the order of CF1 ≥CF2>CF4≥CF3>CF5 and the values ranged from 3.52 to 8.34 mg Ca/g fraction solids.

The endogenous calcium in cucumber tissue was 1.53 mg Ca/g DW tissue (**Table 3.4**), which was around ten times higher than in apple tissue (**Table 3.3**). This ratio was consistent with USDA database for nutritional values of food (USDA, National Agricultural Research Service), but values we detected are lower for both cucumbers and apples. The endogenous calcium in cucumbers and apples is reported to be around 4 and 0.4 mg Ca/g DW tissue without peel and seeds, respectively (USDA, National Agricultural Research Service). As shown in **Table 3.4**, endogenous calcium in CF1, CF2, CF3 and CF4 fractions before binding was at higher levels compared to that of apple tissue. This implies that calcium content in cucumber was naturally higher, or the cucumber was already treated with calcium before sale. As for tissue powder, surprising increases in calcium content were determined in controls after 20 hrs of mixing cucumber powder in the buffer although no calcium was added. Since only alcohol insoluble content in tissue powder was collected for ICP-MS determination, it seems that most of the calcium is bound by the alcohol insoluble compounds and ethanol precipitation concentrated calcium. However, the binding procedure caused marginal increases of total calcium concentration in fraction blanks because all fractions were obtained by precipitation with ethanol during extraction. We did not observe high endogenous free Ca^{2+} concentration in ISE blank suspension (data not shown), which also implied that most endogenous calcium might be present as a bound form as ISE determines the free Ca^{2+} released from tissue powder in the solution. High calcium content in CF1 indicated that cytoplasm polymers had higher affinity to calcium.

Table 3.4 - Calcium in cucumber mesocarp tissue powder and fractions (by ICP-MS).

Source	Calcium (mg/g sample)		
	Before binding	Blank	After binding
Cucumber powder	1.53	14.73±0.90	35.08±3.30
CF1	28.36±10.80	35.50±0.12	44.02±2.96
CF2	7.29±2.75	8.16±0.46	14.61±2.52
CF3	4.38	N/A	6.22
CF4	2.22±0.80	N/A	18.69
CF5	0.06±0.01	0.11±0.07	5.95±0.22

Values were average ± standard deviation (n=2) except values with no standard deviations; N/A represented samples were not determined.

The exogenous calcium bound by cucumber fractions and powders obtained by ICP-MS measurement is presented in **Figure 3.5**. As there was no blank for CF3 and CF4, the exogenous bound calcium was calculated by subtracting the calcium before binding from total calcium after binding in these two fractions instead of calcium in the blank. Since the results showed the level of calcium in the blank mostly was close to the calcium before binding in cucumber fractions (**Table 3.4**), the slight change in calculation should be negligible. Binding capacity of the fractions were in the order of F4>CF1≥CF2≥CF5≥CF3 with the range of 1.84 to 16.47 mg Ca/g fraction solid, but only the value for F4 was significantly higher than the rest of fractions. Although ICP-MS and ISE did not show the same trends in binding capacity, CF1, CF2 and CF4 did show higher binding potential than CF3 and CF5 in both measurements. Much higher value was obtained with ICP-MS compared to ISE for CF4 in which pectic substances were dominant.

The calcium analysis for apple and cucumber samples demonstrated increased calcium content after addition of exogenous calcium. This is consistent with previous studies which reported that application of calcium salts could increase calcium content in fruits and vegetables. The total calcium content in cortical tissue of apples infiltrated with 2% calcium chloride after harvest increased by 4 times (Chardonnet and others 2003). Calcium content in cucumber pickles increased by 3 times when brine contained 100 mM calcium chloride (Buescher and Hudson 1986).

3.4.11 Effect of fraction composition and calcium-binding

Evaluation of each fraction's composition and their ability to bind calcium revealed the relationships in both apples and cucumbers. Thus, fractions that bound the

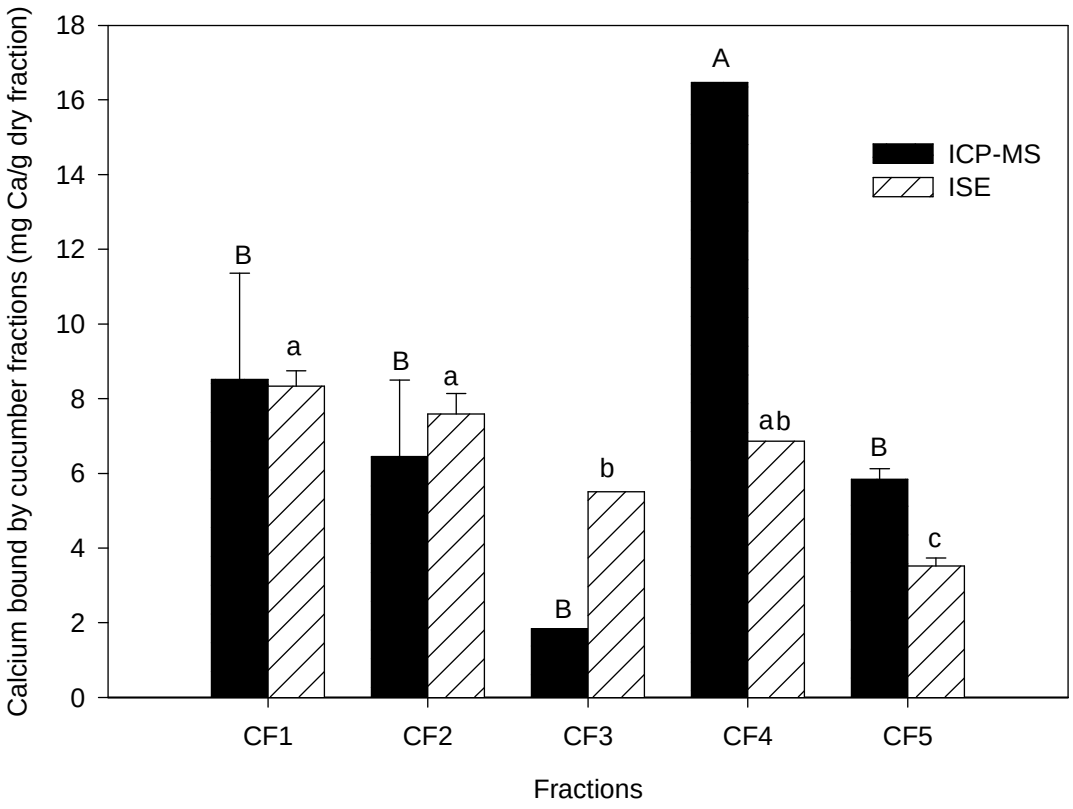

Figure 3.5 - Calcium binding to fractions from cucumber mesocarp tissue. Values were obtained after binding for 20 hrs; Experiments were performed in 25 mL 10 mM Tris-HCl buffer with Ca^{2+}-to-fraction 1:50 (w/w) at 23 °C; Bars represent standard devation (n=2) except CF3 and CF4; Values with different letters within same type of measurement were significant different (Duncan's comparison $P<0.05$).

highest amounts of calcium, AF4 and AF2 from apples, and CF4, CF2, and CF1 from cucumbers had highest level of pectic substances (F4 in apples and cucumbers), cell membrane proteins (F2 in apples and cucumbers), and cytoplasmic proteins (F1 in apples and cucumbers). Fractions that bound calcium the least, AF5 and AF1 for apples, and CF5 and CF3 for cucumbers were mainly composed of cellulose (F5 in apples and cucumbers), had low levels of pectins and proteins (AF1 in apples), or were mainly composed of cell wall proteins (CF3 in cucumbers).

Overall results indicated that the F4 fraction in which pectin was the dominant compound showed higher binding potential to calcium than all other fractions. This re-confirmed the role of pectin in calcium-induced firmness in plant-based products (Poovaiah and others 1988). In addition, this study found that cell membrane proteins and cell cytoplasmic proteins also interacted with calcium. Previous research also has found that calcium could interact with membrane proteins and cause protein pattern changes in apple fruits (Poovaiah and others 1988). Additionally, fractions mainly consisting of cellulose also showed come affinity for calcium ions, which may be attributed to physical adsorption, as reported for calcium binding to cellulose, methyl-cellulose hemicellulose and psyllium (Torre and others 1992; Luccia and Kunkel 2002).

3.5 Conclusion

The involvement of different macromolecules from apple and cucumber tissues in calcium binding was explored by *in vitro* calcium binding with apple cortical and cucumber mesocarp tissue powder and stepwise extract solids. The dominant compounds in the extracted fractions from apple are as follows: pectic substances in F1, F2, and F4,

protein in F3, and polysaccharide in F5. F2 and F4 showed higher binding capacity than F1 and F5. The dominant compounds in the fractions from cucumber are as follows: proteins in F1, F2, and F3. pectic substances in F4, polysaccharide in F5. F1, F2 and F4 showed higher binding capacity than F3 and F5. Results indicated that calcium was bound by all fractions from both apple and cucumber tissues, but those fractions dominantly composed of pectic substances, membrane protein or cytoplasmic protein showed higher binding capacity. The results confirmed the important role of pectic substances in calcium-induced firmness in both cucumber and apple. However, cell membrane, cytoplasm proteins may be involved as well due to the high level interaction between calcium ions and these macromolecules observed in this study.

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Chapter 4 Isothermal Titration Calorimetry Analysis of Calcium-Binding to Pectin and BSA

4.1 Abstract

The binding behaviors of pectin and bovine serum albumin to calcium were investigated by Isothermal Titration Calorimetry (ITC). Both polymers showed binding capacity to calcium with different isothermal patterns and different calcium-binding capacities, indicating that interactions between calcium and biopolymers from vegetables, fruits, and mushrooms can be researched with the aid of ITC technique to further understand the mechanism regarding the textural effect of calcium on these products.

4.2 Introduction

Isothermal Titration Calorimetry (ITC), which can measure enthalpy (ΔH), entropy (ΔS°), free energy (ΔG°), association constant constant (K) upon the reaction occurring between metal and polymers, has been widely employed in studying metal-polymer interactions, (Wilcox, 2008). Interactions of calcium with food substances such as pectin and proteins have been invstigated by ITC (Fang and others 2008).

In previous studies, we quantitatively investigated the calcium binding to macromolecular extracts from apples, cucumbers, and mushrooms, and important information about calcium binding to the extracts obtained by ITC may facilitate the understanding of the role of calcium in tissue firmness improvement. As proteins and pectic substance fractions extracted previously showed the highest capacity for binding with calcium ions, in this experiment we were trying to preliminarily characterize calcium-binding to pectin and bovine serum albumin (BSA) using ITC and gather data for further studies on calcium-binding to extracts from apples, cucumbers, and mushrooms.

4.3 Material and Methods

4.3.1 Materials

Pectin, BSA, calcium chloride ($CaCl_2$), and potassium chloride (KCl) were purchased from Sigma (St. Louis, MO).

4.3.2 ITC Experiment

Pectin, BSA, $CaCl_2$, and KCl were separately dispersed in a 10 mM Tris-HCl buffer (pH 7.0) and stirred overnight to ensure complete dissolution. The titration calorimetry measurements were performed with a TAM III titration calorimeter (TA Instrument Inc. USA) at 25 °C. Aliquots (8 -10 µL) of $CaCl_2$ / KCl ligand solution (25-100 mM) were sequentially titrated into 3 mL reaction cell initially containing 2.7 mL of either 10 mM Tris-HCl buffer (pH 7.0) or sample solutions (0.2%-6.7% w/v). A total of 25-30 injections were performed for each measurement with an interval of 10 mins between two successive injections, and the stirring speed was 60 rpm for all the experiments. Binding constant (K), binding enthalpy (ΔH), and accumulative heat were analyzed by TAM assistant software (TA Instrument Inc. USA).

4.4 Results and Discussion

4.4.1 Calcium binding to pectin

The ITC thermograph for the sequential injection of 25 mM $CaCl_2$ into 0.2% pectin (w/v) solution is displayed in **Figure 4.1 - A**. The heat flow peak, which indicates

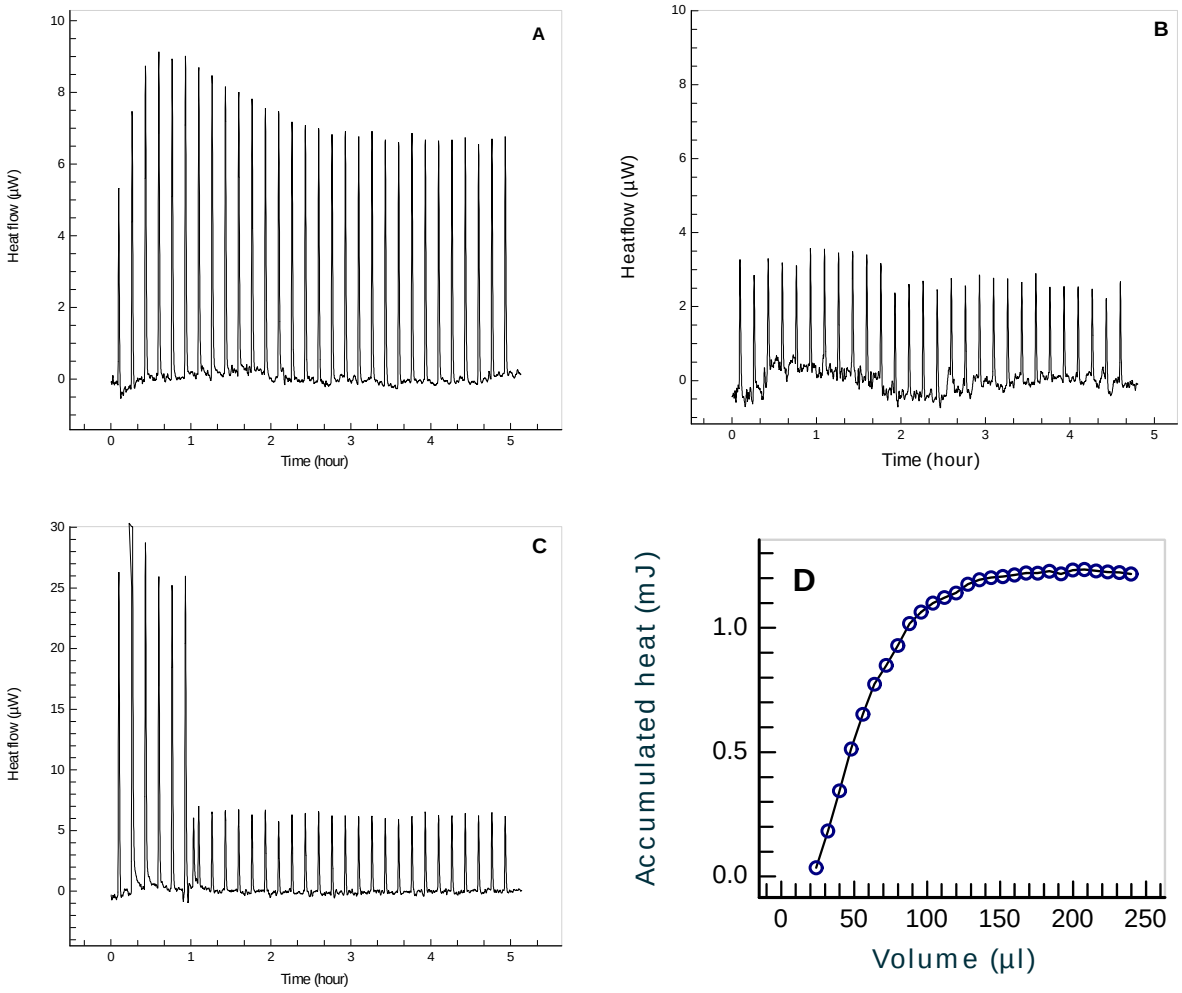

Figure 4.1 - ITC data for $CaCl_2$ binding to pectin (0.2% citrus pectin, 10mM Tris-HCl buffer, pH 7.0, 8µL × 30 injections). A: 25mM $CaCl_2$ into pectin; B: Dilution effect (25mM $CaCl_2$ into Tris-HCl buffer); C: Viscosity effect (The first 6 injections with 100mM $CaCl_2$, the rest 24 injections with Tris-HCl buffer); D: accumulative heat of $CaCl_2$ into pectin (after deduction of dilution and viscosity effect, volume represents the added $CaCl_2$).

the heat release at each injection, increased with injection of $CaCl_2$ in the first 4 titrations, then decreased and achieved a flatted stage indicating the saturation of binding. The dilution effect of calcium into buffer was obtained by using corresponding blank experiment as shown in **Figure 4.1 - B**. It showed the whole pattern was flatted. However, the dilution effect of around 3 µW was not equal to the peak value of 7 µW after saturation of binding. This might be caused by an increased viscosity of pectin solution or other side effects after addition of $CaCl_2$, which was then examined by initially titrating 100 mM $CaCl_2$ into 0.2% pectin (w/v) solution then the Tris-HCl buffer (**Figure 4.1 - C)**. It showed around 6 µW heat released. Nevertheless, the combination of dilution and other side effects (around 7 µW) could not be obtained by simply summing the individual values (around 9 µW). In the present experiment, the heat accumulated from the interaction between $CaCl_2$ and pectin was calculated by subtracting the combination of dilution and other side effects (7 µW) upon titration shown in **Figure 4.1 - D**. The binding saturation was achieved after 15 injections (around 150 µL $CaCl_2$), at which the weight ratio between calcium and pectin was around 28 mg Ca /g pectin solids. The accumulated heat after calcium-binding was saturated was around 1.2 mJ. Additional study on KCl binding to pectin was performed to compare it with calcium binding to pectin showing in **Figure 4.2 - A**. Corresponding blank experiment was shown in **Figure 4.2 - B**. Similar patterns were obtained between KCl binding to pectin and its blank, and the pattern of KCl into pectin was distinctly differed from $CaCl_2$ binding to pectin. Because the molecular weight of source pectin was not determined, the thermodynamic parameters were not calculated.

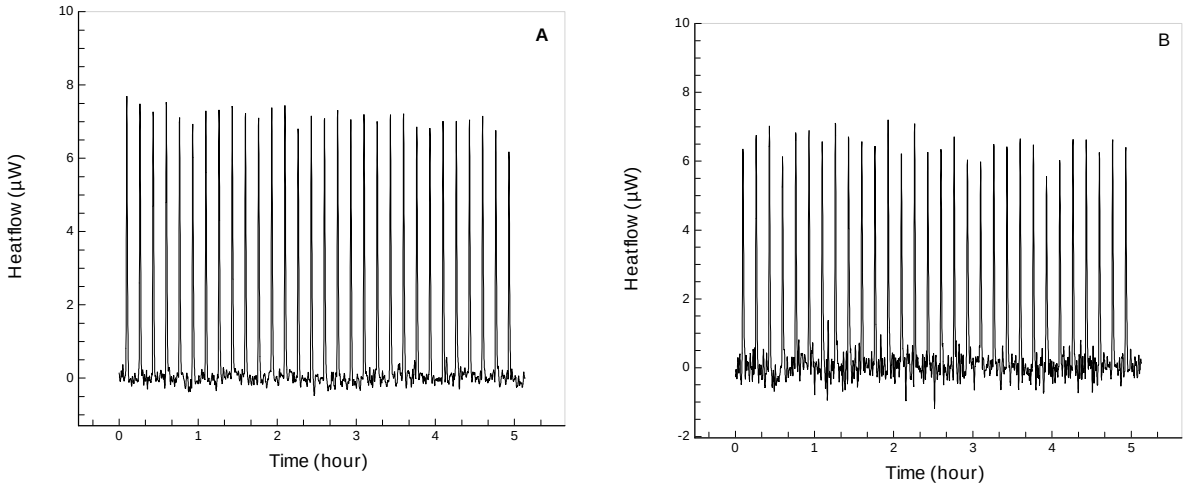

Figure 4.2 - ITC data for KCl titration into pectin at 25 °C in 10 mM Tris-HCl buffer (pH 7.0). A: 50mM KCl titration into 0.2% pectin; B: Dilution effect (50mM KCl titration into Tirs-HCl buffer).

The results showed a weak binding between calcium and pectin. The pattern of $CaCl_2$ binding to pectin obtained in our experiment was different from that obtained by Fang and others using acetate buffer (pH 5.0). The pattern they got was starting from decrease, then increase, decrease, and saturation (Fang et al., 2008). This difference might be caused by different binding buffer and/or pectin source and concentration. The comparison between $CaCl_2$ and KCl binding to pectin suggested the interaction between Ca^{2+} and pectin was significantly different from the interaction between K^+ and pectin.

4.4.2 Calcium binding to BSA

Figure 4.3 - A displays thermograph of the 25 mM $CaCl_2$ titrated into 6.7% BSA. The pattern indicated that a regular shape of isotherm curve occurred under the current system. The accumulated heat after subtracting the dilution effect was shown in **Figure 4.3 -C**. The binding saturation was achieved after adding around 70 µL $CaCl_2$ with accumulated heat of around 42 mJ. The weight ratio of calcium-binding was around 0.4 mg Ca/g BSA solids. As the molecular weight of BSA was known, the data was fitted to calculate the thermodynamic parameters for the interaction (**Figure 4.3 - B, D**). The results showed that binding between BSA and calcium was exothermic and strong ($k \sim 10^4$). The binding ratio of BSA to calcium based on mole concentration is around 1: 6.

4.5 Conclusions

The binding behaviors of pectin and BSA upon addition of calcium were investigated by ITC under the system that was used in previous experiments for fraction binding from cucumbers, apples, and mushrooms (10mM Tris-HCl, pH 7.0). Both polymers showed binding capacity to calcium with different isothermal patterns. The

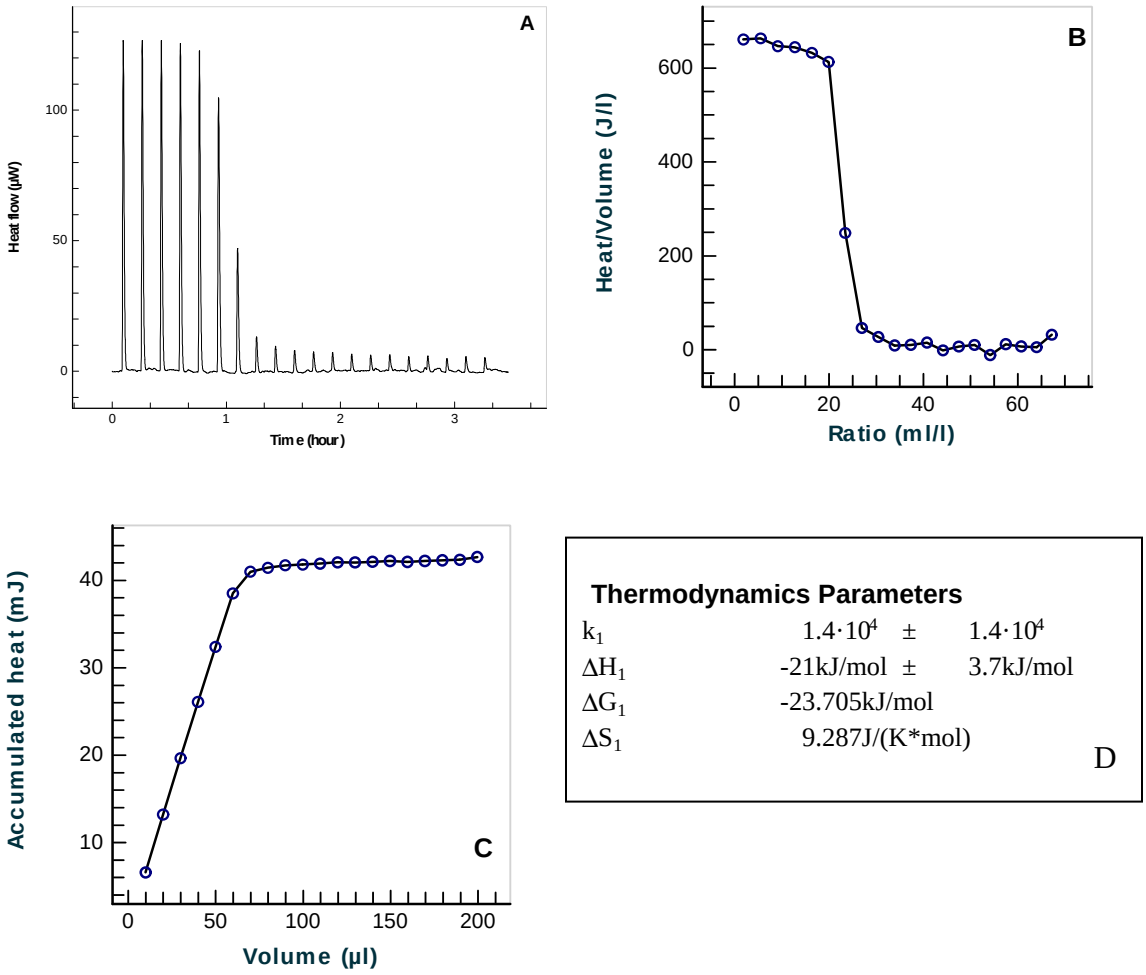

Figure 4.3 - ITC data for 25 mM $CaCl_2$ titration into 6.7% BSA at 25 °C in 10 mM Tris-HCl buffer (pH 7.0) with 10μL × 25 injections: A: $CaCl_2$ into BSA; B: Fitted model for thermodynamic parameters calculation; C: Accumulated heat after deduction of dilution effect; D: Thermodynamic parameter by fitting model.

isotherm curve was different from regular binding curves in calcium-binding to pectin. The binding capacity was around 28 mg Ca/g pectin solids. The isotherm curve obtained from BSA was regular with calcium-binding capacity of 0.4 mg Ca/g BSA solids. The parameters obtained were consistent with previous reported values regarding calcium binding to other proteins. It revealed that further research could be carried out using ITC to elucidate the interaction of calcium to macromolecules in vegetables, fruits, and mushrooms for fully understanding calcium-induced firmness in these products.

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Chapter 5 Conclusions

To fully understand the mechanism of calcium-induced firmness in fruits, vegetables, and mushrooms, potential macromolecules and sites involved in calcium-binding were investigated by *in vitro* calcium-binding with tissue extracts from apple, cucumbers, and mushrooms. Different fractions were stepwise extracted and the dominant compounds in the each fraction were: cytoplasmic proteins in MF1, cell membrane proteins and polysaccharides in MF2, cell wall alkaline soluble polysaccharides in MF3a and MF3b, cell wall alkaline insoluble polysaccharides and chitin in MF4a and MF4b in mushroom fractions; pectic substances in AF1, AF2, and AF4, cell wall proteins in AF3, cell wall cellulose in AF5 for apple fractions; Cytoplasmic proteins, cell membrane proteins, cell wall proteins in CF1, CF2, CF3, respectively, cell wall pectic substances in CF4, cell wall cellulose in CF5 for cucumber fractions. The calcium-binding to extracted fractions revealed that calcium was bound by all fractions from all three materials, but those fractions dominant composed of pectic substances, membrane protein and cytoplasmic proteins, showed higher binding capacity.

Considering chemical composition and calcium binding capacity for each fraction, the potential biopolymers and sites that might contribute to the textural property improvements by calcium treatment in apples, cucumbers, and mushrooms identified with following specific conclusions:

1. Pectic substances play an important role in calcium-induced firmness in both cucumbers and apples, which confirms previously noted “egg-box” model.

2. The possible sites and macromolecules for calcium-binding in mushrooms include cytoplasmic and cell membrane proteins.

3. Cell membrane proteins from apples and cucumbers, and cytoplasmic proteins from cucumbers also showed high calcium-binding capacity, which is consistent with what has been observed in mushrooms and suggests cell membrane and cytoplasmic proteins may contribute to calcium-induced firmness as side effects to the “egg-box” model.

This study identified cytoplasmic and cell membrane proteins as the most potential macromolecules involved in calcium-binding other than pectic substances. However, the binding of calcium to compounds in plants and mushrooms is complicated and remains unclear. Calcium may bind to calcium-related proteins, and negatively charged residues of polysaccharides and proteins. Binding may take place by ion exchange, chelation, and adsorption. High calcium-binding capacity of isolated fractions alone can not explain the contribution of calcium in texture improvement. Considering the effects of calcium on firmness improvement, possible biopolymer-calcium complexes, and the influence of the complexes on cell microenvironment need to be characterized to fully explain the function of calcium in softening retardation.

Vita

Xiaoyun Deng was born in Changyang, Hubei, China on December 19, 1975. She went to Huazhong Agricultural University in Wuhan Hubei in September of 1994. There she received a bachelor of Agronomy degree in Plant Protection in July 1998. After graduation, she worked as Assistant Agronomist in Jingzhou Institute of Agricultural Science in Hubei, China from July 1998 to August 2000. In September of 2000, he enrolled in College of Plant Science in Huazhong Agricultural University and received a Master of Science degree in Plant Pathology in July 2003. In January 2006, she enrolled in Department of Food Science and Technology in University of Tennessee, Knoxville and worked as a graduate research assistant. She will obtain her Master of Science degree in Food Science on August 2008.