Aspergillus niger Control on Strawberries by Recombinant Tobacco Osmotin for Extending Shelf-life

by

Dong Chen

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 6, 2012

Keywords: osmotin, protein purification, protein stability, antimicrobial, strawberry, fungi

Approved by

Tung-Shi Huang, Chair, Professor of Poultry Science
Jean Weese, Professor of Poultry Science
Manpreet Singh, Associate Professor of Poultry Science
Narendra Singh, Professor of Biological Sciences

Abstract

Osmotin, a pathogenesis-related (PR) protein, possesses antimicrobial activity. The objectives of this study were: 1) to determine the stability of the recombinant tobacco osmotin (rOSM), 2) to define the antimicrobial profiles of rOSM, and 3) to control *Aspergillus niger* on strawberries by rOSM. The rOSM was expressed in *Escherichia coli* and stored at -20, 4, and 25 °C for stability study. The antimicrobial activities of rOSM were tested against *A. niger*, *Penicillium aurantiogriseum*, *Cryptococcus neoformans* and 26 foodborne pathogenic bacteria. Strawberries were inoculated with *A. niger* spores and treated with rOSM. The infection rates of strawberries were recorded daily at 25 °C for 8 days. The rOSM was stable for 6 months at -20, 4, and 25 °C. The antimicrobial activity of rOSM was dose and microorganism related. The rOSM treated strawberries showed longer shelf-life. These results indicated that rOSM has a potential use in foods for food safety.

Acknowledgements

I would like to first thank my major advisor, Dr. Tung-Shi Huang, for his selfless help, patience and guidance during the past three years. I am so impressed by Dr. Huang's hard-working spirit, wide range of knowledge, and optimistic perspective of life. Gratitude is extended to the other committee members, Dr. Jean Weese, Dr. Manpreet Singh and Dr. Narendra Singh for their precious time and expertise. I am also grateful to my parents, Ronghui Chen and Qiong Lai, who have sacrificed a lot for my growth. Without the encouragement of my parents, I could not have succeeded in my tasks. I would like to extend special thanks to the previous colleague in my lab, Dr. Ywh-Min Tzou, who has helped me to tackle a lot of problems that I encountered during my research. Dr. Tzou also made the cobalt beads for my research and gave me constructive suggestions. Lastly, I am grateful to our lab technician, Vondraniece Johnson, who helped wash glassware during the past year. Without the help from the people mentioned, I could not have finished my master study.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AMB	Amphotericin B
ATCC	American Type Culture Collection
BPB	Butterfield's phosphate buffer
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FAO	Food and Agriculture Organization
GMP	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HACCP	Hazard Analysis and Critical Control Point
LB	Lysogeny Broth
LBGM	Lysogeny Broth Containing 0.25% Glucose, 10 mM $MgSO_4$
MIC	Minimal Inhibitory Concentration

NASH	Non-Alcoholic Hteatohepatitis
O.D.	Optical Density
PBS	Phosphate Buffer Solution
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEG	Polyethylene Glycol
PR	Pathogenesis Related
PS	Potassium Sorbate
RNA	Ribonucleic Acid
rOSM	Recombinant Tobacco Osmotin
SAS	Statistical Analysis Software
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
*Sn*OLP	*Solanum nigrum* Osmotin-like Protein
STEC	Shiga Toxin-Producing *Escherichia Coli*
TLPs	Thaumatin-like Proteins
UV	Ultraviolet

CHAPTER 1: INTRODUCTION

This chapter addresses the food safety issues in current global food market and food service. Foodborne pathogens are the most notorious sources to compromise the safety of food. The purposes and significances of this study will also be mentioned throughout this chapter.

1. Background

As the demand of food has been growing so rapidly and globally, food safety becomes a great concern around the world. Foods are produced all over the world and traded internationally. Therefore, food safety is not only a regional problem but a global concern. Contamination of foods has raised more negative impacts on the consumers' health and social economy than ever before.

Foodborne illnesses are caused by the consumption of foodborne pathogens, toxins and chemicals contaminated foods. In the United States, the Centers for Disease Control and Prevention (CDC) estimated that there were 1,000 reported disease outbreaks and 48 million foodborne illnesses each year causing 128,000 hospitalizations and 3,000 deaths (CDC 2011). However, these data are considered underestimated, since not all foodborne diseases were reported. The incidence of foodborne illnesses is even more difficult to estimate in developing countries because of the regulations, enforcement, and awareness.

Foodborne diseases bring an enormous social and economic burden to the health systems. Scharff (2012) estimated that $77.7 billion was cost annually by using enhanced models in the United States due to foodborne illnesses associated with 31 identified pathogens and unspecified agents. *Salmonella*, *Listeria*, *Campylobacter*, and *Escherichia coli* O157:H7 are responsible for the most foodborne illnesses. In 2009, peanut butter produced by Peanut Corporation of America in Blakely, Georgia was tested positive for *Salmonella* resulting in the deaths of 9 people and at least 714 sickened (CDC 2009). Fungi such as *Aspergillus* and *Penicillium*, not only cause diseases and allergy to some people, but also negatively affect the yield of crops. Moreover, since September 11th terrorist attacks in 2001, some pathogenic bacteria and fungi became a great concern for being used as bioweapons (Pappas and others 2006).

Although some antimicrobial markers in genetically modified crops can increase the crop yield and reduce the use of pesticides and preservatives, safety is still of public concern. The addition of antibiotics in food production is also worried about the transfer of antibiotic resistance factors to human and animal pathogens. The concepts of “natural” and “minimally processed” foods are currently popular among consumers, who are already questioning the negative impact of applying chemical preservatives, such as sodium benzoate and potassium sorbate to foods.

2. Purpose of Study

Because of the concerns mentioned previously, natural antimicrobial agents have attracted the attention of food safety specialists. One of them is the proteins expressed by plants, since they do not have the animal-like immune system but need to express antimicrobial peptides acting as a defensive system. Osmotin, which belongs to a group of natural and edible

antimicrobial proteins called pathogenesis-related (PR) proteins, has gained a great attention for several reasons. Osmotin was first identified as the most abundant protein in cultured tobacco cells adapted to osmotic pressure (Singh and others 1985). Secondly, regulation of osmotin gene has been studied extensively in plants. Thirdly, osmotin is so unique that it can be induced in response to both biotic and abiotic stresses in plants. Besides, osmotin expressed in transgenic plants had shown antifungal activity (Liu and others 1996). Last but not least, since osmotin and its homologs are believed to be present in most edible plants, this protein is widely thought safe for human consumption (Singh and others 1987).

The purposes of this study are 1) to determine the stability of rOSM stored at different temperatures, 2) to test its antifungal and antibacterial activities against fungi and 26 foodborne pathogenic bacteria, and 3) to apply the recombinant tobacco osmotin on strawberries to control *Aspergillus niger* for extending shelf-life.

3. Significance of Study

Currently, commonly used preservatives in the food industry are artificial chemicals. These chemical preservatives have disadvantages. First, due to the various conditions of food matrices, pHs, temperatures and oxidation-reduction potentials, chemical preservatives cannot fully exhibit their antimicrobial activity. For example, the antimicrobial activity of sorbic acid will be compromised when the pH is above 6.5. Second, some food preservatives may react with other food constituents and produce carcinogens. Nitrite is often employed to inhibit bacterial growth, especially to inhibit clostridium growth in meats. This chemical is also able to stabilize the red color of meat and develop the flavor. However, Nitrite can react with secondary amines, tertiary amines and quaternary ammonium compounds to form nitrosamines, which may be

carcinogens (Jay and others 2005). Third, acceptability of the addition of chemical preservatives to foods has been decreasing among the customers and consumers nowadays. With the concept of “green and organic food”, people tend to prefer natural and minimally processed food.

Osmotin is a natural protein which was first found in tobacco. Then osmotin and its homologs have been found to widely exist in many plants, which people have eaten for hundreds of years (Tzou and others 2011). Osmotin has shown excellent antifungal activity in previous study (Abad and others 1996). But the research of antimicrobial activity of osmotin has not been investigated intensively due to the limited sources of osmotin. Recently, the recombinant tobacco osmotin has been successfully expressed in *E. coli* (Tzou and others 2011). Besides, whether osmotin has synergy with other preservatives or whether osmotin shows antifungal activity at practical settings as well as that at laboratory settings still need further research. This study offers a new perspective to explore the characteristics of osmotin and its potential use as a food preservative.

CHAPTER 2: REVIEW OF LITERATURE

This chapter is a literature review related to current foodborne pathogens, preservatives that are used by people and their unsafe side to human health. Dipping and coating strategies for extending shelf-life of strawberries will be discussed as well. The pathogenesis-related proteins are explored and osmotin is highlighted in this chapter to emphasize its antimicrobial activity. It is important to notice that natural and edible osmotin has the potential to be used as a food preservative substituting the chemical preservatives which have gained more worries.

1. Foodborne Pathogen

Foodborne pathogens include pathogenic bacteria, fungi, viruses, parasites, and prion. Pathogens exist in the environment ubiquitously and are widely found in soil, water, air, animals and plants. There are a lot of possibilities by which pathogens are brought into foods. Raw materials, such as meats and plants, may be contaminated with foodborne pathogens before they are transported into processing plants. Jones and others (2008) estimated that over the past 60 years, about 30% of all emerging infections were caused by foodborne pathogens. This trend might be intensified along with the food industry globalization.

Among these pathogens, pathogenic microorganisms are responsible for most foodborne illnesses and outbreaks. Pathogenic microorganisms can cause diseases by breaching cellular barriers. They can also replicate, persist and even express specific virulent agents in a host for

transmission to a new vulnerable host (Bhunia 2008). There are beneficial and spoilage microorganisms as well. People have hundreds of years of history in applying beneficial microorganisms to make fermented foods such as cheese, beer and kimchi. Food spoilage could occur due to the existence of spoilage microorganisms under poor processing and storage conditions. Foods contaminated with spoilage microorganisms may result in undesirable texture, flavor and appearance, which would not be suitable for human consumption.

Diarrheal diseases are thought to be a symptom of a foodborne infection. Mild to severe complications such as joint infection, kidney failure, paralysis, bloodstream infection, miscarriage and so on could also be developed in more vulnerable people (CDC 2011). The social burden caused by foodborne illnesses could not be neglected. For instance, 1 million of *Salmonella* transmitted foodborne infections were estimated each year (Scallan and others 2011) and all cases of salmonellosis would cost $2,708,292,046 for the medical expense (USDA 2011), and a single lethal case of Shiga Toxin-Producing *Escherichia Coli* (STEC) O157 would cost $7 million (Frenzen and others 2005).

In 2010, a total of 19,089 infections with 4,247 hospitalizations and 68 deaths were confirmed by FoodNet (CDC 2011). Among these infections, *Salmonella* led to 8,256 infections and the largest number of hospitalizations (2,290) and deaths (29). Compared to the 2006-2008 period, the incidence of *Salmonella* infection in 2010 increased by 10%. Among the most common serotypes, Typhimurium did not change significantly, while Enteritidis and Newport increased by 36% and 47%, respectively (CDC 2011).

Significant declines in incidences for some common bacterial pathogens have been achieved in 2010 when compared to 1996-1998. The incidence of *Campylobacter* decreased by

27%, *Listeria* by 38%, *Salmonella* by 10%, *Shigella* by 57%, STEC O157 by 44%, and *Yersinia* by 52%. Nevertheless, the incidence of *Vibrio* species had increased by 115%, and also 39% and 16% more infections than 2006-2008 period and 2009, respectively (FoodNet 2010). Although it is not common the estimated incidence of the *Vibrio* infection has been increasing for almost the past 10 years.

Another main source of foodborne pathogen is fungi. Fungi are classified as a kingdom which is separate from animals, plants and bacteria. The accurate number of fungal species is still unknown. Most fungi can produce spores, which could be transported by water, air, soil and insects. The large portion of fungi is called *mycelium* which is composed of branch-like *hyphae*. Some fungi are beneficial to humans, such as edible mushrooms, but there are some species which can cause allergic reactions and respiratory problems. Certain fungal species can produce toxic substances called mycotoxins under proper conditions. Most mycotoxins are believed to be carcinogens and suspected as mutagenic and teratogenic compounds. Due to the production of mycotoxins, *Aspergillus* and *Penicillium* are two main genera of research interest in the food safety. Aflatoxin is one of the most potent carcinogens (Stark 1980). The Food and Agriculture Organization (FAO) of the United Nations estimated that 25% of global crops are contaminated by mycotoxins, and the aflatoxin is the most common. Even though aflatoxin is a well known cancer-causing mycotoxin, it is considered unavoidable in foods and animal feeds. Aflatoxins are divided into B_1, B_2, G_1, G_2, and M_1 chemical forms by the color of fluorescence released when they are exposed to UV light. In the United States, aflatoxin limit is regulated as less than 0.5 µg/kg for M_1 in milk and 20 µg/kg in all other foods. The best way to prevent and control mycotoxins is by strictly following Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP). Sensitive methods for mycotoxins detections are now

available and products containing mycotoxins over regulatory limit will be immediately excluded from the processing line (Bhunia 2008).

Yeasts are eukaryotic microbes belonging to the fungi kingdom. Like bacteria, yeasts could also be divided into useful and pathogenic ones. Useful yeasts are widely used in winemaking and bakery industries due to their different metabolic pathways of sugar fermentation under low or high oxygen conditions. However, some species of yeasts are opportunistic pathogens which threaten the safety of food. Among the pathogenic yeasts, *Cryptococcus neoformans*, a facultative intracellular pathogen, is characterized as an encapsulated budding yeast. It is a common cause of human disease, especially in immunecompromised people. The infection of *C. neoformans* is termed cryptococcosis. The mechanism of virulence is still elusive, but phenol oxidase production, growth at 37 °C and a capsule seems the requirements of pathogenicity (Mitchell and Perfect 1995). Cryptococcosis is reported as the leading mycological cause of mortality and first indication among Acquired Immunodeficiency Syndrome (AIDS) patients (Chuck and Sande 1989). The incidence of cryptococcosis among AIDS patients has been estimated as 6 to 10% in the United States, Western Europe, and Australia and 15-30% in sub-Saharan Africa (Powderly 1993).

Currently, resistances of *C. neoformans* to amphotericin B (AMB), flucytosine, and azoles have been widely investigated *in vitro* and *in vivo*. The MICs of these chemicals are low but lack standardization (Ghannoum and others 1992). The azole compounds have been used clinically to treat cryptococcosis and the usage has been accelerated since the advent of AIDS epidemic. However, the research on their side effects and potential toxicities to humans are needed (Mitchell and Perfect 1995). Combination of these chemicals has shown potential to fight

against *C. neoformans* and the following combinations have shown positive interactions: AMB-flucytosine, AMB-azole, AMB-rifampin, flucytosine-azole and even triple combination of AMB-flucytosine-azole (Medoff and others 1972; Polak and others 1982). Nonetheless, these combinations need to be standardized and more *in vitro* and *in vivo* antifungal studies.

2. Current Food Preservatives and Their Disadvantages

Adding food preservatives into foods is one of the essential ways to preserve foods and extend the shelf-life. Food preservatives are used to stop or inhibit the growth of spoilage and pathogenic microorganisms thus enhancing storage time. Currently, several chemicals, which serve as antimicrobial agents or antioxidants, are widely used. However, these chemicals have recently raised a lot of controversies about their negative health impacts.

Sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) are commonly used in cured meat to stabilize color, improve flavor and inhibit bacterial growth (Shahidi and Pegg 1992). However, when nitrites meet secondary amines, they react and form nitrosamines — well-known carcinogens. Studies have shown that cured meats containing nitrite and nitrosamines associate with higher incidence of gastric, oesophageal (Jakszyn and Conzalez 2006) and colorectal cancers (Cross and others 2010). Chronic exposure to cured meats with nitrite or nitrate contributes to the pathogenesis of insulin-resistance diseases, including neurodegeneration, diabetes mellitus (DM), and non-alcoholic steatohepatitis (NASH) (de la Monte and others 2009).

Sulfites have a long history of being used as food preservatives. They include sulfur dioxide (SO_2), sodium and potassium salts of sulfite (Na_2SO_3 and K_2SO_3), bisulfate ($NaHSO_3$ and $KHSO_3$) and metabisulfite ($Na_2S_2O_5$ and $K_2S_2O_5$). They are widely used in beverages, beers, fruits, vegetables, meats and pharmaceuticals. Nevertheless, Gunnison and Jacobsen (1987)

reported that 5 to 10% of chronic asthmatics were very sensitive to the sulfites, which might cause difficulty in breathing immediately after eating a food preserved by sulfites. Vitamin B_1, also known as thiamine, was believed to decompose at the presence of sulfites (Dwivedi and Arnold 1973). The sulfites are therefore not allowed to be added in foods considered as thiamine sources.

Benzoic acid, sorbic acid and propionic acid are also commonly used as preservatives in foods. They are generally recognized as safe (GRAS) and are able to possess their antimicrobial activity only when they are undissociated. They exert higher antimicrobial activity at lower pH values. The pK of benzoate, sorbate and propionate are 4.2, 4.8 and 4.87, respectively. At a pH of 4.0, 60% of benzoate, 86% of sorbate and 88% of propionate are undissociated. Nonetheless, when the pH is 6.0, only 1.5% of benzoate, 6% of sorbate and 6.7% of propionate remain undissociated (Jay and others 2005). Thus they are thought to perform effectively below a pH 6.0. However, a low pH could inhibit the growth of most bacteria. Addition of these acids in acidic foods could inhibit the growth of yeasts and molds, but their applications are limited.

3. Current Strategies to Extend Shelf-life of Strawberries

Strawberries are a good source of vitamin C and phenolic compounds but they have a short post-harvest shelf-life (Aguayo and others 2006). Except mechanical damage and physiological deterioration, water loss is the key factor which facilitates the deficit of nutrients and sensory properties (Nunes and others 1998). Strategies have been developed to extend the shelf-life of strawberries, including low temperature storage, modified atmosphere packaging, improvement of washing and sanitizing operations, dipping and coating treatments. In this

literature review, dipping and coating treatments are the main focus since these treatments parallel the application of osmotin on strawberries for this study.

Dipping treatments could be better for protecting the quality of strawberries by rinsing off the bacteria, molds, enzymes and other degradative agents from the fruit surface. Dipping time often ranges from 1 to 5 min. Luna-Guzman and others (1999) reported that the dipping time did not improve firmness of fresh-cut cantaloupe after dipped in $CaCl_2$. Unlike dipping time, dipping temperature is widely thought to play an important role in this treatment. Lurie and Klein (1992) also found that apple (*Malus domestica* Borkh. cv. Anna) quality would be better for those treated with $CaCl_2$ and heat than that with individual treatment alone. This synergistical phenomenon was also reported by Garcia and others (1996). These two treatments may provide the energy to form the salt bridge cross-links with demethylated pectin, resulting in less accessible cell wall to the decay enzymes (Sams and others 1993). Dipping temperature of 60 °C is believed beneficial to the fruits (Soliva-Fortuny and Martin-Belloso 2003), but to the strawberries, 45 °C is the best temperature to extend shelf-life without damaging fruit quality (Garcia and others 1996). Drying of wet surfaces is recommended by draining with caution. During drying, the strawberries are suggested to be handled carefully to avoid mechanical bruises and contamination with microbial spoilage (Soliva-Fortuny and Martin-Belloso 2003).

Fungal infection, physical injuries and physiological disorders are three major factors threatening the quality of strawberries (Sanz and others 1999). The edible coatings act as a barrier which could retard the microbial contamination, prevent loss of volatile compounds, improve selective gas permeability and reduce respiration rate. They also serve as a carrier of food additives, such as antimicrobial agents, flavors, colors and antioxidants (Kester and

Fennema 1986). Compared to the conventional synthetic films, more and more natural and edible coatings have been studied for the concerns of the food safety by treating with synthetic chemicals. Del-Valle and others (2005) reported that prolonged shelf-life of strawberries was fulfilled by the application of a cactus-mucilage (*Opuntia ficus indica*) edible coating. In recent years, chitosan, a modified natural biopolymer derived by deacetylation of chitin which is a major component of the shells of crustacean, has gained a lot of attention to be used as coating to extend shelf-life of strawberries. Chitosan has two characteristics, which are antimicrobial activity and film-forming properties, making it a potential source for coating (No and others 2007). El Ghaouth and others (1991) first reported to apply chitosan coating to control decay of strawberries. Chitosan coating was found effective to inhibit the infection of *Botrytis cinerea*, *Cladosporium* sp. and *Rhizopus stolonifer* on strawberries due to its fungistatic property (El Ghaouth and others 1992; Park and others 2005). The addition of calcium, vitamin E (Han and others 2004; Hernandez-Munoz and others 2006, 2008) and oleic acid (Vargas and others 2006) to the chitosan coating formulation can increase the nutritional value and intensify the antimicrobial activity of chitosan. The trend of using natural antimicrobial substances in food preservation will be prevalent in the future.

4. Pathogenesis-related (PR) Proteins

In response to pathogen infections, plants, which lack an immune system, are able to accumulate a set of proteins, called pathogenesis-related (PR) proteins (Kononowicz and others 1992). Originally, five families of PRs (PR-1 to -5) were identified in tobacco by both molecular and biochemical techniques (Van Loon and others 1987; Bol and others 1990). In 1994, 11 families of PRs were characterized in tobacco and tomato, and there are more PR-like proteins to be classified. Standards for inclusion of new PRs were developed as follows: 1) Protein must be

induced only under the condition of pathogen invasion, and 2) induced protein must be confirmed in different laboratories independently if the expression has been in only one plant-pathogen combination, or must be proved to exist in at least two different plant-pathogen combinations (Van Loon and Van Strien 1999). Based on these criteria, due to their different primary structures, serological relationships, amino acid sequence similarities and functions, PR proteins have been grouped into 17 families thus far (Van Loon and others 2006). However, only group 1 through 5 have shown antifungal activity both *in vivo* and *in vitro* (Abad and others 1996). The common properties of PRs are: 1) they are easily separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under native conditions; 2) high proportion of PRs has been found to be secreted in extracellular spaces between cells; 3) PRs are highly resistant to digestion of proteolysis; 4) low pH is the preferable condition to extract PRs selectively (Legrand and others 1987).

Most PRs have a molecular weight of 5-70 kD and they are highly resistant to proteolytic enzymes. These characteristics help plants to resist the biotic and abiotic stresses. The highly proteolysis-resistant PRs also play an important role to trigger an allergic response, while humans consume the PRs-containing plants. PR genes transgenic economical plants have been widely developed for their relatively high resistance to pathogen infections. However, it should be carefully evaluated due to their possibility of allergen sources (Hoffmann-Sommergruber and others 2000).

A typical member of each PR family has been well researched, which is helpful to search homologs in the same or related plant species. Among the families, the mechanism of antifungal effect of PR-1 family members is still not totally understood, but its antifungal activity was demonstrated both *in vitro* and *in vivo* (Niderman and others 1995). Expression of PR-1 eased

the symptoms in tobacco which was infected by *Peronospor tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander and others 1993). PR-2 consists of β-1,3-glucanases. PR-3, -4, -8 and -11 are mainly chitinases, which exhibit both antifungal and lysozyme activities. Chitinases and β-1,3-glucanases could work synergistically to boost their antifungal activity both *in vitro* and *in vivo*. Therefore, the resistance against *Cercospora nicotianae* was greatly enhanced in tobacco which expressed both chitinase and glucanase (Zhu and others 1994; Jach and others 1995). In tomato, both protein expressed plants gave an apparent inhibition against *Fusarium oxysporum* f.sp. *lycopersici*, but transgenic plants expressing either one of the two proteins did not show any antifungal activity (Jongedijk and others 1995). The structures of PR-5 proteins are very similar to thaumatin and they are homologs to permatins, which are able to penetrate fungal membranes. Like some PR-1 proteins, basic PR-5 proteins also exhibited antifungal activity against *P. infestans* (Vigers and others 1992; Zhu and others 1996). PR-6 is a group of proteinase inhibitors which are in defense against foreign invaders, such as microbes and insects (Van Loon 1997). PR-7 serves as endoproteinase, which are thought to hydrolyze the cell walls of fungi. PR-9 family consists of peroxidases which might strengthen cell walls by forming lignin in response to pathogen invasion (Van Loon and Van Strien 1999). The PR-10 family is like ribonucleases at the structural level and it consists of cytoplasmic proteins (Moiseyev and others 1997), but whether it has the ability to cleave RNA is still unclear. All the plant defensins (PR-12), thionins (PR-13), and thaumatin-like proteins (LTPs, PR-14) have been shown to possess antifungal and antibacterial activity due to their effect on plasma membrane of invaded pathogens (Bohlmann 1994; Garcia-Olmedo and others 1995; Broekaert and others 1997). PR-15 and -16 are two PR proteins that are mainly found in monocots. They are two families of germin-like oxalate oxidases and oxalate oxidase-like proteins which have superoxide

dismutase activity, respectively (Bernier and Berna 2001). These two families of PRs either stimulate defense responses of plants directly or indirectly generate H_2O_2 which would intoxicate foreign invaders (Van Loon and others 2006). The mechanism of action of PR-17 proteins is still elusive, but it was believed that they contained sequences very close to the active site of zinc-metalloproteinases (Christensen and others 2002).

Acidic PRs are mainly induced and secreted into the intercellular space of the leaf upon infection, while basic counterparts are expressed at a relatively low level in healthy plants and transported to the vacuole by a signal located at the C-terminus. In addition, basic PR proteins are controlled in a tissue-specific manner (Eyal and Fluhr 1991; Linthorst 1991). Gaseous phytohormone ethylene and methyl jasmonate are able to regulate the expression of basic PR proteins while signaling molecules like salicylic acid and reactive oxygen species could mediate the expression of acidic PR genes upon pathogen invasion (Hoffmann-Sommergruber 2000). Mauch and Staehelin (1989) suggested the extracellular PRs would set the first defense line to the pathogen invasion and if it failed, the basic PRs in vacuole could act as second line to encompass the pathogen by lytic process.

5. PR-5 Family

PR-5 Proteins are also called thaumatin-like proteins (TLPs) since this group of proteins has a high level of similarity to the sequence of thaumatin (Hoffmann-Sommergruber 2000). Thaumatin tastes sweet and it originated from a tropical plant named *Thaumatococcus daniellii* (Daniell 1852). PR-5 proteins were detected expressed in leaves of young tobacco, but would accumulate to a high level in a short period if the plant underwent pathogen infection or abiotic stress, such as salinity (LaRosa and others 1986). Therefore, based upon the PR inclusion

standards mentioned earlier, some PR-5 proteins are not PRs, but PRs-like. This group of proteins was also detected developmentally expressed at a high level in overripe fruits of *Diospyros texana* and cherries, and flower buds of *Brassica* (Vu and Hyunh 1994; Fils-Lycaon and others 1996; Cheong and others 1997). The molecular weight of PR-5 ranges from 22 to 26 kD, while there are also 16kD proteins belonging to this family since 58 amino acids are deleted. Among the 58 amino acids, 16 cysteines which are highly conserved between the higher molecular weight PR-5 proteins form 8 disulfide bridges.

Although PR-5 proteins share high similarity in sequence, even small differences in amino acids leads to different functions. Compared to sweet tasting thaumatin which exhibited nearly no antifungal activity (Ogata and others 1992; Zemanek and Wasserman 1995), zeamatin possessed relatively high antifungal activity without sweet taste (Malehorn and others 1994).

All the PR-5 proteins have shown antifungal activity, but the mechanism of action is not totally clear. One hypothesis was that PR-5 proteins acted like β-1,3-glucanase to hydrolyze β-1,3-glucans from invading pathogens (Grenier and others 1999); the other hypothesis was that PR-5 proteins could insert into the fungal membrane resulting in forming a transmembrane pore, which would cause a destabilized membrane and result in cell rupture (Roberts and others 1990).

Transgenic plants overexpressing PR-5 proteins do not surely increase biotic resistance. In transgenic tobacco and potato, overexpression of osmotin showed more resistance to *P. infestans* (Zhu and others 1996). However, transgenic tobacco overexpressing osmotin did not show any enhanced resistance to *Phytophtera parasitica* var. *nicotianae*. The symptoms of transgenic rice plants overexpressing a 23 kD rice PR-5 protein were like those of control plants

under the infection of *Rhizoctonia solani* except the transgenic plants showed smaller lesions (Datta and others 1997).

6. Osmotin

Osmotin was first identified as a protein synthesized in tobacco cells which were adapted to the osmotic stress (Singh and others 1985), but was later found it had antifungal activity *in vitro* against a series of fungi, including economic plant pathogens (Woloshuk and others 1991; Vigers and others 1992; Liu and others 1994, 1996; Abad and others 1996; Tzou and others 2011). Therefore, osmotin could be induced by both biotic and abiotic stresses, including high salinity, and abscisic acid (Singh and others 1989).

In the osmotin asymmetric units, there is a noncrystallographic dimer. The tertiary structures in the two monomers are a little different. There are three domains in the osmotin crystals, and some PR proteins including thaumatin and osmotin share a similar fold. Domain I is the core of the whole molecule, which is composed of an 11-strand, flattened β-sandwich (residues 1-53, 82-125 and 175-205); domain II is composed of several loops stabilized by four disulfide bonds (residues 126-174). There is an apparent cleft formed between domain I and II. Domain III is composed of a small loop (residues 54-81) which is stabilized by two disulfide bonds (Min and others 2004).

Osmotin and thaumatin have a lot of similarities, including structure, molecular weight, the presence of several disulfide bonds, the lack of sulfhydral residues, a high proportion of proline, and a basic pI. However, thaumatin is a sweet protein, while osmotin does not exhibit any sweetness. Besides, antiosmotin-I did not cross react with thaumatin. There were two forms of osmotin purified from tobacco cell suspension cultures adapted to NaCl, named Osmotin I,

aqueous soluble form and II, detergent soluble form. These two forms of osmotin were accumulated in adapted cells at an approximate radio of 2:3 (Singh and others 1987). The pI of Osmotin-I was 7.8 while the pI of Osmotin-II was over 8.2. A 26 kD precursor of osmotin was synthesized but not accumulated in control cells and its pI value was lower than both Osmotin-I and Osmotin-II purified from adapted S-25 cells (Singh and others 1985, 1987). The molecular weight of mature form of osmotin was 24 kD (Min and others 2004). Both the Osmotin-I and Osmotin-II shared the same first 22 amino acids on the N-terminal end. Osmotin-I was more susceptible to protease digestion than Osmotin-II, which was inexorable to protease due to its presumed posttranslation modifications. However, as explained earlier, osmotin was highly resistant to proteolysis. Based on this premise, even Osmotin-I could only be partially digested by *Staphalococcus* V-8 protease and trypsin. N-terminal sequence of Osmotin-I was found still intact after proteolysis. This might indicate different osmotin forms were translated from different genes or at least processed differently (Singh and others 1987).

Vigers and others (1991) indicated that zeamatin, an antifungal protein expressed in corn, had homologous N-terminal sequence with osmotin, so osmotin might have antifungal properties. Also, Woloshuk and others (1991) identified antifungal protein AP24 was actually osmotin by using the spore germination bioassay with *P. infestans*. Osmotin was also induced strongly during the hypersensitive response to tobacco mosaic viruses by Stintzi and others (1991). After that, it has been found that the expression of osmotin was not only induced by virus infection but also by other biotic factors, such as bacteria and fungi. Osmotin gene transgenic tobacco and potato were developed and osmotin production was equivalent to those induced by fungal infection in nontransformed plants. However, only transgenic potato plants exerted disease resistance to spore inoculation of *P. infestans*, which is the cause of late blight disease. Purified

osmotin was observed more effective against *P. infestans in vitro*. Certain inhibition of *P. nicotianae* was also found *in vitro* even though no *in vivo* effect could be observed (Liu and others 1994). To determine spectrum of *in vitro* antifungal activities of osmotin, Abad and others (1996) reported osmotin showed some degree of inhibition to the growth of 31 isolates representing 18 fungal genera by inducing spore lysis, inhibiting spore germination or decreasing germing viability. Among these fungi assayed, hyphal growth of *Trichoderma longibrachiatum*, *Bipolaris*, *Fusarium* and *Phytophthora* species was very sensitive to the osmotin, while *Aspergillus flavus*, *A. parasitica*, *Phizoctonia solani* and *Macrophomina phaseolina* was least sensitive to osmotin treatment.

Osmotin is a basic PR-5 protein which is mostly secreted in intracellular compartments, probably in vacuoles. However, compared to the basic, vacuolar forms of PR-5 proteins with a C-terminal extension, all C-terminal 20 amino acids truncated osmotin was secreted extracellularly as acidic forms of PR-5 proteins and they still possessed the antifungal activity to *P. infestans* (Liu and others 1996). Since many fungi initially invade into the extracellular space of host plants, the presence of truncated osmotin secreted extracellularly might show its antifungal properties to retard disease symptoms.

Roberts and Selitrennikoff (1990) proposed that zeamatin, which was homologous to osmotin, might insert directly into the membrane to form transmembrane pores resulting in amino acid leakage. Abad and others (1996) confirmed this hypothesis and further showed osmotin only exerted its antifungal activity under hypotonic conditions. A member of PIR proteins, induced by heat and nitrogen limitation, was the determinant in the cell wall of the yeast *Saccharomyces cerevisiae* to resist osmotin (Yun and others 1997; Narasimhan and others 2003). Osmotin was afterwards reported involving in subverting target cell signal transduction

pathway in yeast (Yun and others 1998). Osmotin could induce apoptosis in *Saccharomyces cerevisiae* and its mammalian homolog is adiponectin, which regulates cellular sugar and lipid metabolism (Narasimhan and others 2001, 2005). Although the sequences of these two homologs are totally different, they share same β barrel domain.

Since osmotin and its homologs are present in most edible plants, osmotin is supposed to be nontoxic to humans. Massive expression of osmotin is a great potential in the future for food safety application. However, expression of osmotin has failed due to its toxicity to microorganisms and biochemical properties. *Solanum nigrum* osmotin-like protein (*Sn*OLP) was reported to be expressed successfully in *E. coli* (Campos and others 2008), but it was still different from tobacco osmotin at some critical surface amino acids (Min and others 2004). Tzou and others (2011) developed a protocol by which truncated tobacco osmotin could be successfully expressed in *E. coli* and the recombinant osmotin showed antifungal activity *in vitro*. In the present study, osmotin was expressed by using the protocol developed by Tzou and others (2011) and further antimicrobial activities of recombinant osmotin were tested.

To make the wholesome and safe foods, manufacturers are trying to find more natural antimicrobials without using chemical preservatives. Natural antimicrobial peptides have gained more attention in the food industry to extend the shelf-life. Osmotin, a member of PR-5 proteins, has showed antifungal activity *in vitro*. Besides, osmotin exists in tobacco and some other edible plants, which means this protein has been eaten by people for hundreds of years and it should be safe for human consumption. Thus, osmotin has the potential to become a food preservative. This study will not only investigate its antifungal activity to three common fungi, but also determine the antibacterial activity to common foodborne pathogenic bacteria. Its practical application on strawberries to control *Aspergillus niger* infection will be also explored. Therefore,

this study can provide antimicrobial activity information of osmotin for its future application as a preservative.

CHAPTER 3: ANTIMICROBIAL ACTIVITY OF OSMOTIN

1. Materials and Methods

1.1. Pathogen Strains and Cultures Preparation

1.1.1. Yeast

Cryptococcus neoformans (ATCC 13690) was inoculated in YPAD liquid medium (1% yeast extract, 2% peptone, 2% glucose, and 0.01% adenine) and incubated in Environ Shaker (Lab-line Instruments, Melrose Park, IL) at 30 °C, 150 rpm for 16 h. The culture was streaked on YPAD plates and incubated at 30 °C overnight.

One single colony of *Cryptococcus neoformans* was transferred to YPAD medium and incubated at 30 °C with 150 rpm shaking overnight. The culture was then washed twice with PBS (phosphate buffered saline, pH 7.2) through centrifugation at 2,800 × *g* for 8 min at 4 °C. The yeasts were re-suspended in YPAD medium and the population was estimated by $O.D._{640nm}$ and adjusted to 10^2 CFU/mL for use.

1.1.2. Molds

Cultures of *Aspergillus niger* and *Penicillium aurantiogriseum* (Microbiologics® Inc., St. Cloud, MN) were maintained at 4 °C on potato dextrose agar (PDA, OXOID, Hampshire, England) slant test tubes. One week before use, 2 mL of autoclaved deionized water was added

to the tube and vortexed slowly to remove spores from mycelia. The spore suspension was spread on PDA plates and incubated at 25 °C for spore production.

The spores were harvested by adding 5 mL sterilized water to a plate and gently scraping off spores from mycelia with a spatula. The spore concentration was determined by hemacytometer (Hausser Scientific, Horsham, PA) and diluted to 10^6 conidia/mL for use.

1.1.3. Bacteria

The following bacteria were used in this study: *Bacillus cereus*, *Citrobacter amalonaticus* (SA 5615), *Enterobacter aerogenes* (ATCC 13048), *Escherichia coli* O157:H7 (204 P and ATCC 43895), *Klebsiella Oxytora* (ATCC 13182), *Listeria innocua* (ATCC 33090), *Listeria monocytogenes* (ATCC 19111 and G 3990 4b), *Morganella margani* (CDC 8103-85), *Salmonella dublin* (S 2424), *Salmonella enteritidis* (H 4638 and H 4639), *Salmonella mission*, *Salmonella mission* (NA resistant), *Salmonella montevideo*, *Salmonella montevideo* (rif-R), *Salmonella paratyphi*, *Salmonella paratyphimurium*, *Salmonella typhi* (CDC 3434-73 and CDC 9032-85), *Salmonella typhimurium* (ATCC 13311), *Shigella boydii* (ATCC 9207), *Staphylococcus aureus* (ATCC 12600), *Vibrio cholera* (M 802), and *Yersinia kritensenii* (PL 115185).

Each strain grew in Lysogeny Broth (LB, USB Corporation, Cleveland, OH) at 37 °C with 200 rpm shaking overnight and streaked on LB plates subsequently. The bacteria from single colony were cultured at 37 °C with 200 rpm shaking overnight in LB medium. The culture was then washed twice with PBS by centrifugation at 2,800 × *g* for 8 min at 4 °C. The bacteria

were re-suspended in LB medium and population was calculated by the pre-constructed standard curve. The concentration was further diluted to 10^2 CFU/mL.

1.2. Preparation of Recombinant Tobacco Osmotin

1.2.1. Expression of recombinant tobacco osmotin

Recombinant tobacco osmotin (rOSM) was expressed from constructed *Escherichia coli* as described by Tzou and others (2011). An *E. coli* single colony was selected and grown overnight at 37 °C in 250 mL LBGM medium (LB medium added with 0.25% glucose, 10 mM $MgSO_4$), containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol with shaking at 200 rpm. Then the culture was inoculated in 2,500 mL ZYM505 medium (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 0.05% glucose, 50 mM NH_4Cl, 25 mM KH_2PO_4, 25 mM Na_2HPO_4, 5 mM Na_2SO_4, 50 µM $FeCl_3$, 20 µM $CaCl_2$, 10 µM each of $MnCl_2$ and $ZnSO_4$, and 2 µM each of $CoCl_2$, $CuCl_2$, $NiCl_2$, Na_2MoO_4, Na_2SeO_3 and H_3BO_3) (Studier 2005; Tzou and Others 2011) containing 50 µg/mL ampicillin and 25 µg/mL chloramphenicol. When the $O.D._{600nm}$ of the culture reached to 2.5 ~ 4.0, the lactose at 0.2% (wt/vol) was added and the culture was incubated at 37 °C for another 8 hours to induce the recombinant protein production. The pH of the culture was adjusted to 6.7 ~ 6.8 with 10 M NaOH at 10 min, 1 and 4 h after adding lactose. Cells were collected through centrifugation at 4,500 × *g* for 30 min at 4 °C. Before sonicated, cells were suspended in 20 mM Tris/HCl, pH 8.0 buffer with 1 mg/mL lysozyme and incubated at 37 °C for 30 min to lyse the cells. The inclusion bodies were collected as a pellet by centrifugation at 15,000 × *g* for 10 min at 4 °C and then washed twice with 20 mM Tris/HCl, 1% Triton X-100, pH 8.0. The inclusion bodies were dissolved in 8 M urea for further rOSM purification.

1.2.2. Purification and refolding of recombinant tobacco osmotin

Cobalt (Co)-agarose affinity beads were used to bind rOSM by gentle rotation for 30 min at room temperature. The rOSM bound Co-agarose beads were collected by centrifugation at 700 × *g* for 3 min at 10 °C and then washed twice with 30 mL washing buffer (8 M urea, 10 mM NaH_2PO_4, 1% Triton X-100, 10 mM imidazole, pH 7.0). The protein was eluted 7 times at room temperature with one bed volume of elution buffer (8 M urea, 10 mM NaH_2PO_4, 1% Triton X-100, 200 mM imidazole, pH 7.0). The eluents were pooled and dialyzed in 50 mM sodium acetate (pH 5.2) for 48 h with 4 changes. The purified rOSM was stored at 4 °C for further use.

1.2.3. Optimization of refolding efficiency

In order to improve the recombinant protein refolding efficiency, a refolding kit (Thermo Scientific Pierce® Protein Refolding Kit, Product NO. 89867, Thermo Fisher Scientific Inc., Rockford, IL) was used. This refolding kit consisted of 9 base buffers (pH 8.2, Table 1) and 7 buffer additives (Table 2) for dialysis. These buffers included various denaturant conditions (0 to 1.1 M guanidine and 0 to 0.88 M arginine) for reducing protein aggregation. The additives were used to promote the refolding of native structure protein (Thermo Scientific 2008). The concentrations of rOSM dialyzed in these different combinations (Table 3) of buffers and additives were measured and antifungal activities were compared.

Table 1-Nine Base Buffers (pH 8.2) and Their Components (Thermo Scientific 2008).

Buffer	Components
1	55 mM Tris, 21 mM NaCl, 0.88 mM KCl
2	440 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
3	880 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
4	550 mM guanidine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
5	550 mM guanidine, 440 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
6	550 mM guanidine, 880 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
7	1.1 M guanidine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
8	1.1 M guanidine, 440 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl
9	1.1 M guanidine, 880 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl

Table 2-Buffer Additives (Thermo Scientific 2008).

Buffer Additives
Dithiothreitol (DTT)
Reduced Glutathione (GSH)
Oxidized Glutathione (GSSG)
10 mM Polyethylene Glycol (PEG)
Divalent Cation Stock
5 M NaCl
100 mM EDTA

Table 3-Different Combinations of 9 Base Refolding Buffers and Buffer Additives (Thermo Scientific 2008).

Base Refolding Buffer ID	Base Refolding Buffer Volume (µL)	100 mM EDTA (µL)	200 mM GSH (µL)	100 mM GSSG (µL)	H_2O (µL)	rOSM Eluent (µL)
1	900	10	10	2	28	50
2	900	10	10	4	26	50
3	900	10	5	10	25	50
4	900	10	10	4	26	50
5	900	10	5	10	25	50
6	900	10	10	2	28	50
7	900	10	5	10	25	50
8	900	10	10	2	28	50
9	900	10	10	4	26	50

1.2.4. Determination of recombinant tobacco osmotin concentration

The purity of the rOSM was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Favre 1973) and the concentration of rOSM was determined by Lowery protein assay (Lowery and others 1951).

1.3. Antimicrobial Assay

1.3.1. Assay of anti-yeast activity

The yeast growth medium was prepared by mixing equal volume of two times strength YPAD medium with rOSM solutions (0 to 1,400 µg/mL), respectively. Each mixed medium was subsequently inoculated by adding 100 µL of 10^2 CFU/mL *Cryptococcus neoformans*. Each treatment was triplicated. All the tubes were incubated at 30 °C with 150 rpm shaking. The $O.D._{640nm}$ of culture in each tube was measured at 12 h interval for 7 days.

1.3.2. Assay of anti-mold activity

1.3.2.1. Assay of mycelial growth inhibition

PDA plates were spread with 200 µL of rOSM solutions (0 to 1,400 µg/mL). Each concentration was triplicated. Agar disks (diameter = 0.8 cm) contained tested fungal mycelia were placed at the center of PDA plates. Hyphae were allowed to grow at 25 °C and the diameter of the colony was measured at 24 h interval for several days.

Recombinant tobacco osmotin and its combination with potassium sorbate were also used to test the antifungal growth. PDA plates containing rOSM, potassium sorbate and their combinations were prepared. The treatments were as follows: 350 µg/mL rOSM only, 700

µg/mL rOSM only, 100 parts per million (ppm) potassium sorbate only, 500 ppm potassium sorbate only, 350 µg/mL rOSM plus 100 ppm potassium sorbate, 350 µg/mL rOSM plus 500 ppm potassium sorbate, 700 µg/mL rOSM plus 100 ppm potassium sorbate, and 700 µg/mL rOSM plus 500 ppm potassium sorbate. The fungal spores were prepared as mentioned in section 1.1.2. and 100 µL of spore suspension (ca. 10^2 conidia/mL) was spread evenly on each plate. The plates were incubated at 25 °C. From each plate, 5 colonies were selected and their diameters were measured at 24 h interval for several days.

1.3.2.2. Inhibition of spore germination

Spore suspensions (ca. 10^6 conidia/mL) of *Aspergillus niger* and *Penicillium aurantiogriseum* were prepared and 100 µL of each suspension was added in 400 µL 2.25 times strength potato dextrose broth (PDB, Neogen Corporation, Lansing, MI) mixed with 500 µL 50 mM sodium acetate solution (control) or rOSM, resulting in final treatments containing 10^5 conidia/mL with 0, 300, and 700 µg/mL rOSM. Each treatment was tested in triplicate. Spore suspensions were incubated at room temperature for 20 to 30 h. After incubation, the total number of spores and the number of germinated spores were recorded by hemacytometer and the inhibition of spore germination was calculated.

1.3.3. Assay of antibacterial activity

1.3.3.1. Liquid medium method

The culture medium was prepared by mixing equal volume of 2 times strength LB medium with 50 mM sodium acetate buffer containing 0 or 1,400 µg/mL rOSM. The final concentration of rOSM in the medium was 700 µg/mL and the medium without rOSM was the control.

The bacterial cultures and preparation used for this test were the same as mentioned in section 1.1.3.. The medium was inoculated with 100 µL of bacterial suspensions (10^2 CFU/mL). Each treatment contained 10 CFU of bacteria with 0 (control) or 700 µg/mL rOSM. The enrichment was tested triplicatedly. Antibacterial activity was calculated by measuring $O.D._{640nm}$ after 72 h incubation at 37 °C with 200 rpm shaking. The $O.D._{640nm}$ of the control group which grew in the absence of rOSM was taken as 100%.

1.3.3.2. Spread plate assay method

The inoculum of *Listeria monocytogenes* (G 3990 4b) was prepared as mentioned in section 1.1.3.. After washed twice and the bacteria were re-suspended in BPB (Butterfield's phosphate buffer), the population of *L. monocytogenes* was adjusted to 10^8 and 10^5 CFU/mL for use. Then 100 µL of each suspension (10^8 and 10^5 CFU/mL) was added in 1.5 mL micro-centrifuge tube containing 900 µL rOSM solution (0 to 1,400 µg/mL). The final bacterial populations were 10^7 and 10^4 CFU/mL. The cultures were incubated on a mini labroller (Labnet International Inc., Edison, NJ) for gently rolling 1 h at room temperature. The cultures were then made 10-fold serial dilution and spread out on LB plates. These plates were incubated at 37 °C for 18 h and colonies were recorded for analysis.

1.4. Determination of Stability of rOSM Stored at Different Temperatures

Mycelial growth inhibition assay was conducted monthly up to 6 months by using rOSM solutions stored at -20, 4, and 25 °C, respectively. Each time, 200 µL of rOSM solutions (1,400 µg/mL) was spread evenly on PDA plates. Agar disks containing mycelia of the tested fungi were placed at the center of PDA plates. Plates were incubated at 25 °C for mycelia growth and the diameters of colonies were measured after 120 h incubation.

1.5. Data Analysis

One-way ANOWA tests (SAS Enterprise Guide version 4.2, SAS Institute Inc., Cary, NC) were applied in this study to determine stability and antimicrobial activity of recombinant tobacco osmotin.

2. Results and Discussion

2.1. Preparation of Recombinant Tobacco Osmotin

2.1.1. Expression, purification and refolding of recombinant tobacco osmotin

Recombinant tobacco osmotin was expressed successfully in *E. coli*. After purification with Co-agarose affinity beads, the purity of rOSM was close to 100% (Figure 1). Lowery protein assay was employed after dialysis and the protein concentration was about 1,400 µg/mL. This is the highest concentration that has been obtained in our lab and it is similar to those data from the literature.

2.1.2. Optimization of refolding efficiency

A protein refolding kit was used to optimize the components of refolding dialysis buffer. Based on the solubility of rOSM, the following combination was thought as the best: in 1 mL optimized dialysis buffer, it contains 900 µL of 550 mM guanidine, 55 mM Tris, 21 mM NaCl, and 0.88 mM KCl, 10 µL 100 mM EDTA, 10 µL 200 mM GSH, 4 µL 100 mM GSSG, 26 µL H_2O and 50 µL rOSM eluted sample. Compared to the rOSM dialysis buffer of 50 mM sodium acetate (pH 5.2) used by Tzou and others (2011), the rOSM solution was clear after dialyzed in the optimized buffer, indicating the aggregation of protein was tremendously suppressed (Figure

2). The molarity ratio of GSH : GSSG was 5 : 1 which showed the best oxidation-reduction potential for rOSM refolding. From the SDS-PAGE analysis, the solubility of rOSM in optimized buffer was dramatically more increased than that dialyzed in 50 mM sodium acetate, pH 5.2 (Figure 3). After centrifugation, the majority of rOSM precipitated out in the sodium acetate dialysis buffer, while the concentrations of rOSM in optimized dialysis buffer were close before and after centrifugation. However, there was no significant difference ($P < 0.05$) between the antifungal activities of rOSM dialyzed in both buffers to *Aspergillus niger* (Figure 4, 5 and 6), which indicated that aggregated rOSM also had antifungal activity.

2.2. Assay of Anti-yeast Activity

As shown in Figure 7, recombinant tobacco osmotin possessed excellent anti-yeast activity to *Cryptococcus neoformans*. The higher concentration of rOSM showed higher inhibitory efficacy to this pathogenic yeast. The growth rate of *Cryptococcus neoformans* was slow and at 5 CFU/mL inoculum level, it required 24-36 h before turbidity was observed. In the control, it took 60 h to reach the stationary phase, while 72, 108, 132, 156, and 156 h were needed for the concentrations of rOSM at 22, 44, 87, 175, 350 µg/mL, respectively. With 700 µg/mL rOSM, the growth of *Cryptococcus neoformans* was completely inhibited during the tested 7 days.

2.3. Assay of Anti-mold Activity

2.3.1. Assay of mycelial growth inhibition

Two fungal isolates, *Aspergillus niger* and *Penicillium aurantiogriseum* were tested for mycelial growth inhibition at the presence of various concentrations of rOSM. As shown in

Figure 8 and 9, rOSM inhibited mycelial growth of both tested fungi. The antifungal activity of rOSM to two tested fungi was dose dependent and fungal species specific. As to *Aspergillus niger*, it grew so rapidly that it covered the control PDA plates in 144 h, while the diameter of the mycelia was still around 4.0 cm on rOSM (from 1,000 to 1,400 µg/mL) treated PDA plates after 216 h incubation. Although the inhibition of mycelial growth of the two tested fungi was more efficient with higher concentration of rOSM, it did not grow at the same pace to *Aspergillus niger* when the concentration of rOSM increased from 1,000 to 1,400 µg/mL.

To determine the minimum 50% inhibition concentrations (MIC_{50}) of rOSM to the two tested fungi, mycelial growth inhibition of *Aspergillus niger* and *Penicillium aurantiogriseum* was conducted (Figure 10). After 120 h incubation at presence of various concentrations of rOSM, both of the two tested fungi showed different degrees of mycelial growth inhibition. The MIC_{50}s of rOSM to *Aspergillus niger* and *Penicillium aurantiogriseum* were 800 and 1,200 µg/mL, respectively. Therefore, *Aspergillus niger* was more sensitive to rOSM than *Penicillium aurantiogriseum*. Both of the tested fungi formed thick mycelia on the control PDA plates while the mycelial growth on the rOSM treated PDA plates was sparse, especially at the edge of the mycelial colonies.

Potassium sorbate is a commonly known food preservative and widely used to inhibit yeasts and molds in the food industry. Since rOSM showed antifungal activity and its potential application in the food industry, combinations of rOSM and potassium sorbate were investigated in this study. As shown in Figure 11, 12, 13 and 14, antifungal activity of potassium sorbate was concentration dependent. Potassium sorbate at 100 ppm showed almost no antifungal activity during the test. Combination of rOSM and 100 ppm potassium sorbate showed almost the same

antifungal effect as the same concentration of rOSM without potassium sorbate. At initial 96 h incubation, the antifungal activity of 500 ppm potassium sorbate was similar to those of rOSM treatments. However, the antifungal activity of 500 ppm potassium sorbate was less than that of 350 µg/mL rOSM which was the lowest rOSM concentration tested. Although colonies were not observed during the initial 96 h at the rOSM and 500 ppm potassium sorbate treatments, rOSM and 500 ppm potassium sorbate combination did not show synergistic antifungal activity after that, especially to *Penicillium aurantiogriseum*.

2.3.2. Inhibition of spore germination

The effect of rOSM on spore germination of *Aspergillus niger* and *Penicillium aurantiogriseum* was investigated in PDB containing 0 to 700 µg/mL of rOSM after 20-30 h incubation. The rOSM had shown different effects on the spore germination rate for different fungi at various concentrations. The higher rOSM concentration resulted in the lower spore germination rate shown in the treatment. However, around 50% spore germination of both the tested fungi was inhibited at 700 µg/mL of rOSM (Figure 15), which indicated that the spores were more sensitive to rOSM than mycelium did since MIC_{50} of mycelial growth of the tested two fungi was 800 and 1,200 µg/mL, respectively. The germination time for *Penicillium aurantiogriseum* spores was also delayed under the rOSM treatment.

2.4. Assay of Antibacterial Activity

2.4.1. Liquid medium method

In this study, antibacterial activity of recombinant tobacco osmotin was conducted by measuring the O.D. at 640nm. The medium without rOSM was the control and its $O.D._{640nm}$ was

taken as 100% growth at the end of incubation. There were 26 bacteria used for testing the antibacterial activity of rOSM (Table 4). After 72 h incubation, all tested bacterial strains had different degrees of sensitivity to rOSM at 700 µg/mL. *Enterobacter aerogenes* (ATCC 13048), *Listeria monocytogenes* (ATCC 19111), *Salmonella enteritidis* (H 4638), *Salmonella typhi* (CDC 9032-85), *Salmonella typhimurium* (ATCC 13311), and *Yersinia kritensenii* (PL 115185) were the most sensitive bacterial strains to rOSM, while *Listeria innocua* (ATCC 33090), *Salmonella mission*, *Salmonella mission* (NA resistant), *Salmonella montevideo* (rif-R), and *Salmonella montevideo* were the least sensitive. Both *Listeria monocytogenes* (ATCC 19111) and *Listeria monocytogenes* (G 3990 4b) showed relatively high sensitivity (36.2% and 49.7% growth, respectively), while *Listeria innocua* (ATCC 33090) was one of the least sensitive bacteria (94.9% growth). The variability of sensitivity was more apparent within the *Salmonella* family, in which *Salmonella enteritidis* (H 4638) was very sensitive (27.1% growth) but *Salmonella montevideo* (rif-R) was the least (95.8% growth). Besides, there were no apparent evidences to show different sensitivities between Gram positive and negative bacteria or between spore-forming and non spore-forming bacteria to rOSM.

The unexplained observation was that before the stationary phase, the $O.D._{640nm}$ of both rOSM treated medium and control was almost the same. Once the bacteria growth reached the maximum, the $O.D._{640nm}$ dropped sharply for the rOSM treated samples while that of control maintained the same high level of $O.D._{640nm}$.

2.4.2. Spread plate assay method

After treated with 1,400 µg/mL rOSM, populations of *Listeria monocytogenes* (G 3990 4b) were reduced 2.4 log at low (10^4 CFU/mL) inoculum level and 1.8 log at high (10^7 CFU/mL)

inoculum level (Table 5). The higher concentration of rOSM was helpful to lower the survival of *Listeria monocytogenes* (G 3990 4b) after 1 h treatment. Combined with Table 4, processing time and working concentrations were the essential factors which could affect the antibacterial activity of rOSM.

Table 4-Antibacterial Activity of rOSM (700 µg/mL). After 72 h incubation, the $O.D._{640nm}$ of all the cultures was measured and the $O.D._{640nm}$ of control cultures growing in the absence of rOSM was taken as 100%.

Bacteria Strains	**Antibacterial Activity of rOSM** (% of growth)
Bacillus cereus	58.1
Citrobacter amalonaticus (SA 5615)	64.5
Enterobacter aerogenes (ATCC 13048)	36.3
E. coli O157:H7 (ATCC 43895)	70.7
E. coli O157:H7 (204 P)	62.6
Klebsiella Oxytora (ATCC 13182)	44.8
Listeria innocua (ATCC 33090)	94.9
Listeria monocytogenes (ATCC 19111)	36.2
Listeria monocytogenes (G 3990 4b)	49.7
Morganella margani (CDC 8103-85)	60.1
Salmonella dublin (S 2424)	83.4
Salmonella enteritidis (H 4638)	27.1
Salmonella enteritidis (H 4639)	58.7
Salmonella mission	93.8
Salmonella mission (NA resistant)	95.6
Salmonella montevideo	91.4
Salmonella montevideo (rif-R)	95.8
Salmonella paratyphi	47.1
Salmonella paratyphimurium	85.6

(Continued)

Bacteria Strains	**Antibacterial Activity of rOSM** (% of growth)
Salmonella typhi (CDC 9032-85)	31.2
Salmonella typhi (CDC 3434-73)	76.9
Salmonella typhimurium (ATCC 13311)	36.1
Shigella boydii (ATCC 9207)	45.7
Staphylococcus aureus (ATCC 12600)	73.5
Vibrio cholera (M 802)	47.7
Yersinia kritensenii (PL 115185)	35.2

Table 5-Antibacterial Activity of rOSM to *Listeria monocytogenes* (G 3990 4b).

Inoculum Level (CFU/mL)	Concentration of rOSM (µg/mL)	Final Population ($\bar{X}$ ± STD, CFU/mL)
10^4	0	$2.65 \pm 0.21 \times 10^4$
	50	$6.85 \pm 0.64 \times 10^2$
	350	$1.85 \pm 0.21 \times 10^2$
	700	$1.35 \pm 0.49 \times 10^2$
	1,400	40 ± 14
10^7	0	$3.47 \pm 0.59 \times 10^7$
	50	$2.93 \pm 0.31 \times 10^7$
	350	$2.03 \pm 0.26 \times 10^7$
	700	$2.63 \pm 0.21 \times 10^7$
	1,400	$1.72 \pm 0.09 \times 10^5$

2.5. Determination of Stability of rOSM Stored at Different Temperatures

After 120 h incubation, the colony diameters of *Aspergillus niger* and *Penicillium aurantiogriseum* were about 6.0 and 2.7 cm on control PDA plates, respectively; while the diameter of colonies were about 2.3 and 1.3 cm, respectively on treatments (Figure 16 and 17). All rOSMs which were stored at -20, 4, and 25 °C, showed stable and consistent antifungal activity to mycelial growth of *Aspergillus niger* and *Penicillium aurantiogriseum* during 6 months. The antifungal efficacy of rOSM indicates that it has the potential for commercial use in many foods which are stored at -20, 4, and 25 °C.

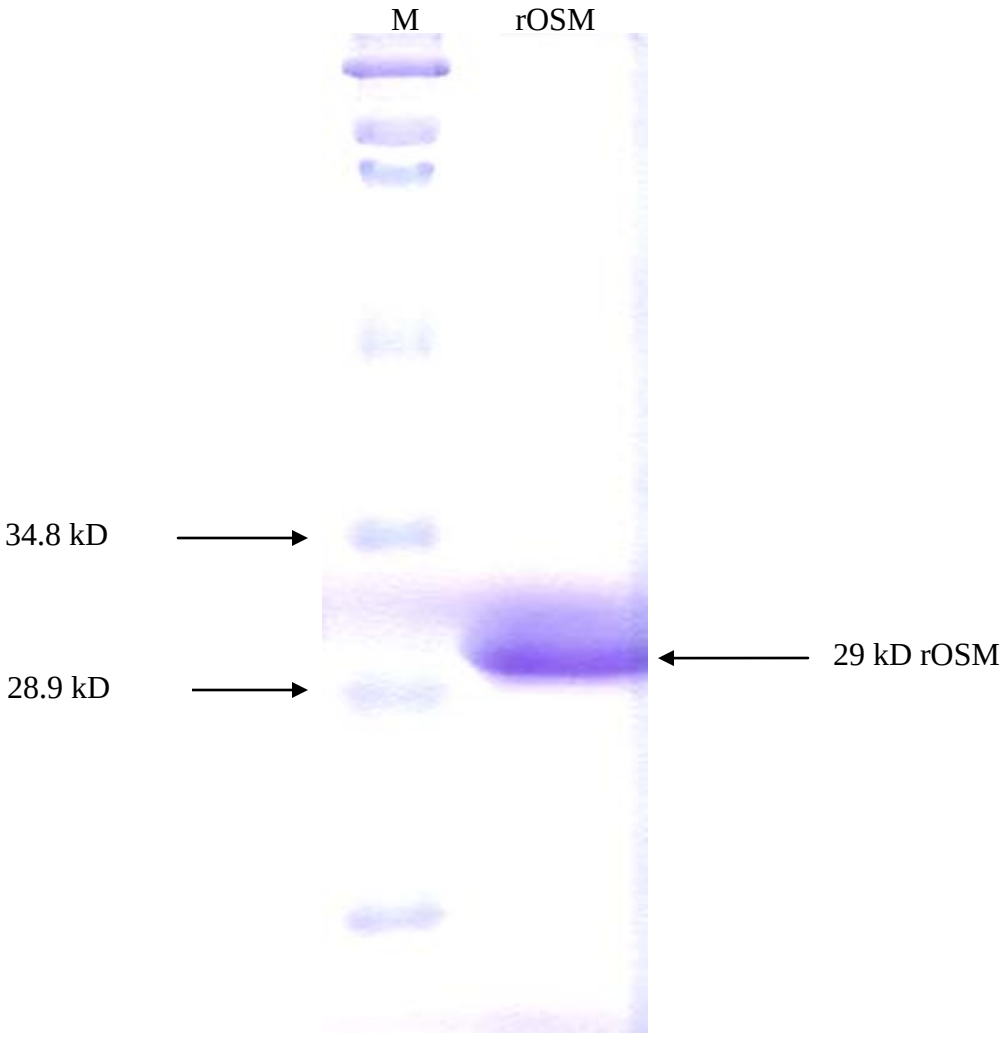

Figure 1-SDS-PAGE of Cobalt-agarose beads purified rOSM. Lane M is molecular weight markers.

Figure 2-Dialysis of rOSM in 50 mM sodium acetate, pH 5.2 (left) and optimized dialysis buffer (right).

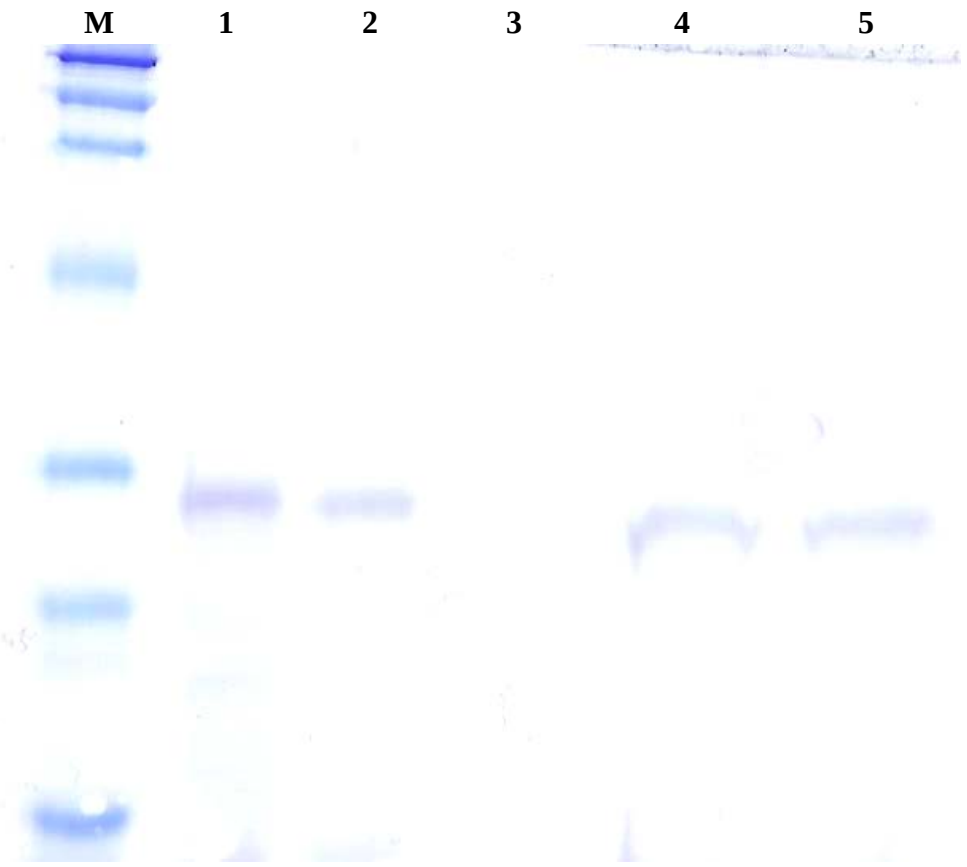

Figure 3-SDS-PAGE of rOSM dialyzed from 50 mM sodium acetate and optimized buffer. Lane M: molecular weight markers; lane 1: rOSM before dialysis; lane 2: rOSM dialyzed in 50 mM sodium acetate; lane 3: supernatant of dialyzed rOSM after centrifugation at 3,000 × *g* for 5 min at 4 °C; lane 4: rOSM solution dialyzed in optimized buffer; lane 5: supernatant of rOSM dialyzed in optimized buffer after centrifugation at 3,000 × *g* for 5 min at 4 °C.

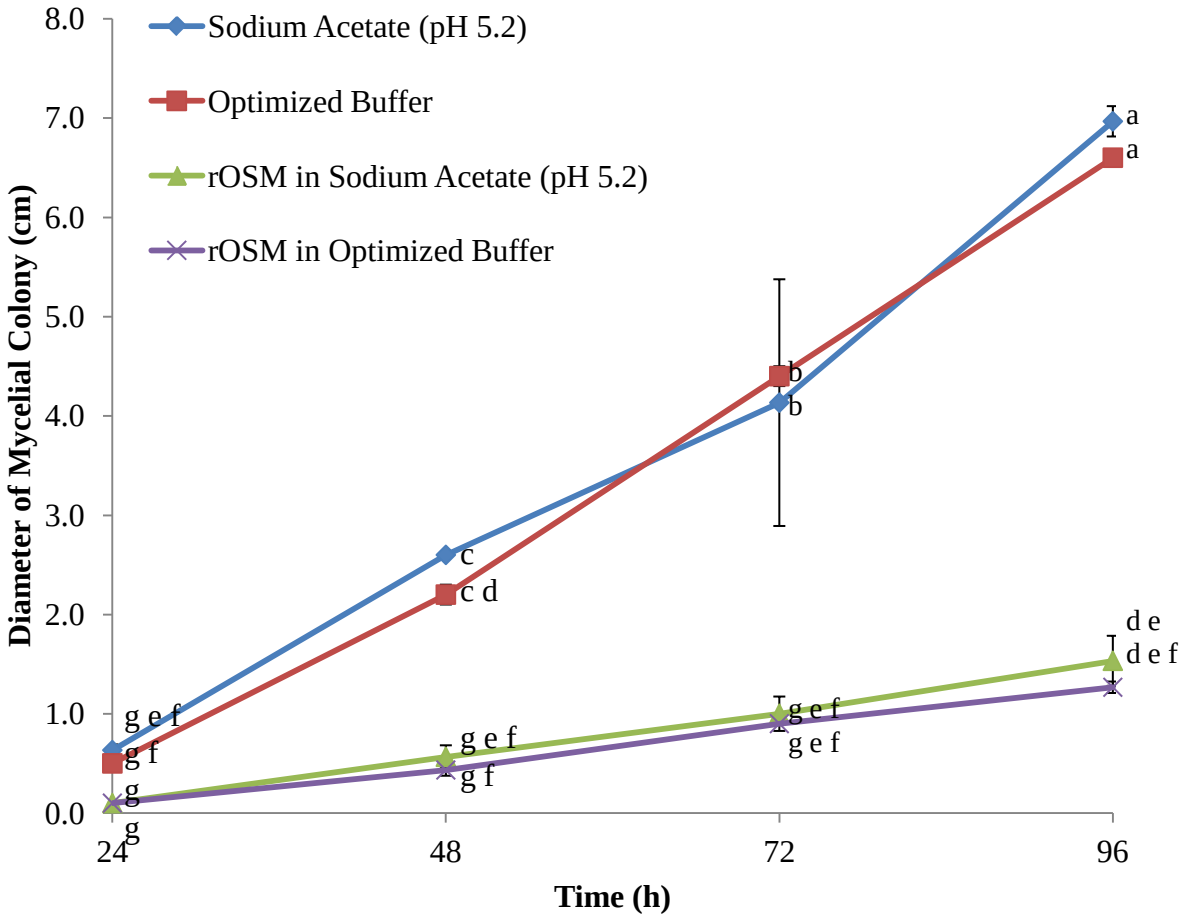

Figure 4-The inhibition of mycelial growth of *Aspergillus niger* by rOSM dialyzed in sodium acetate and optimized buffer on PDA plates. Bars represent standard deviations (*n* = 3). Different letters mean significant differences (P > 0.05).

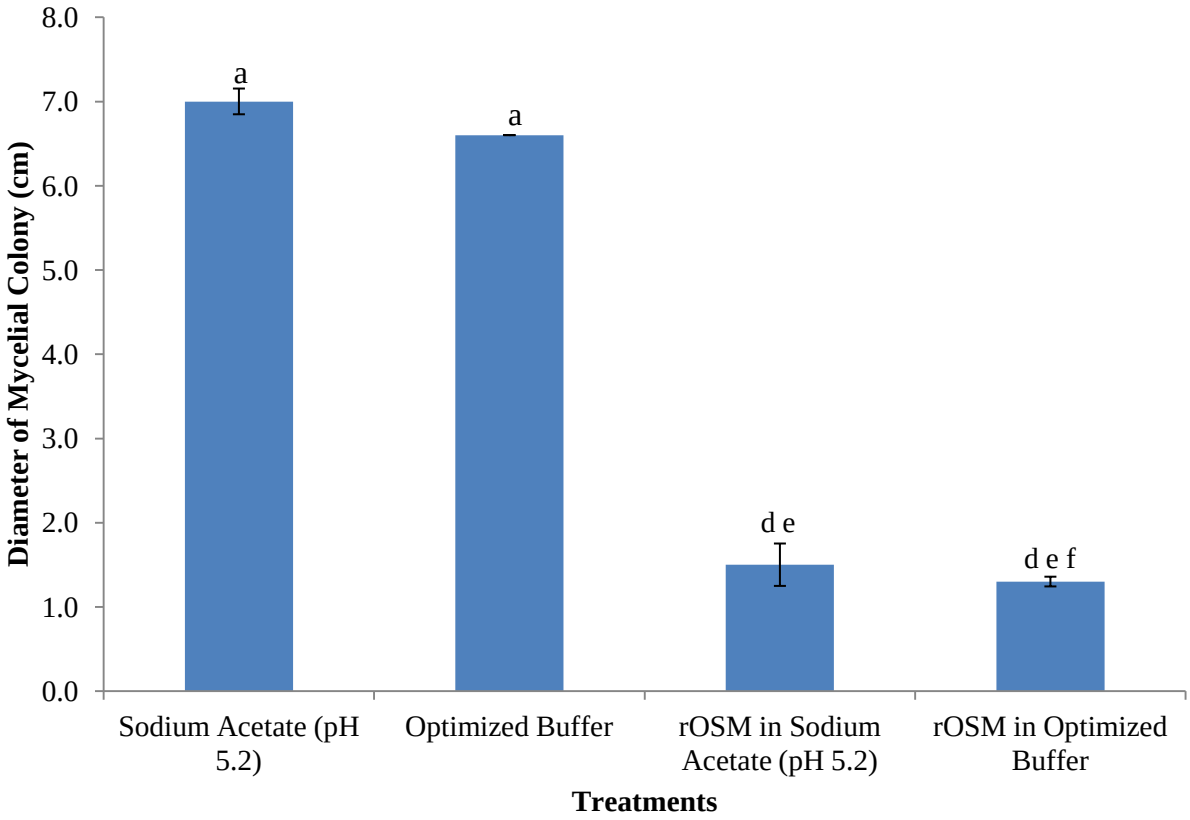

Figure 5-Inhibitory effect of rOSM dialyzed in sodium acetate and optimized buffer on mycelial growth of *Aspergillus niger* on PDA plates after 96 h incubation. Bars represent standard deviations (n = 3). Different letters mean significant differences (P > 0.05).

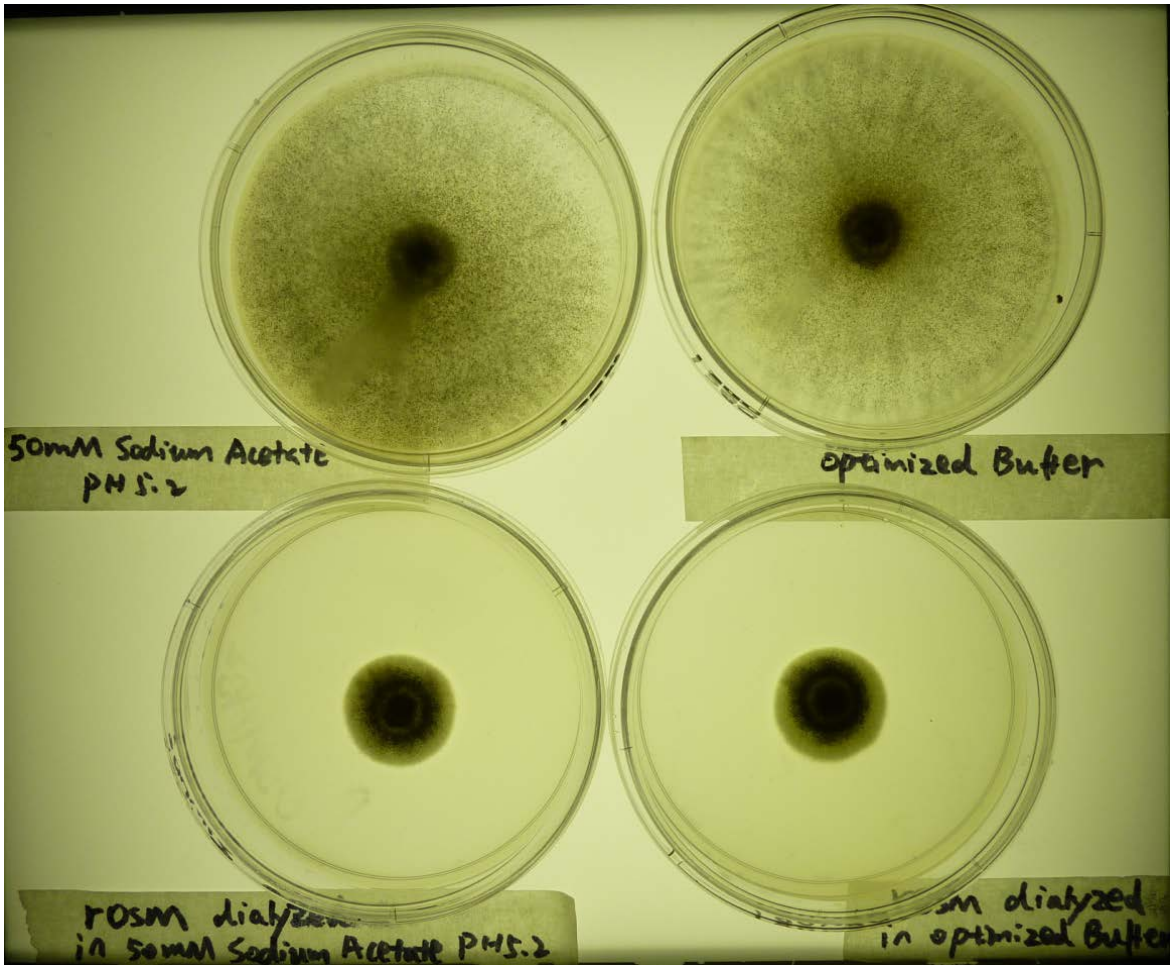

Figure 6-Morphology of *Aspergillus niger* on PDA plates spread by rOSM dialyzed in sodium acetate and optimized buffer at 96 h incubation.

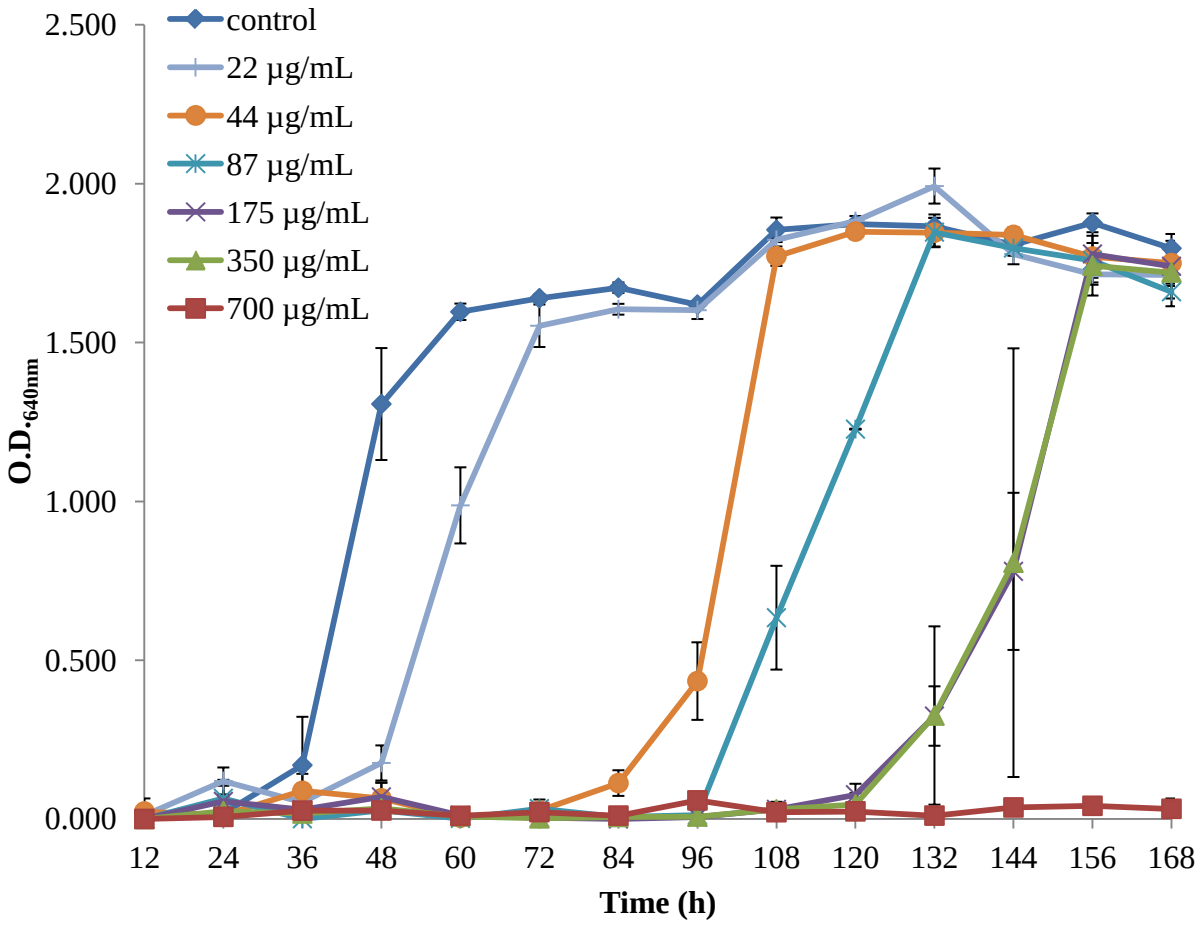

Figure 7-The anti-yeast activity of rOSM to *Cryptococcus neoformans* (ATCC 13690). Bars represent standard deviations (n = 3).

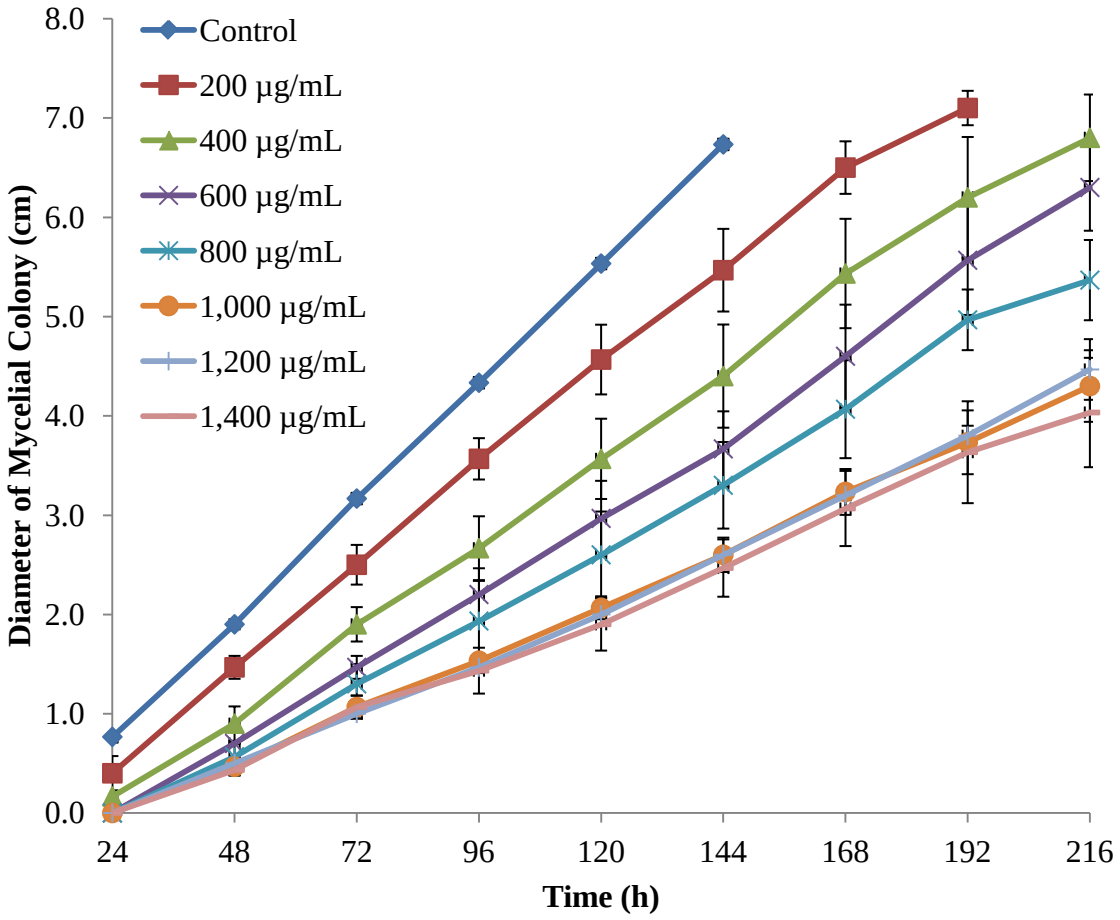

Figure 8-The inhibition of mycelial growth of *Aspergillus niger* by rOSM on PDA plates. Bars represent standard deviations (n = 3).

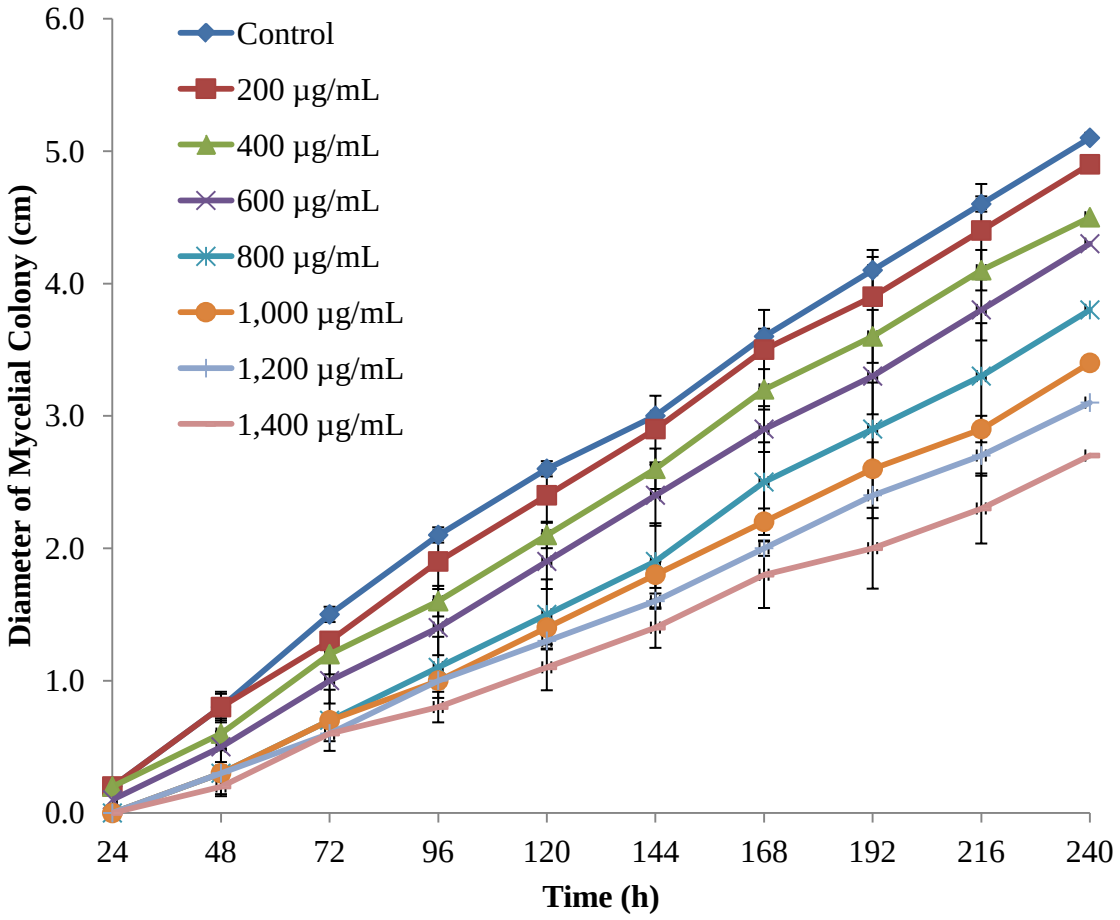

Figure 9-The inhibition of mycelial growth of *Penicillium aurantiogriseum* by rOSM on PDA plates. Bars represent standard deviations (n = 3).

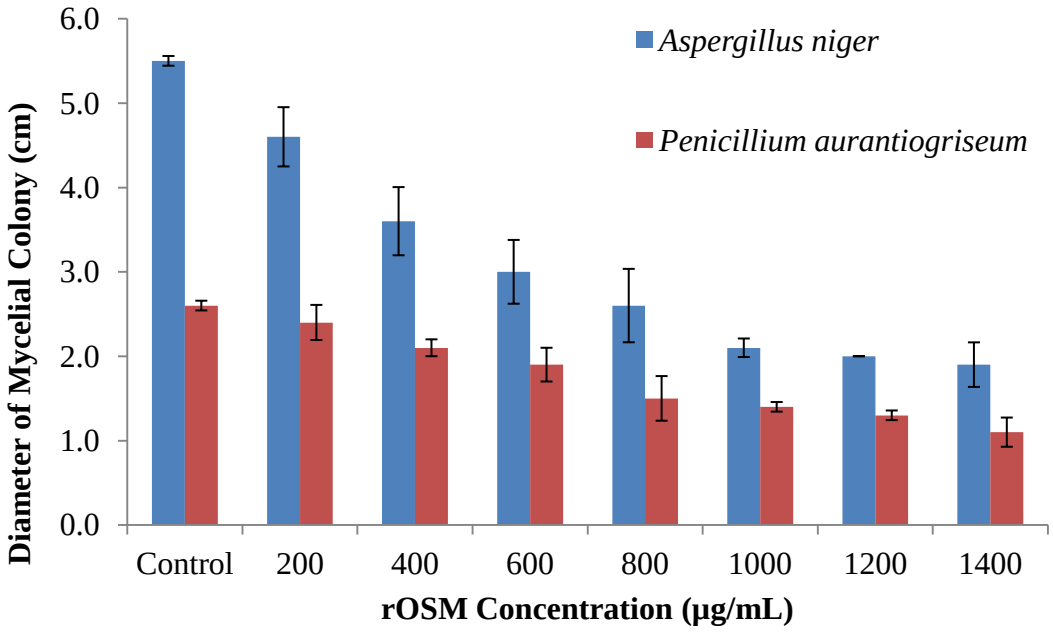

Figure 10-Inhibitory effect of rOSM on mycelial growth of *Aspergillus niger* and *Penicillium aurantiogriseum* on rOSM PDA plates after 120 h incubation. Bars represent standard deviations (n = 3).

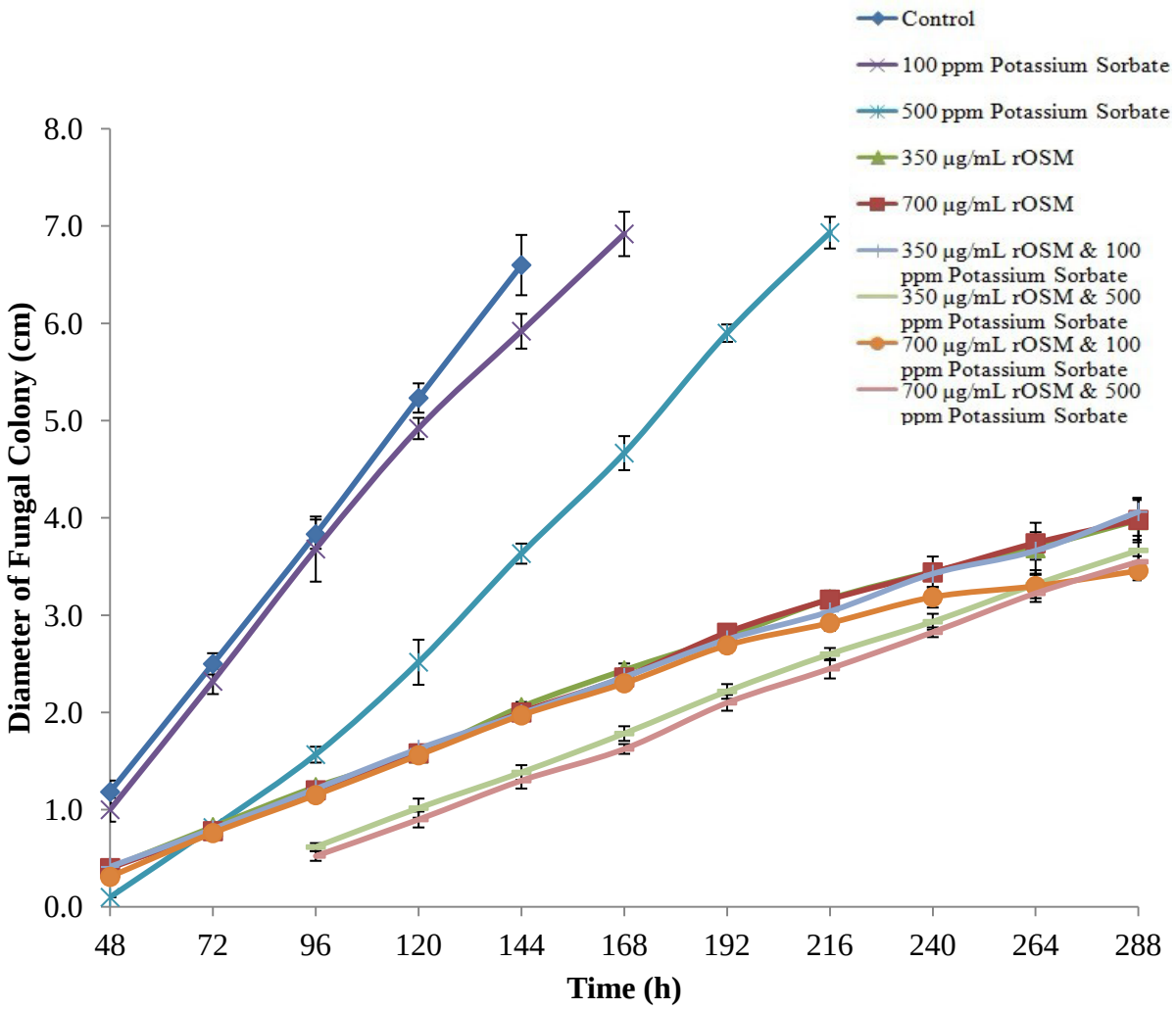

Figure 11-Mycelial growth of *Aspergillus niger* on PDA plates containing rOSM, potassium sorbate and their combinations. Bars represent standard deviations (n = 5).

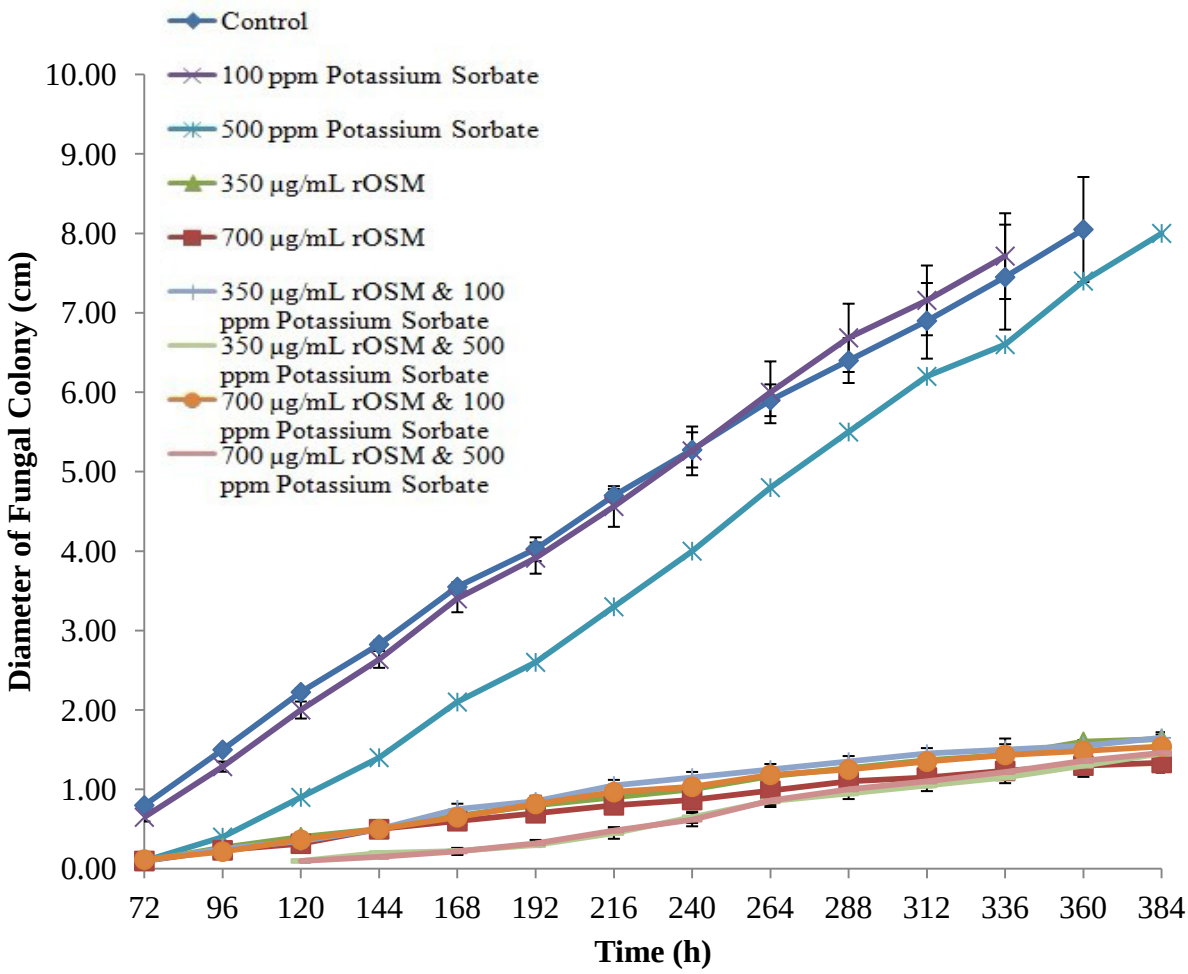

Figure 12-Mycelial growth of *Penicillium aurantiogriseum* on PDA plates containing rOSM, potassium sorbate and their combinations. Bars represent standard deviations (*n* = 5).

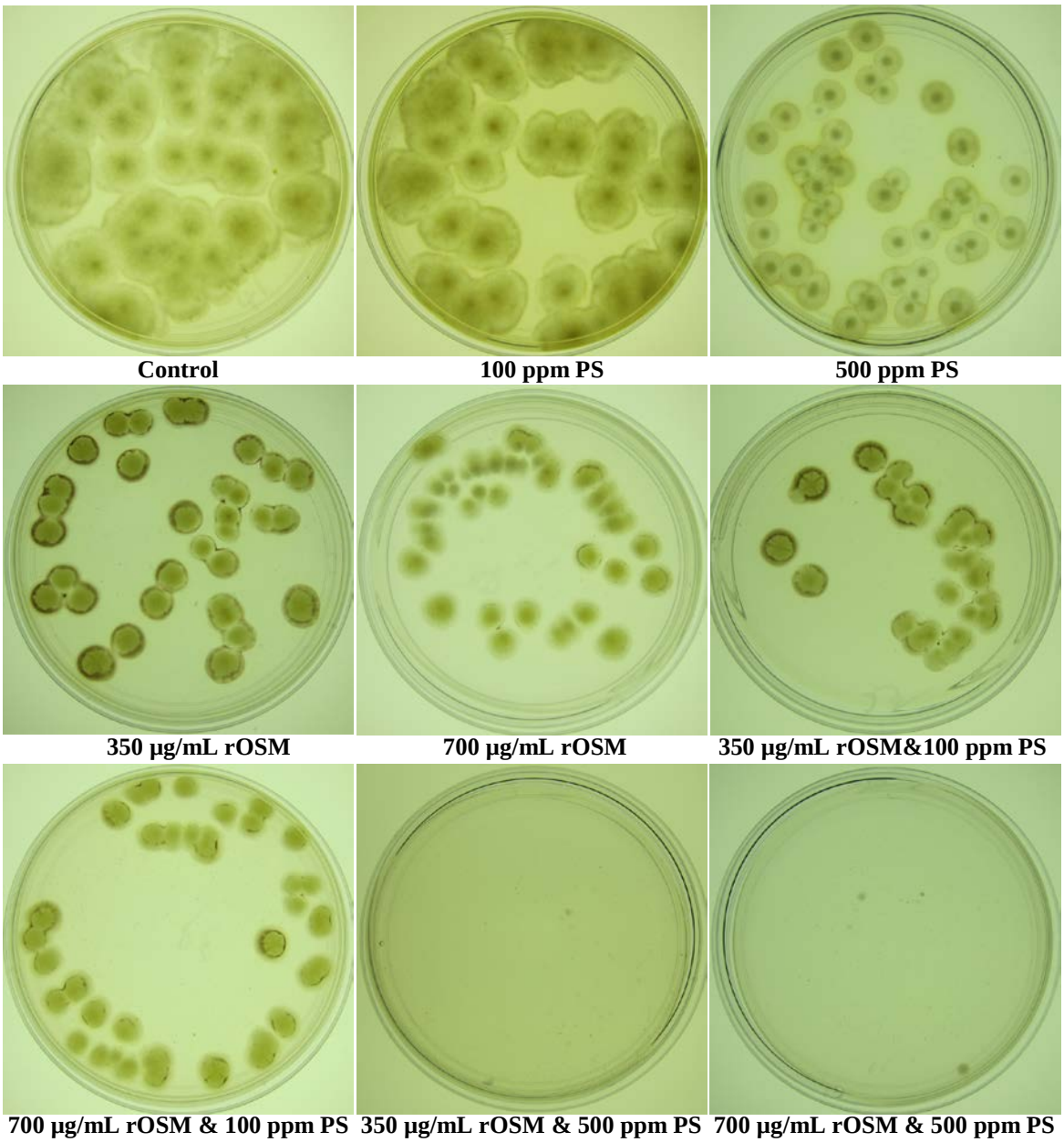

Figure 13-Morphology of *Aspergillus niger* on PDA plates containing rOSM, potassium sorbate and their combinations after 96 h incubation. PS: potassium sorbate.

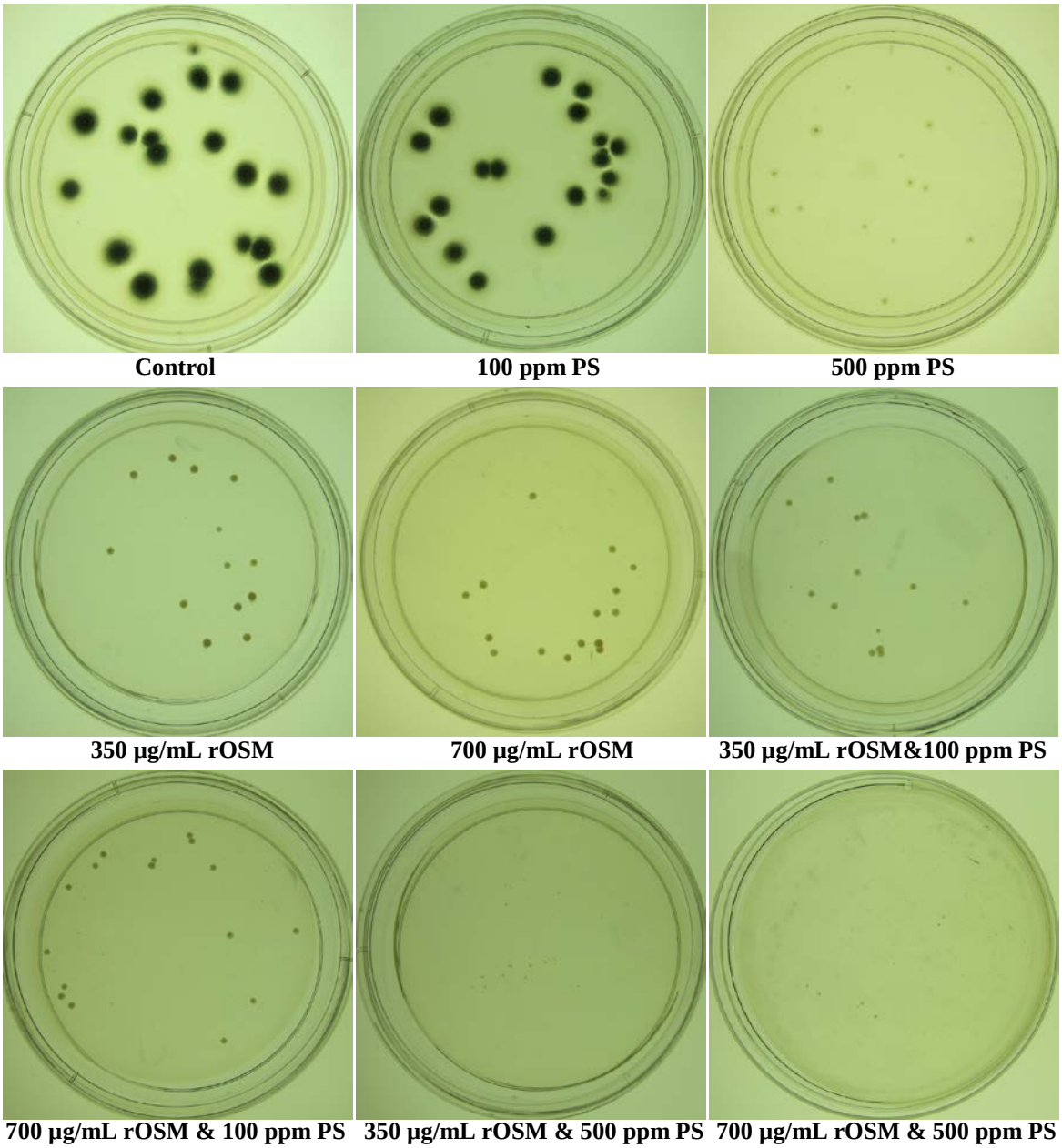

Figure 14-Morphology of *Penicillium aurantiogriseum* on PDA plates containing rOSM, potassium sorbate and their combinations after 96 h incubation. PS: potassium sorbate.

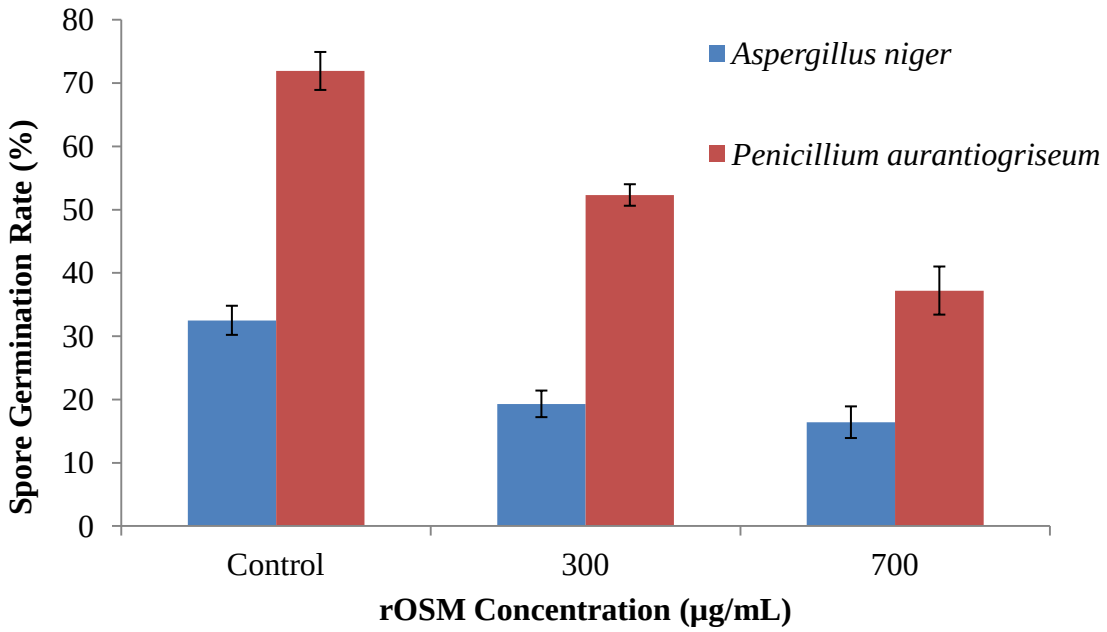

Figure 15-Spore germination rates of *Aspergillus niger* and *Penicillium aurantiogriseum* at different rOSM concentrations. Bars represent standard deviations (*n* = 3).

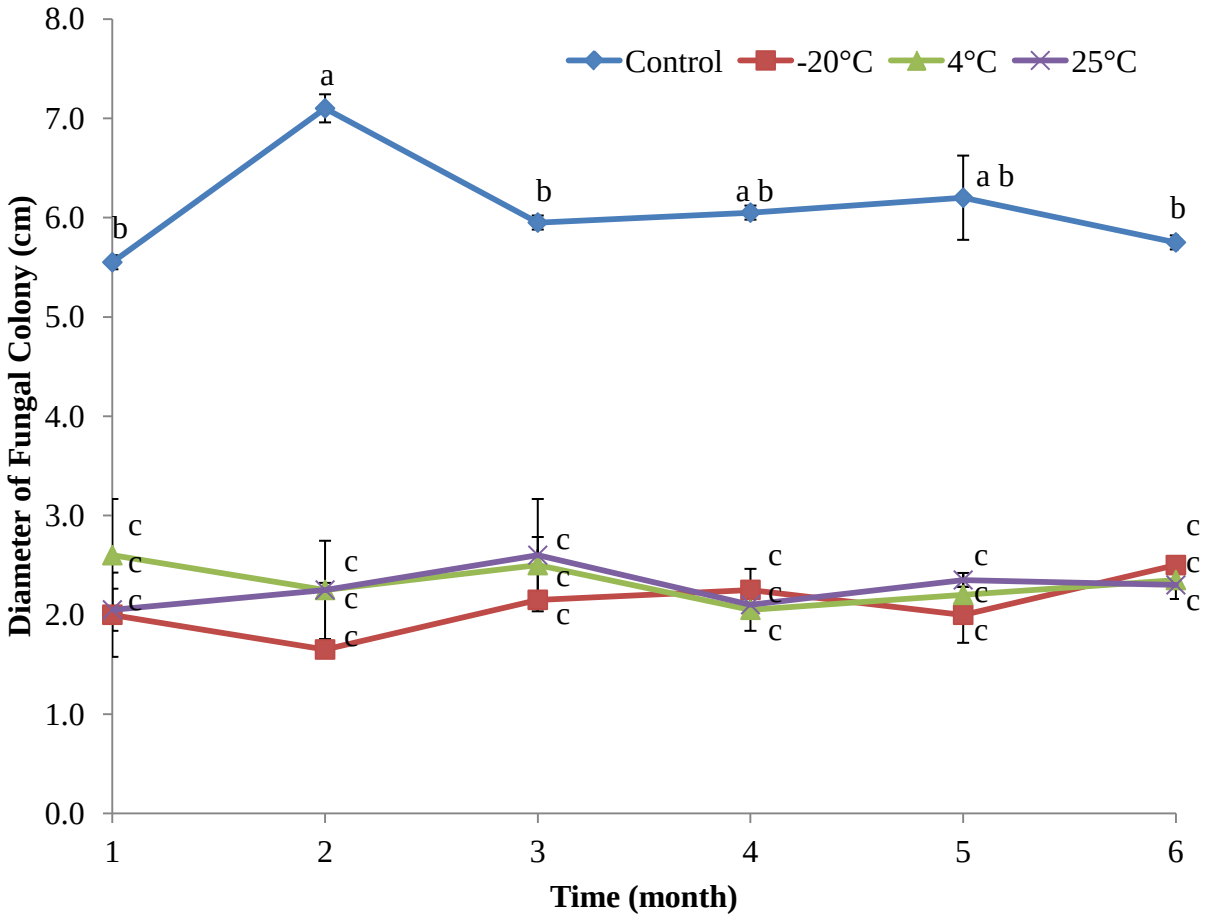

Figure 16-Mycelial growth inhibition of *Aspergillus niger* by rOSM on PDA plates after 120 h incubation. The rOSM solutions were stored at -20, 4, and 25 °C. Bars represent standard deviations (*n* = 3). Different letters mean significant differences (P > 0.05).

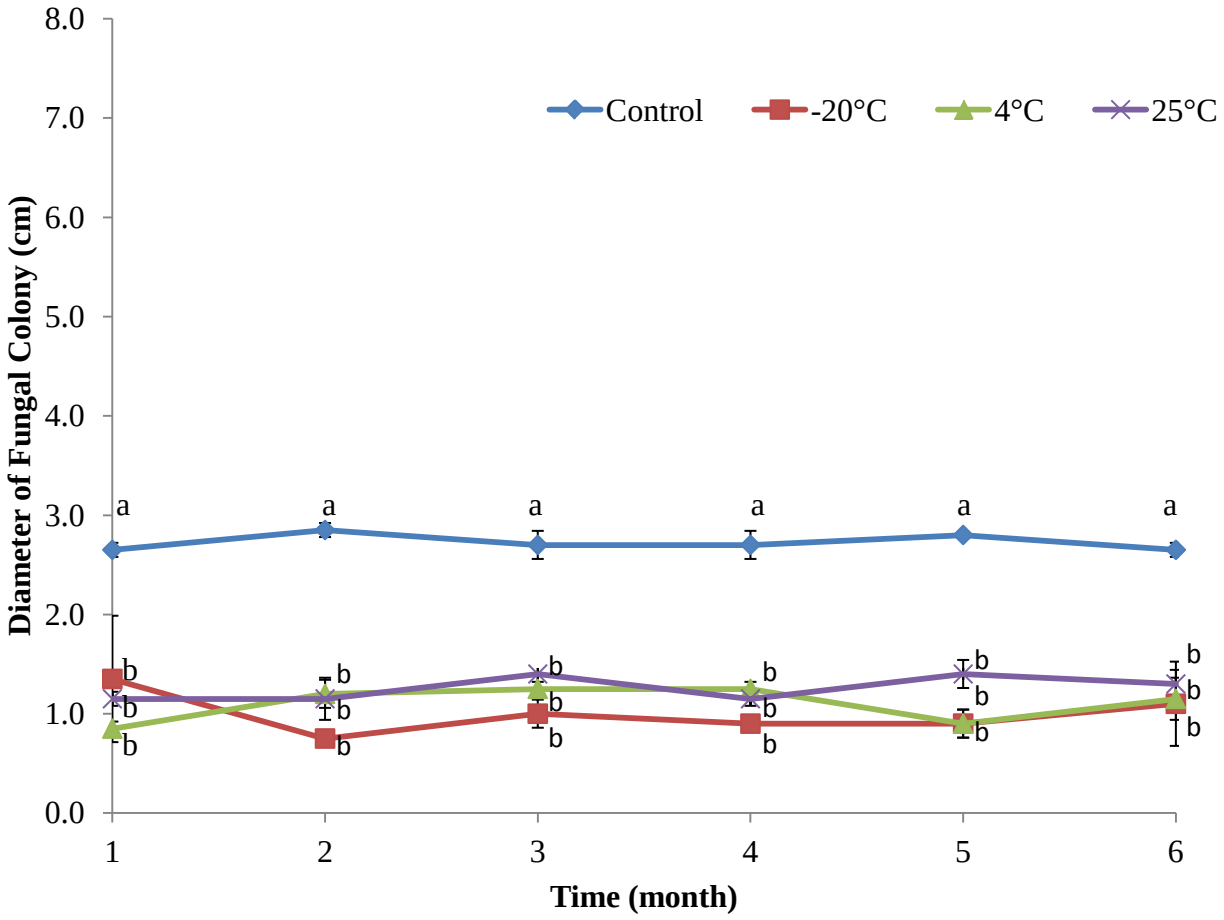

Figure 17-Mycelial growth inhibition of *Penicillium aurantiogriseum* by rOSM on PDA plates after 120 h incubation. The rOSM solutions were stored at -20, 4, and 25 °C. Bars represent standard deviations (*n* = 3). Different letters mean significant differences (P > 0.05).

CHAPTER 4: *Aspergillus niger* CONTROL ON STRAWBERRIES BY RECOMBINANT TOBACCO OSMOTIN

1. Materials and Methods

1.1. Preparation of *Aspergillus niger* Spore Suspension

Aspergillus niger spores were used in this study. In one slant PDA culture, 2 mL sterilized water was added to make the spore suspension. After vortexed slowly to remove spores from mycelia, 200 µL of spore suspension was taken out and the concentration of spores was determined by hemacytometer. The spore concentration was further diluted to the designated working concentration of 10^3 conidia/mL for use.

1.2. Preparation of Strawberries

Strawberries were purchased from a local grocery store before use. The fresh strawberries without observed diseases and wounds were first immersed in 70% ethanol, 30 min for disinfection before rOSM treatment. All alcohol treated strawberries were put in a biosafety hood (NuAire, Inc., Plymouth, MN) 1 h for drying. Then, they were immersed in sterile water to wash off ethanol residual and put them back in the biosafety hood for moisture removal.

1.3. Strawberry Inoculation

After the strawberries were disinfected and air dried, 10 µL of spore suspension (ca. 10^3 conidia/mL) of *Aspergillus niger* was spot-inoculated on the surface and two spots for each strawberry.

After inoculation, the strawberries were placed on the aluminum foil in a biosafety hood to allow spores attachment. This static condition lasted 2-4 h which allowed the surface of strawberries to totally dry.

1.4. Dip Treatment by Applying Recombinant Tobacco Osmotin

The inoculated strawberries were dipped in 350, 700, and 1,400 µg/mL rOSM and 500 ppm potassium sorbate solutions for 1 min, respectively. For the control, the strawberries were dipped in sterile 50 mM sodium acetate solution. Each treatment contained 5 strawberries. After the treatment, the strawberries were put into a biosafety hood for drying. Dried strawberries were then put into plastic bags individually and stored at 25 °C.

1.5. Data Analysis

The growth of mold was recorded daily by measuring the area covered by the mold after treatment. The degree of mold coverage rate was evaluated by using the following visual rating scale: 0, no mycelial growth on the surface of strawberries; 1, less than 20% surface covered by mycelium; 2, 20-40% surface covered by mycelium; 3, 40-60% surface covered by mycelium; 4, 60-80% surface covered by mycelium; 5, more than 80% surface covered by mycelium.

2. Results and Discussion

The recombinant tobacco osmotin showed the inhibition to *Aspergillus niger* growth on strawberries (Figure 18). The potassium sorbate of 500 ppm slightly inhibited *A. niger* growth on inoculated strawberries, but it did not extend the shelf-life. In the rOSM treatments, the growth of *A. niger* was inhibited and the shelf-life of strawberries was extended. Recombinant tobacco osmotin could reduce the infection rate. The inhibitory effect of rOSM was dose dependent with higher concentration exerting more antifungal activity. After 156 h incubation at 25 °C, the degrees of mold coverage on strawberries under control and potassium sorbate treatments were 4.9, but it was only 2.8 on the strawberries treated with 1,400 μg/mL rOSM. The mold covered on the rOSM-treated strawberries was much less than those in the control group.

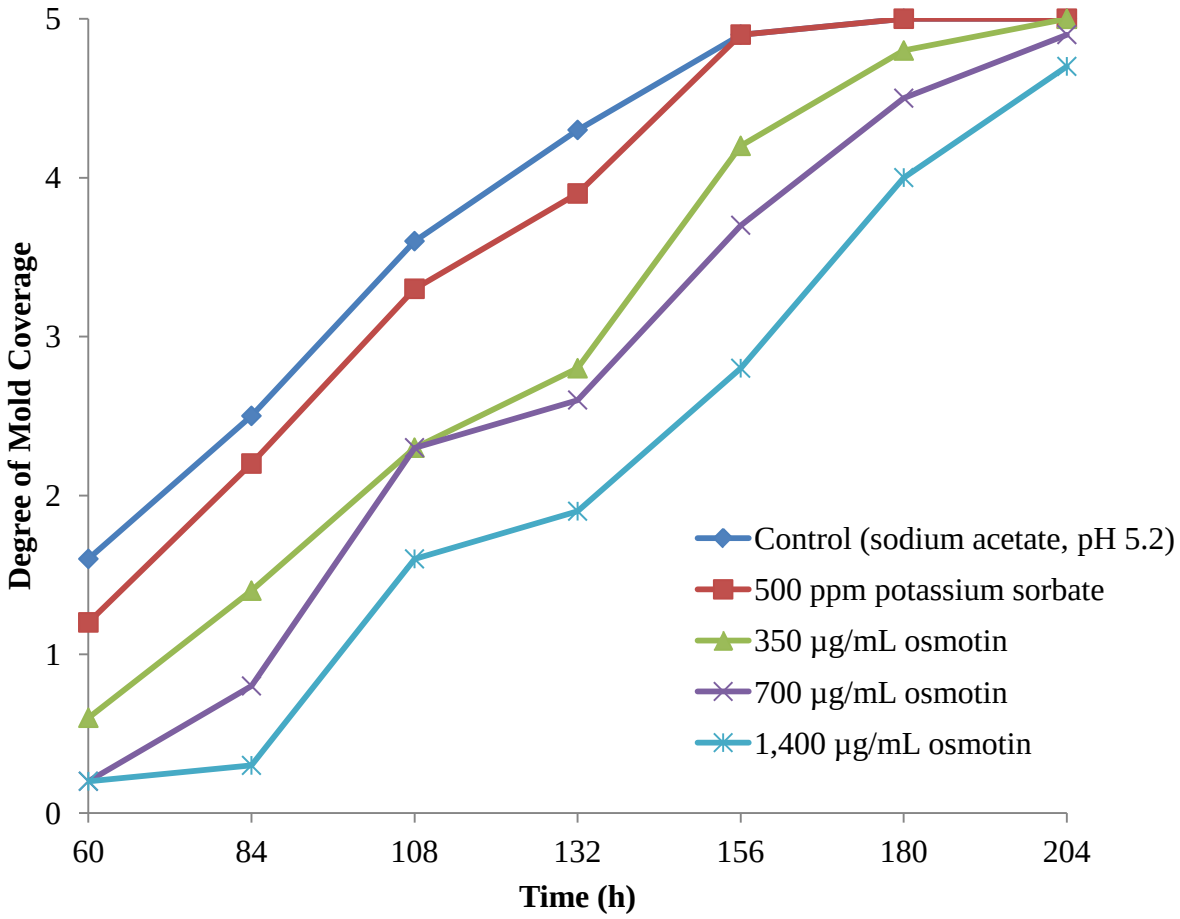

Figure 18-Inhibitory effect of rOSM and potassium sorbate to *Aspergillus niger* growth on strawberries. The degree of mold coverage rate was evaluated by using the following visual rating scale: 0, no mycelial growth on the surface of strawberries; 1, less than 20% surface covered by mycelium; 2, 20-40% surface covered by mycelium; 3, 40-60% surface covered by mycelium; 4, 60-80% surface covered by mycelium; 5, more than 80% surface covered by mycelium.

CHAPTER 5: CONCLUSIONS

In this study, recombinant tobacco osmotin was expressed, purified and refolded successfully and its concentration was 1.4 mg/mL. The rOSM showed good antimicrobial activities against bacteria, yeasts and molds.

A commercial refolding kit was used to find an optimal refolding dialysis buffer. Among the tested buffers, the formula of the optimal dialysis buffer was: 495.0 mM guanidine, 49.5 mM Tris, 18.9 mM NaCl, 0.8 mM KCl, 1.0 mM EDTA, 2.0 mM GSH, and 0.4 mM GSSG. This refolding buffer could improve the solubility and stability of rOSM. However, the rOSM dialyzed in both 50 mM sodium acetate (pH 5.2) and optimized buffer showed similar antifungal activity to *Aspergillus niger*. This phenomenon indicated that aggregated rOSM still possessed antifungal activity, and it was suggested that the antimicrobial activity would be less affected by the complex food systems.

Cryptococcus neoformans (ATCC 13690) was tested in the study and its growth was delayed with the presence of rOSM. The higher concentration of rOSM exerted more inhibition and 700 µg/mL rOSM could delay the yeast growth for at least 7 days.

Recombinant tobacco osmotin showed inhibition to both mycelial growth and spore germination of *Aspergillus niger* and *Penicillium aurantiogriseum,* and its inhibitory efficacy was dose dependent. The MIC_{50} of rOSM to the mycelial growth of *Aspergillus niger* and

Penicillium aurantiogriseum was 800 µg/mL and 1,200 µg/mL, respectively, while 700 µg/mL of rOSM could effectively inhibit 50% spore germinations of both molds. The spore germination was more sensitive than the mycelial growth to rOSM. Compared to the thickness of mycelial colonies, it was sparser and less on rOSM spread PDA plates than on the control PDA plates, especially at the edge of the colonies. Combination of rOSM and 500 ppm potassium sorbate was found exerting stronger inhibitory efficacy than individuals to both tested molds. Fungal growth was not observed for the initial 96 h on the combined treatments (rOSM with 500 ppm potassium sorbate). However, after 96 h, the mycelial growth rates were similar to those treatments with rOSM only.

Antibacterial activity of rOSM has been performed the first time. After 72 h incubation at the presence of 700 µg/mL rOSM, 26 tested bacteria showed various degrees of growth inhibition. Antibacterial activity of rOSM was conducted by comparing the $O.D._{640nm}$ of rOSM contained medium to the control medium which was taken as 100% growth. *Salmonella enteritidis* (H 4638) was the most sensitive bacterium to rOSM (27.1% growth), while *Salmonella montevideo* (rif-R) was the least sensitive and had 95.8% growth. However, there was no clear relationship between the members of the same genus and their sensitivity to rOSM. The sensitivity of bacteria to rOSM was also not related to the Gram (+)/(-) or spore-forming features.

The recombinant tobacco osmotin was shelf stable. After stored at -20, 4, and 25 °C up to 6 months, all rOSM samples showed the same antifungal activity ($P < 0.05$). It will increase the potential use for inhibiting microbial growth in foods, because most foods are stored at one of these three temperatures.

Finally, implementation of rOSM on strawberries to control *Aspergillus niger* for extending shelf-life was investigated. Dipping of rOSM could effectively delay the mold coverage rate resulting in extensive shelf-life. Higher concentration of rOSM showed more inhibition to *Aspergillus niger* infection. The antifungal activity of 350 μg/mL rOSM, the lowest concentration of rOSM tested, was higher than that of 500 ppm potassium sorbate, which indicated the potential application of rOSM in the food industry.

Overall, recombinant tobacco osmotin could be expressed in large quantity and could inhibit the growth of a wide range of microbes, including yeast, molds and bacteria. Recombinant tobacco osmotin was stable in the temperature between -20 to 25 °C for at least 6 months. The rOSM could delay *Aspergillus niger* infection and extend the shelf-life of strawberries. Based on this study, osmotin has the potential to serve as a food preservative in the future for the food industry.

REFERENCES

Abad LR, D'Urzo MP, Liu D, Narsimhan ML, Ruveni M, Zhu JK, Niu X, Singh NK, Hasegawa PM, Bressan RA. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci 118:11-23.

Aguayo E, Jansasithorn R, Kader AA. 2006. Combined effects of 1-metylcyclopropene, calcium chloride dip, and/or atmospheric modification on quality changes in fresh-cut strawberries. Postharvest Biol Technol 40:269-78.

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weyman K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc Natl Acad Sci USA 90:7327-31.

Bernier F, Berna A. 2001. Germins and germin-like proteins: plant do-all proteins. But what do they do exactly? Plant Physiol Biochem 39:545-54.

Bhunia AK. 2008. Foodborne Microbial Pathogens. New York: Springer. p 1-4, 42-47.

Bohlmann H. 1994. The role of thionins in plant protection. Crit Rev in Plant Sci 13:1-16.

Bol JF, Linthorst HJM, Cornelissen BJC. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol 28:113-38.

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW, Osborn RW. 1997. Antimicrobial peptides from plants. Crit Rev in Plant Sci 16:297-323.

Campos Mde A, Silva MS, Magalhaes CP, Ribeiro SG, Sarto RP, Vieira EA, Grossi de Sa MF. 2008. Expression in *Escherichia coli*, purification, refolding and antifungal activity of an osmotin from *Solanum nigrum*. Microb Cell Fact 7:7.

[CDC] Centers for Disease Control and Prevention. 2009. Investigation Update: Outbreak of *Salmonella Typhimurium* Infections, 2008-2009. Available from: http://www.cdc.gov/salmonella/typhimurium/update.html. Accessed Nov 10, 2011.

[CDC] Centers for Disease Control and Prevention. 2011. Vital Signs: Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food --- Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996 —2010. MMWR 60:749-55.

Cheong NE, Choi YO, Kim WY, Chang S, Cho MJ, Lee SY. 1997. Purification of an antifungal PR-5 protein from flower buds of *Brassica campestris* and cloning of its gene. Physiol Plantarum 101:583-7.

Christensen AB, Ho Cho B, Naesby M, Gregersen PL, Brandt J, Madriz-Ordenana K, Collinge DB, Thordal-Christensen H. 2002. The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins. Mol Plant Pathol 3:135-44.

Chuck SL and Sande MA. 1989. Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. N Engl J Med 321:794-9.

Cross AJ, Ferrucci LM, Risch A, Graubard BI, Ward MH, Park Y, Hollenbeck AR, Schatzkin A, Sinha R. 2010. A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. Cancer Res 70:2406-14.

Daniell WF. 1852. On the *Synsepalum dulcificum*, Decand; or miraculous berry of Western Africa Pharm J 11:445.

Datta K, Tu J, Alam MF, Oliva N, Vasquez A, Abrigo E, Mew T, Muthukrishnan S, Datta SK. 1997. Expression of chitinase and thaumatin like protein gene in transgenic rice for sheath blight resistance (abstract). General Meeting of the International Program on Rice Biotechnology Malacca Sept 15-17 1997, p 353.

de la Monte SM, Neusner A, Chu J, Lawton M. 2009. Epidemilogical Trends Strongly Suggest Exposures as Etiologic Agents in the Pathogenesis of Sporadic Alzheimer's Disease, Diabetes Mellitus, and Non-Alcoholic Steatohepatitis. J Alzheimers Dis 17:519-29.

Del-Valle V, Hernandez-Munoz P, Guarda A, Galotto MJ. 2005. Development of a cactus-mucilage edible coating (*Opuntia ficus indica*) and its application to extend strawberry (*Fragaria ananassa*) shelf-life. Food Chem 9:751-6.

Dwivedi BK, Arnold RG. 1973. Chemistry of Thiamine Degradation in Food Products and Model Systems: A Review. J Agric Food Chem 21:54-60.

El Ghaouth A, Arul J, Ponnampalam R, Boulet M. 1991. Chitosan coating effect on storability and quality of fresh strawberries. J Food Sci 56:1618-20.

El Ghaouth A, Arul J, Grenier J, Asselin A. 1992. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology 82:398-402.

Eyal Y and Fluhr R. 1991. Cellular and molecular biology of pathogenesis related proteins. Oxf Surv Plant Mol Cell Bio 7:223-54.

Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P. 1996. A cherry protein and its gene abundantly expressed in ripening fruit have been identified as thaumatin-like. Plant Physiol 111:269-73.

FoodNet C. 2010. Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food --- 10 States, 2009. MMWR 59:418-22

Frenzen PD, Drake A, Angulo FJ. 2005. Economic cost of illness due to *Escherichia coli* O157 infections in the United States. J Food Prot 68:2623-30.

Garcia JM, Herrera S, Morilla A. 1996. Effects of postharvest dips in calcium chloride on strawberry. J Agric Food Chem 44:30-33.

Garcia-Olmedo F, Molina A, Segura A, Moreno M. 1995. The defensive role of nonspecific lipid-transfer proteins in plants. Trends Microbiol 3:72-74.

Ghannoum MA, Ibrahim AS, Fu Y, Shafiq MC, Edwards JE, Jr., Criddle RS. 1992. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. J Clin Microbiol 30:2881-6.

Grenier J, Potvin C, Trudel J, Asselin A. 1999. Some thaumatin-like proteins hydrolyze polymeric beta-1,3-glucans. Plant J 19:473-80.

Gunnison AF, Jacobsen DW. 1987. Sulfite hypersensitivity. A critical review. CRC Crit Rev Toxicol 17:185-214.

Han C, Zhao Y, Leonard SW, Traber MG. 2004. Edible coatings to improve storability and enhance nutritional value of fresh and frozen strawberries (*Fragaria* × *ananassa*) and raspberries (*Rubus ideaus*). Postharvest Biol Technol 33:67-78.

Hernandez-Munoz P, Almenar E, Ocio MJ, Gavara R. 2006. Effect of calcium dips and chitosan coatings on postharvest life of strawberries (*Fragaria* × *ananassa*). Postharvest Biol Technol 39:247-53.

Hernandez-Munoz P, Almenar E, Del Valle V, Velez D, Gavara R. 2008. Effect of chitosan coating combined with postharvest calcium treatment on strawberry (*Fragaria* x *ananassa*) quality during refrigerated storage. Food Chem 110:428-35.

Hoffmann-Sommergruber K. 2000. Plant allergens and pathogenesis-related proteins—What do they have in common? Int Arch Allergy Immunol 122:155-66.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant Journal 8:97-109.

Jakszyn P, Gonzales CA. 2006. Nitrosamine and related food intake and gastric and esophafeal cancer risk: a systematic review of the epidemiological evidence. World J Gastroenterol 12:4296-303.

Jay JM, Loessner MJ, Golden DA. 2005. Modern Food Microbiology. 7th Ed. New York: Chapman & Hall. p 302-9.

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL. 2008. Global trends in emerging infectious diseases. Nature 451:990-3.

Jongedijk E, Tigelaar H, Van Roekel JSC, Bres-Vloemans SA, Dekker I, Van den Elzen PJM, Cornelissen BJC, Melchers LS. 1995. Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173-80.

Kester JJ, Fennema OR. 1986. Edible films and coatings: a review. Food Technol 60:47-59.

Kononowicz AK, Nelson DE, Singh NK, Hasegawa PM, Bressan RA. 1992. Regulation of the Osmotin Gene Promoter. Plant Cell 4:513-24.

Laemmli UK, Favre M. 1973. Gel electrophoresis of proteins. J Mol Biol 80:575-99.

LaRosa PC, Singh NK, Hasegawa PM, Bressan RA. 1986. Stable NaCl tolerance of tobacco cells is associated with enhanced accumulation of osmotin. Plant Physiol 91:855-61.

Legrand M, Kauffmann S, Geoffroy P, Fritig B. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA 84:6750-4.

Linthorst HJM. 1991. Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10:123-50.

Liu D, Raghothama KG, Hasegawa PM, Bressan RA. 1994. Osmotin overexpression in potato delays development of disease symptoms. Proc Natl Acad Sci USA 91:1888-92.

Liu D, Rhodes D, D'Urzo MP, Xu Y, Narsimhan ML, Hasegawa PM, Bressan RA, Abad L. 1996. In vivo and in vitro activity of truncated osmotin that is secreted into the extracellular matrix. Plant Sci 121:123-31.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-75.

Luna-Guzman I, Cantwell M, Barrett DM. 1999. Fresh-cut cantaloupe: effects of $CaCl_2$ dips and heat treatments on firmness and metabolic activity. Postharvest Biol Tec 17:201-13.

Lurie S, Klein JD. 1992. Calcium and heat treatments to improve storability of 'Anna' apples. HortScience 27:36-39.

Malehorn DE, Borgmeyer JR, Smith CE, Shah DM. 1994. Characterization and expression of an antifungal zeamatin-like protein (*Zlp*) gene from *Zea mays*. Plant Physiol 106:1471-81.

Mauch F, Staehelin LA. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. The Plant Cell 1:447-57.

Medoff G, Kobayashi GS, Kwan CN, Schlessinger D, Venkov P. 1972. Potentiation of rifampicin and 5-fluorocytosine as antifungal antibiotics by amphotericin B. Proc Natl Acad Sci USA 69:196-9.

Min K, Ha SC, Hasegawa PM, Bressan RA, Yun DJ, Kim KK. 2004. Crystal structure of osmotin, a plant antifungal protein. Proteins 54:170-3.

Mitchell TG, Perfect JR. 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 8:515-48.

Moiseyev GP, Fedoreyeva LI, Zhuravlev YN, Yasnetskaya E, Jekel PA, Beintema JJ. 1997. Primary structures of two ribonucleases from ginseng calluses. New members of the PR-10 family of intracellular pathogenesis-related plant proteins. FEBS Lett 407:207-10.

Narasimhan ML, Damsz B, Coca MA, Ibeas JI, Yun DJ, Pardo JM, Hasegawa PM, Bressan RA. 2001. A plant defense response effector induces microbial apoptosis. Mol Cell 8:921-30.

Narasimhan ML, Lee H, Damsz B, Singh NK, Ibeas JJ, Mastmuto TK, Woloshuk CP, Bressan RA. 2003. Overexpression of a cell wall glycoprotein in *Fusarium oxysporum* increases virulence and resistance to a plant PR-5 protein. Plant J 36:390-400.

Narasimhan ML, Coca MA, Jin J, Yamauchi T, Ito Y, Kadowaki T, Kim KK, Pardo JM, Damsz B, Hasegawa PM, Yun DJ, Bressan RA. 2005. Osmotin is a homolog of mammalian adiponectin and controls apoptosis in yeast through a homolog of mammalian adiponectin receptor. Mol Cell 17:171-80.

Niderman T, Genetet I, Bruyere T, Gees R, Stintzi A, Legrand M, Fritig B, Mosinger E. 1995. Pathogenesis-related PR-1 proteins are antifungal; isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol 108:17-27.

No HK, Meyers SP, Prinyawiwatkul W, Xu Z. 2007. Applications of chitosan for improvement of quality and shelf life of foods: a review. J Food Sci 72:87-100.

Nunes MCN, Brecht JK, Morais AMMB, Sargent SA. 1998. Controlling temperature and water loss to maintain ascorbic acid in strawberries during postharvest handling. J Food Sci 63:1033-6.

Ogata CM, Gordon PF, deVos AM, Kim SH. 1992. Crystal structure of a sweet tasting protein thaumatin I at 1.65 A resolution. J Mol Biol 228:893-908.

Pappas G, Panagopoulou P, Christou L, Akritidis N. 2006. Category B potential bioterrorism agents: bacteria, viruses, toxins, and foodborne and waterborne pathogens. Infect Dis Clin North Am 20:395-421, x.

Park SI, Stan SD, Daeschel MA, Zhao Y. 2005. Antifungal coatings on fresh strawberries (*Fragaria* × *ananassa*) to control mold growth during cold storage. J Food Sci 70:M202-7.

Polak A, Scholer HJ, Wall M. 1982. Combination therapy of experimental candidiasis, cryptococcosis and aspergillosis in mice. Chemotherapy 28:461-79.

Powderly WG. 1993. Cryptococcal meningitis and AIDS. Clin Infect Dis 17:837-42.

Roberts WK, Selitrennikoff CP. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J Gen Microbiol 136:1771-8.

Sams CE, Conway WS, Abbot JA, Lewis RJ, BenShalom N. 1993. Firmness and decay of apples following postharvest pressure infiltration of calcium and heat treatment. J Am Soc Hortic Sci 118:623-7.

Sanz C, Perez AG, Olias R, Olias JM. 1999. Quality of strawberries packed with perforated polypropylene. J Food Sci 64:748-52.

Scallan E, Hoekstra RM, Angulo FJ. 2011. Foodborne illness acquired in the United States---major pathogens. Emerg Infect Dis 17:7-15.

Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75:123-31.

Shahidi F, Pegg RB. 1992. Nitrite-free meat curing systems: update and review. Food Chem 43:185-91.

Singh NK, Handa AK, Hasegawa PM, Bressan RA. 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol 79:126-37.

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA. 1987. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85:529-36.

Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA. 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol 90:1096-101.

Soliva-Fortuny RC, Martin-Belloso O. 2003. New advances in extending the shelf-life of fresh-cut fruits: a review. Trends Food Sci Tech 14:341-53.

Stark AA. 1980. Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. Annu Rev Microbiol 34:235-62.

Stintzi A, Heitz TS, Kauffmann S, Legrand M, Fritig B. 1991. Thaumatin-like protein of virus-infected tobacco osmotin. Physiol Mol Plant Pathol 38:137-46.

Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41:207-34.

Thermo Scientific. 2008. Thermo Scientific Pierce® Protein Refolding Kit Guide Product No. 89867. Thermo Fisher Scientific Inc..

Tzou YM, Huang TS, Huggins KW, Chin BA, Simonne AH, Singh NK. 2011. Expression of truncated tobacco osmotin in *Escherichia coli*: purification and antifungal activity. Biotechnol Lett 33:539-43.

[USDA] US Department of Agriculture. 2011. Foodborne illness cost calculator: *Salmonella*. Available from: http://www.ers.usda.gov/data/foodborneillness/salm_intro.asp. Accessed Mar 8 2012.

Van Loon LC, Gerritsen YAM, Ritter CE. 1987. Identification, purification, and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol Biol 9:593-609.

Van Loon LC. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103:753-65.

Van Loon LC, Van Strien EA. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85-97.

Van Loon LC, Rep M, Pieterse CM. 2006. Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135-62.

Vargas M, Albors A, Chiralt A, Gonzalez-Martinez C. 2006. Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings. Postharvest Biol Technol 41:164-71.

Vigers AJ, Roberts WK, Selitrennikof CP. 1991. A new family of plant antifungal proteins. Mol Plant Microbe In 4:315-23.

Vigers AJ, Weidmann S, Roberts WK, Legrand M, Selitrennikoff CP, Fritig B. 1992. Thaumatin-like pathogenesis related proteins are antifungal. Plant Sci 83:155-61.

Vu L, Hyunh Q. 1994. Isolation and characterization of a 27 kDa antifungal protein from the fruits of *Diospyros texana*. Biochem Biophys Res Commun 202:666-71.

Woloshuk CP, Meulenhoff SJ, Sela-Burlage M, Van den Eltzen PJM, Cornelissen BJC. 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3:619-28.

Yun DJ, Zhao Y, Pardo JM, Narasimhan ML, Damsz B, Lee H, Abad LR, D'Urzo MP, Hasegawa PM, Bressan RA. 1997. Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. Proc Natl Acad Sci USA 94:7082-7.

Yun DJ, Ibeas JI, Lee H, Coca MA, Narsimhan ML, Uesono Y, Hasegawa PM, Pardo JM, Bressan RA. 1998. Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Mol Cell 1:807-17.

Zemanek E, Wasserman BP. 1995. Issues and advances on the use of transgenic organisms for the production of Thaumatin, the intensely sweet protein from *Thaumatococcus daniellii*. Crit Rev Food Sci Nutr 35:455-66.

Zhu BL, Chen THH, Li PH. 1996. Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. Planta 198:70-77.

Zhu Q, Maher EA, Masoud S, Dixon RA and Lamb CJ. 1994. Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. Nat Biotechnol 12:807-12.