ISOLATION OF EXTRACELLULAR PROTEINS FROM *OPHIOSTOMA ULMI* AND THEIR EFFECT ON TENSILE PROPERTIES OF THERMOPLASTIC STARCH

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Starch-derived bioplastics are an inexpensive, renewable and environmentally-friendly alternative to traditional petroleum-based plastics. Proteins secreted by *Ophiostoma ulmi*, were investigated for their application in bioplastic product. Proteins were isolated from fungal cultures by anion exchange chromatography and used to treat starch. Subsequently, plastic films were generated by solution casting, with glycerol as plasticizer. Tensile strength of the films was found to increase significantly compared to the control. The relative water holding capacity of the treated starch also decreased dramatically. Attempts to identify fungal proteins by MALDI-TOF MS/MS did not result in positive matches, mainly due to lack of fungal sequence information. Additionally, the effect of non-specific proteins resulted in a modest increase in tensile strength and a slightly greater effect on water absorption. Proteins secreted by *O. ulmi* were therefore implicated in improving properties of starch-based plastics. Investigation into the role of an extracellular polysaccharide is also suggested.
Acknowledgements

I would like to thank Dr. Dinesh Christendat for his guidance and supervision over the course of my research and for his patience and help in completion of my thesis. I am also grateful to my co-supervisor Dr. Mohini Sain for his support and knowledgeable input into the project. I would like to thank all my lab mates; especially Leah Draper and Van Quach for their friendship and always being there for me, Geoff Fucile for his insight, Jimmy Poulin for keeping me informed about world events and James Peek for the humor. I am also thankful to May Aldea, Jignasha Patel and Shu Hui for their wisdom and optimism and to Vibha Tyagi and Smith Sundar for their support and technical help. Finally, I would like to thank my family; my mom and Asad, for being extremely patient and for all their love and confidence in me.
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List of Abbreviations

ASTM - American Society for Testing and Materials
BSA - Bovine Serum Albumin
C.I. % - Confidence Interval %
DEAE – Diethylaminoethyl
DTT - Dithiothreitol
EDTA – Ethylenediaminetetraacetic acid
EPS – Extracellular Polysaccharide
FT-IR – Fourier Transform-Infrared spectroscopy
GPS – Global Protein Server
His – Histidine
IEC – Ion Exchange Chromatography
IPTG - Isopropyl β-D-thiogalactopyranoside
kD/kDa – kiloDalton
LB – Luria-Bertani medium
MALDI-TOF - Matrix Assisted Laser Desorption Ionization-Time-of-Flight
MDa – MegaDalton
MS - Mass spectrometry
MS/MS – tandem MS
Mw – Molecular weight
MWCO – Molecular Weight Cut Off
MYA - Million Years Ago
NCBI – National Centre for Biotechnology Information
Ni-NTA – Nickel Nitrilotriacetic acid
OD – Optical Density
O. ulmi – Ophiostoma ulmi
PAGE – Polyacrylamide gel electrophoresis
PI – Protease Inhibitor
PMF – Protein Mass Fingerprint
PMSF - Phenylmethyl sulfonyl fluoride
PLA - poly(lactic acid)
PHA - polyhydroxyalkanoate
Pv - pathovar
PVC – Polyvinyl Chloride
RH – Relative Humidity
SDH – Shikimate dehydrogenase
SDS – Sodium dodecyl sulfate
spp - species
TPS – thermoplastic starch
TS – Tensile stress
XPS – X-ray photoelectron spectroscopy
1. Introduction

1.1 The need for biodegradable polymer

The widespread use of petroleum-based plastics has raised environmental concerns and instigated efforts towards developing alternatives. The term ‘plastic’ is defined as any of numerous organic synthetic or processed materials that are mostly thermoplastic or thermosetting polymers of high molecular mass and that can be made into objects, films or filaments (Merriam–Webster Dictionary definition). The most commonly used plastic materials such as polyethylene, polyvinylchloride (PVC), polystyrene and polypropylene are synthesized from petrochemicals and are often referred to as traditional plastics (Nawrath et al., 1995 and Mooney, 2009).

Traditional plastics can release toxic byproducts into the environment. For example, PVC plastics are used to make food containers and contain plasticizers called phthalates that can leach out into food (Meeker et al., 2009). Phthalates, bisphenol A or polybrominated diphenyl ethers, as well as other chemicals found in plastics, may be harmful to humans by altering endocrine function or interfering with other biological mechanisms (Heudorf et al., 2007).

Toxicity, depleting petroleum resources as well as waste accumulation has made it imperative to rethink synthetic polymers. Biopolymer-based plastics (bioplastics) can mitigate many of these problems. For example, a major concern with traditional plastic usage is greenhouse gas emissions; 2-3 kg CO₂ emissions are estimated to be produced per kg of resin used while polyhydroxyalkanoate (PHA), a biopolymer-based plastic, only causes
about 0.45kg of CO₂ emissions. Therefore, using bioplastics like PHA can lead to about 80% reduction in global warming potential. Traditional plastics not only use fossil fuels as feedstock but also as an energy source for their manufacture. Bioplastics would reduce reliance on fossil fuels; PHA requires 0.44MJ less fossil fuel in its processing compared to its petroleum-based counterparts (Yu and Chen, 2008).

Another concern is plastic disposal; traditional plastics are designed to be stable in different conditions and to persist in the environment for many years. The ubiquitous use of plastics has contributed to about 12% of the 227 metric tons of municipal waste produced annually in the United States. About 30% of plastic is recovered from the waste stream by recycling, but that still allows plastics to accumulate in the environment at a rate of more than 18.2 metric tons per year (Mooney, 2009).

Furthermore, the large increase in oil prices translates to a higher production cost of petroleum-based plastics, and has led some biopolymer-based plastics or bioplastics to compete with traditional plastics (van Beilen and Poirier, 2007). Much of the research aimed at developing bioplastics has focused on using plant matter as raw material; this is well reviewed by Nawrath et al. (1995), Flieger et al. (2003) and van Beilen and Poirier, (2007) among others.

Plants produce enormous amounts of biomass by carbon dioxide fixation during photosynthesis. Plant biomass synthesis is estimated to be about 140 billion tons annually (Bouws, et al., 2008). Biomass includes materials such as lignin, cellulose and starch which
are produced continuously and are almost inexhaustible, unlike fossil fuels. Lignocellulose and starch have been used in various ways in the industry, the most notable of which are food, paper, textile and adhesives (Flieger et al., 2003). It is not surprising that plastics are also being made from plant polymers since the first known man-made plastic material (in 1862) was cellulose nitrate (Nawrath et al., 1995) and another one of the earliest plastics used in the mid-nineteenth century, collodion, was made with cotton cellulose (van Beilen and Poirier, 2007). More recently, starch has emerged as one of the primary raw materials for eco-friendly plastics.

1.2 Starch

1.2.1 Properties of Starch

Starch is a unique carbohydrate that is found in discrete structures called granules: the primary form of energy storage in plants. Rich sources of starch include cereal plants such as corn and rice, tubers including potato and tapioca. Starch usually exists as a combination of two \( \alpha \)-glucose polymers; amylose, which has a linear structure and amylopectin, which is branched. In native starch granules, amylose exists in an amorphous, non-crystalline state while amylopectin forms crystalline regions. Consequently concentric regions of alternating amorphous and crystalline structure are formed. The relative content of amylose and amylopectin varies amongst the different starch sources (Hui, 2006). The typical amounts of the two polymers in common starch sources are shown in table 1.
Table 1. Approximate amylopectin and amylose composition of various types of starch

<table>
<thead>
<tr>
<th>Type</th>
<th>% Amylopectin</th>
<th>% Amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>Waxy Corn</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>High Amylose</td>
<td>20-45</td>
<td>55-80</td>
</tr>
<tr>
<td>Potato</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>Rice</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>Tapioca</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Wheat</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

(Source: Hui, 2006; Mishra and Rai, 2006)
1.2.2 Amylose and Amylopectin

Amylose is a linear chain where \( \alpha \)-glucose molecules are linked together by 1,4-glycosidic bonds (van Bailein and Poirier, 2007). While amylopectin has a branched structure that contains additional 1,6-glycosidic bonds associated with about one in every twenty glucose residues (figure 1) (van Bailein and Poirier, 2007).

Amylose forms a single helix that has hydrogen-bonding oxygen atoms on the outside surface with only the ring oxygen pointing inwards. The helix possesses a relatively hydrophobic inner surface that holds a spiral of water molecules, which are easily lost to be replaced by hydrophobic lipid or aromatic molecules (Hui, 2006). This property of the amylose molecule is also useful in detection of starch using iodine solution. In this test, iodide ions are sequestered in the central tunnel of amylose helices and produce a blue color which indicates amylose content as detectable at 650 nm (Herrero-Martinez, 2004). Unlike amylose, the branched amylopectin molecule contains up to two million glucose residues in a compact cluster and can form only limited associations with other molecules (Hui, 2006).
a) α-Glucose

b) Amylose

![Amylose Diagram]

1,4 glycosidic bonds

1,6 glycosidic bond

1,4 glycosidic bonds

c) Amylopectin

![Amylopectin Diagram]

Figure 1. Structure of (a) α-glucose, (b) amylose showing 1-4-glycosidic linkage and (c) amylopectin showing 1-4 and 1-6 glycosidic linkage.
1.3 Plastics Derived From Starch

Starch is the largest agricultural commodity after cellulose. Its high abundance (57 x 10^6 tons/year production worldwide) and low cost and has permitted its use in non-food applications. Currently about 40,000 tons of starch/year are converted to plastic materials worldwide (van Beilen and Poirer, 2007).

1.3.1 Plasticization of Native Starch

Native starch is obtained by separation of naturally occurring, raw starches from root or cereal crops and still retains the original structure and characteristics. It is the basic starch product that is marketed in the dry powder form under different grades for various applications. Starch can be processed into a plastic simply by applying heat in the presence of water. During the plasticization process, starch undergoes a number of changes, namely gelatinization, pasting and retrogradation. The first two are temperature-dependent, irreversible modifications and enable the starch granules to be solublized hence playing a role in its properties such as texture, viscosity and moisture retention. (Atwell et al, 1988).

1.3.1.1 Gelatinization

Starch is almost insoluble in cold water; however, when the granules are heated in water, the hydrogen bonds (H-bonds) within the granules are disrupted and the free OH groups of the glucose units interact with water molecules, causing the granules to swell. The initial swelling takes place in the amorphous regions of the starch granule where fewer H-bonds exist and therefore are more susceptible to dissolution (Lozano, 2000).
The temperature at which starch begins to undergo these changes is called its gelatinization temperature. Since all granules in one type of starch do not begin to gelatinize at the same time, the gelatinization temperature for each type of starch is defined by a temperature range (table 2) (Roos, 1995). According to analysis conducted at the Starch Science and Technology Conference in 1988, the gelatinization process differs not only with respect to the source of starch but also on cooking conditions (e.g. pH, temperature, amount of moisture) (Atwell et al., 1988).
Table 2. Gelatinization temperatures of common types of starch

<table>
<thead>
<tr>
<th>Type</th>
<th>Gelatinization Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>62-80</td>
</tr>
<tr>
<td>Waxy Corn</td>
<td>63-72</td>
</tr>
<tr>
<td>Potato</td>
<td>58-65</td>
</tr>
<tr>
<td>Tapioca</td>
<td>52-65</td>
</tr>
<tr>
<td>Wheat</td>
<td>52-85</td>
</tr>
</tbody>
</table>

(Source: Roos, 1995)
1.3.1.2 Pasting

As a starch solution is heated further, the amylose chains begin to leach out of the granules and the viscosity of the solution increases. With continued heating, even more amylose molecules as well as amylopectin leave the granule and are solubilized. This process is called pasting. The viscosity of the medium is highest when the maximum number of swelled, intact granules is present; this is referred to as its peak viscosity. The pasting process is completed once the peak viscosity is reached. It should be noted that pasting is not a completely distinct phenomenon compared to gelatinization but that both processes occur somewhat concomitantly. Further heating after pasting causes more and more granules to break down and an even greater increase in polymer solubilization occurs while viscosity decreases. The textural and viscous properties of the resulting paste change upon cooling and are dependent on the amylose content. (Whistler and BeMiller, 2009).

1.3.1.3 Retrogradation

Retrogradation describes the process whereby solubilized starch molecules re-associate upon refrigeration or cooling to room temperature. Initially, two or more starch molecules may link together followed by the formation of a more highly ordered structure. Under favorable conditions, a crystalline structure is formed (Atwell et al., 1988). During cooling, less thermal energy is available for the starch molecules to remain solubilized, hence causing them to form crystalline aggregates. Retrogradation is especially evident in higher amylose pastes since linear amylose chains can form H-bonds much more readily than the bulkier amylopectin molecules. Starches from different sources retrograde at varying rates and to different extents. For example, tapioca starch (about 18% amylose) sets to a soft gel over
time while high-amylose corn starch (about 40% amylose) sets to a firm gel relatively quickly upon cooling after pasting (Whistler and BeMiller 2009).

1.3.2 Limitations of Starch Plastics

One of the main problems with starch-based plastics is their susceptibility to moisture. Starch is hydrophilic due to the hydroxyl groups projecting outwards from starch chains. Consequently, starch-based plastics swell and deform under high moisture conditions (Mooney, 2009). The other issue with starch-based polymers, is their brittleness, poor strength and lack of stiffness as compared to traditional, petroleum-based plastics (Fang and Fowler, 2003). The brittleness is due to the crystallinity of starch once it has undergone retrogradation. This is usually countered by addition of glycerol.

1.3.3 Starch Plastic Technology

Various strategies have been used to produce starch-based plastics with desired properties. Some of these include blending with petroleum-based resins or other biodegradable plastics, chemical derivitization, fermentation by microbes and subsequent polymerization to polylactic acid (PLA) or polyhydroxyalkanoates (PHA) as well as genetic modification of starch synthesis in plants (Mooney, 2009).

Production of various thermoplastic starch composites is based on mixing starch with vinyl alcohols. These types of polymers tend to be more stable and their biodegradability is inversely proportional to their starch content. (Mooney, 2009). One of the technologies developed to improve starch properties was siliconization of starch granules. This caused a
reduction in moisture absorption and allowed small amounts of starch (5-15%) to be blended with synthetic polymers such as polyethylene, polyvinyl chloride (PVC) and polystyrene. These efforts were aimed at improving biodegradability of synthetic polymers but were met with failure according to biodegradability standards. In fact, the addition of synthetic polymers decreased the biodegradation process of starch itself, possibly due to inaccessibility to microbial enzymes (Nawrath et al, 1997).

Gelatinization of starch prior to blending, is another method developed to allow for a higher starch content to be incorporated into composites. Degradation of these materials is improved compared to blends made with siliconized starch granules. However, they are only partially susceptible to degradation and leave behind a porous mass which may be more prone to erosion (Nawrath et al, 1997).

Starch can be chemically modified to improve its physical properties. For example starch has been hydroxypropylated with a low degree of derivitization with a ratio of 1:100 group being derivitized. This low level derivitization enhances the biodegradability of the composite material. Evercorn plastic materials have developed a modified starch resin that is water resistant but remains biodegradable and compostable (Nawrath et al., 1995).

Fermentation technology has been used to produce polylactic acid (PLA) from starch. PLA was first developed by Dow Chemicals in the 1950s but had limited application in medical devices due to high cost of production. Recent technological advances have reduced cost of production and allowed PLA to be commonly used in packaging. Lactic acid bacteria
break down starch to sugars, which are fermented to pyruvate. Conversion of pyruvate to lactic acid occurs by the action of microbial lactate dehydrogenases. The lactic acid molecules can be condensed to lactide (circularized lactic acid) followed by a ring-opening polymerization reaction to produce high molecular mass PLA (Mooney, 2009).

Genetic engineering of plants is also being used to produce starch materials with desired properties. Antisense down-regulation of granule bound starch synthase I enzyme has been used to produce potato starch that is amylose-free as certain applications require amylopectin only (Nawrath et al., 1995). In cases where higher amylose is needed, similar strategies can be applied. However, transgenic plants grown solely for the purpose of production of novel biopolymers have not been commercialized, mainly due to the time and cost associated with their development.

1.3.4 Tensile Properties

The usefulness of starch and other plastics is evaluated by measuring mechanical or tensile properties. Tensile properties indicate how the material will react to forces being applied. The ability of a material to resist breaking under mechanical stress is one of the most important properties and is widely used as a measure of strength for structural applications. The force (F) per unit cross-sectional area (A) required to break a material in this manner is called its maximum tensile strength (TS). The tensile strength is defined as: \( TS = \frac{F}{A} \) (Davis, 2004 and El Zohairy et al., 2004). The cross-sectional area is a product of the width (w) and thickness (t) of the specimen (A = wt) (Yang et al., 1997).
A tensile test is a fundamental mechanical test where a specimen is subjected to a uniformly increasing force and its behaviour is monitored over time until fracture occurs (Cornish, 1987). At first, the material undergoes uniform elastic deformation (Hooke’s law) followed by plastic deformation. This leads to ‘necking’, which is the reduction in cross-sectional area undergone by a material under stress, and finally fracture occurs. Brittle materials undergo a brief period of elastic deformation and break soon after, while ductile materials go through a long period of deformation and necking before breakage. Universal stress testing machines, such as the Instron, measure the stress-strain relationship of the material under tension (Davis, 2004). Although the application of a material dictates the preferred properties, for our purpose a high tensile strength and low water absorption properties are desired.

1.4 Filamentous Fungi and Ophiostoma

1.4.1 Industrial Application of Filamentous Fungi
The earliest industrial application of filamentous fungi was in the food and beverage sector. However, their ability to produce of large quantities of various extracellular hydrolytic enzymes has warranted a wide range of industrial applications of filamentous fungi, such as in the textile, detergent, pulp and paper and pharmaceutical industries, to name a few. Industrial strains of Aspergillus are available that can produce over 30 g/l of a specific protein, such as glucoamylase which is used for fermentation, primarily for alcohol production (Finkelstein et al., 1989). Extracellular enzyme yields for industrial strains of Trichoderma reesei have been reported to be as high as 100 g/l (Cherry and Fidantsef, 2003). Other fungal enzymes like proteases have also found use in the food and detergent industries.
1.4.2 Application of Fungi in Development of Novel Starch Plastics

Treatment of starch using *Ophiostoma ulmi* (an ascomycete fungus that causes Dutch Elm Disease) was found to increase the molecular weight (Mw) and strength of the polymer as well as its water-barrier properties, thus making it suitable for industrial applications such as packaging. *O. ulmi* was shown to cause the percentage of molecules with Mw >10MDa to increase from 25% to 89% after 3 days (Huang et al, 2006). Additionally, mechanical tests showed that the tensile strength and modulus of the films cast with the modified biopolymer increased about 10 and 40 times, respectively as compared to films made with the “unmodified” starch (Huang et al, 2006). However, the nature and mode of the modification are unknown.

1.4.3 *Ophiostoma* and its Secreted Molecules

*Ophiostoma* is an ascomycete fungal genus that consists of causal agents of the Dutch Elm Disease (DED). This genus was responsible for two massive DED pandemics that ravaged elm trees across the northern hemisphere. The first pandemic, caused by *Ophiostoma ulmi*, began in 1910 in north-west Europe and spread over Europe and North America (Pipe et al. 2000). The emergence of a far more destructive species called *Ophiostoma novo-ulmi* is responsible for the second pandemic (Brasier et al. 2000). Despite their economic importance, these fungi are not very well studied. A universally accepted mechanism for pathogenesis needs to be established; however, the pathogenesis of DED is suggested to involve fungal toxins (mainly cerato-ulmin), growth substances, or cell wall degradation (Takai 1974,; Scheffer et al., 1987; Temple et al., 2000; Keon et al., 1990).
1.4.3.1 Secreted Enzymes

In general, *Ophiostoma* spp do not degrade cellulose and lignin and it has been shown that starch and soluble sugars are their main carbon sources. Thus, enzymes such as amylases are important for their colonization. Other major enzymes secreted by *Ophiostoma* are proteinases and lipases that enable it to hydrolyze proteins and triglycerides, respectively (Wu *et al.*, 2006). Lipase activity has been demonstrated in *O. piliferum* (Brush *et al.*, 1999) and sap-staining *O. piceae*, where hydrolysis of triglycerides results in melanin production (Gao and Breuil, 1998). Furthermore, lipases are important in resin duct colonization (Wu, 2007). Plant pathogenic species of *Ophiostoma* also secrete laccase and cellulolytic enzymes in order to establish and maintain growth in wood (Binz and Canevascini, 1997).

1.4.3.2 Secreted Polysaccharide

Several microbes are known to produce polysaccharides. The ones that are associated with the cell surface are known as capsular polysaccharides (CPS) while those that are secreted into the environment are referred to as extracellular polysaccharides (EPS). *Ophiostoma* is known to produce an EPS (Jeng, 2006) which was believed to help in the infection process by blocking the xylem vessels and preventing water conduction in Elm trees, causing them to die. Recently, Jeng *et al.* (2006) have suggested that the EPS may not be involved in pathogenesis since it is produced in larger amounts in the non-aggressive strain *O. ulmi* compared to the aggressive *O. novo-ulmi*. The EPS can be synthesized intracellularly using sugar nucleotide precursors or extracellularly with exogenous substrates. Many EPS consist of repeating units of sugars that are linked by the action of enzymes such as glycosyltransferases that also attach them to lipid carriers (Boels *et al.* 2001).
1.4.3.3 Hydrophobins

Filamentous fungi are known to secrete small proteins called hydrophobins (Stringer and Timberlake, 1993). These are about 100 amino acids long, amphiphilic proteins that are characterized by eight consecutive cysteine residues (Scholtmeijer et al., 2001). Hydrophobins self assemble at hydrophobic-hydrophillic interfaces in the form of amphipathic films. They are utilized by fungi to adapt to their environment by coating various fungal structures, mediating surface attachment and lowering the surface tension of water (Szilvay et al., 2006).

*Ophiostoma ulmi* also produces hydrophobins for example, cerato-ulmin (CU) which is a toxin involved in DED. Its peptide sequence has been published and shares the main structural characteristics of hydrophobins; a length of about 75 amino acids, eight conserved cysteine residues, and similar hydrophobic domains (Stringer and Timberlake, 1993).

1.5 Hypotheses for the role of *Ophiostoma* proteins in starch modification

In order to explain the starch modification caused by *O. ulmi*, two main hypotheses were made. One hypothesis was that the observed effect may be enzymatic in nature where the exposed hydroxyl (OH) groups of the starch chains are modified. Replacement with more hydrophobic groups could cause the improved water repulsion of the starch plastics.

Another mode of modification could be adsorption of protein to the starch molecule which may block off the hydroxyl groups to reduce interaction with water. Additionally, they could also behave as cross-linking agents that may join starch molecules together in a
manner that improves the strength of the molecule. However, the scope of this particular study is a proteomic investigation to verify that secreted *O. ulmi* proteins are involved in starch modification and not the nature of modification.

### 1.6 Commonly Occurring Enzymatic Modification of Starch

Although the nature of modification mediated by *O. ulmi* is unknown, enzymatic modification of starch merits some discussion here. Enzymatic modification of starch most commonly involves hydrolysis and transglycosylation by carbohydrate enzymes. For example, 4-α-glucanotransferase (αGT) catalyzes the transfer of an α-glucan chain from one α-glucan molecule to another in a reaction called disproportionation. Such enzymes can catalyze intermolecular glucan transfer reactions, as well as intramolecular disproportionation transfers within a single linear glucan molecule (Takaha and Smith, 1999). Another example is branching enzyme (BE, 1,4-α-glucan, 1,4-α-glucan 6-glucosyltransferase), which is also widely used to enzymatically modify starch. BE catalyzes the formation of α-1,6 branching points and converts amylose into branched glucans containing cyclic forms (Takata *et al.*, 1996). A third enzyme is maltogenic amylase, a microbial glycoside hydrolase, which hydrolyzes starch to maltose by the cleavage of α-1,4-glycosidic bonds. It also exhibits high transglycosylation activity via the formation of various glycosidic linkages, such as α-1,6- and α-1,3-bonds, which produce branched oligosaccharides from liquefied starch. Recent literature suggests that maltogenic amylase produces branched side chains when incubated with amylopectin clusters (Lee *et al.* 2008).
1.7 Proteomics

1.7.1 Concept of Proteomics

Proteins are recognized as the key players that mediate biological and metabolic processes in an organism. Over a decade ago, the term proteome was coined to describe the entire set of proteins expressed in an organism under specific conditions. Proteomics, by extension, refers to the large-scale study of proteins, their identification and functional characterization. The assessment of a particular subset of the proteome, for example cell envelope, mitochondrial or secreted proteins, is referred to as subproteomics. Most filamentous fungi subproteomic studies carried out thus far have focused on membrane or organelle proteins. For example, Grinyer et al. (2007) separated and identified 13 subunits of the *T. reesei* 20S proteasome, providing the first filamentous fungal proteasome proteomics. Kim et al. (2007) have reviewed this and other filamentous fungal proteomic studies. Our study focused on the secreted proteins of *O. ulmi*.

Theoretically, MALDI-TOF MS is sufficient for identification of proteins from sequenced genomes (Medina et al., 2005). However when limited or no sequence information is available MS/MS may be required for protein identification. Proteins can be identified by MS/MS of single peptides that coincide exactly with peptides found in protein sequences known from other sequenced organisms. For this study, we have attempted to isolate and identify secreted proteins from *O. ulmi*. Only a partial EST sequence database is available for *O. ulmi*. Therefore 1DE MS/MS was selected as this approach has been shown to be successful in the identification of proteins from large genomes with little or no annotation (Gao and Breuil, 1998 and Medina et al., 2005). Medina et al (2005) identified a
total of 51 unique secreted proteins using this approach from *Aspergillus flavus*, which is also a filamentous fungus with little partial sequence information.

### 1.7.2 Mass Spectrometry as a Tool for Protein Identification

Mass Spectrometry (MS) has emerged as a key tool for proteomics that allows protein identification. Prior to MS, complex mixtures of proteins such as secreted molecules isolated from fungal cultures, need to be separated. This is primarily done by polyacrylamide gel electrophoresis (PAGE) either in one dimension by SDS-PAGE based on difference in mass or in two dimensions by first separating proteins based on their isoelectric point (pI) and then by difference in mass. Individual protein bands/spots can be digested using proteases. The resulting peptides can be subjected to any of the various MS technologies. MALDI-TOF or Matrix Assisted Laser Desorption Ionization- Time-of-Flight MS is relevant to our study and is briefly discussed here.

In MALDI-TOF MS, the sample is spotted onto a metal plate along with a matrix and allowed to crystallize. The plate is inserted into a high vacuum chamber and the sample is irradiated with a laser. The energy from the laser releases protons from the matrix molecules that are acquired by the peptides resulting in singly charged positive ions (in positive mode). The peptide ions are then accelerated by an electric field to the same kinetic energy and are resolved according to their mass-to-charge ratio (m/z). At the end of the flight, the ions reach a detector at different times depending on their m/z ratio. The signal is amplified and transmitted to a data system. Consequently mass spectra called protein mass fingerprints (PMF) are generated where a peak, whose height indicates its relative abundance, represents
each ion. The mass data from these spectra can be correlated to the masses of known proteins in various proteomic databases such as NCBI and Swissprot and the identity of the unknown protein determined (Griffiths and Wang, 2008).

If the information obtained from PMF is insufficient to make a positive identification, tandem MS (MS/MS) is used. Certain peptide ions (usually the most abundant ones) are trapped and fragmented into shorter amino acid sequences by collisions with air or an inert gas like argon. The peptide fragments undergo a similar flight path as in MS where ions with the lowest m/z are detected first. The mass data from MS/MS can be used to identify proteins with a higher confidence than with MS alone.

1.7.3 Genetic diversity in Fungi and Implications for Proteomics Studies

In comparison with animals and plants, fungal genomes are more streamlined. The average genome size is about 37Mb and ranges between 6.5Mb to 795Mb for Scutellospora castanea and Pneumocystis carnii, respectively (Gregory et al., 2007). Gene densities across the genome differ significantly, between 37 and 61 genes per 100kb as compared to 10 genes per 100kb in Drosophila and about 1 gene per 100kb in humans (Aguileta et al., 2008). Chromosome numbers also vary greatly from the smallest number known to be 3 in Sacizosaccharomyces pombe, and the largest 20, in the basidiomycete Ustilago hordei and the chytrid Batrachochytrium dendrobatidis (Aguileta et al., 2008).

Fungi are extremely diverse at the genomic level; the DNA sequences as well as the organization of homologous genes among genomes, tends to be highly divergent. For
example, *Neurospora crassa* and *Magnaporthe grisea* are related ascomycetes that diverged around 200 MYA but their genomes share only about 74% identity at the amino acid level (similar to the distance between mammals and fish) and almost no co-localization of conserved genes within genomes (Aguileta et al., 2008).

*S. cerevisiae*, *S. pombe* and various other filamentous fungi have been sequenced and are well-established organisms for research; however, the vast majority of fungi including *Ophiostoma* are poorly understood. Divergence from the common ancestor of the fungal kingdom occurred over 800MYA (Lutzoni et al., 2004); this signifies the fact that these few well-studied organisms are not truly representative of all the fungi. Some studies have overcome the scarcity of genome annotation; for example, one study made use of activity probes to directly identify serine proteases through covalent enzyme labeling (Wu et al., 2007). Additionally, mass spectrometric techniques may be employed and cross-species matches may be made in some cases. However, given the diversity among fungi, any study (such as this one) involving identification of the secreted proteome would ultimately require specific gene sequence and annotation information.

### 1.7.4 Sequencing information from *Ophiostoma*

An initiative for large-scale sequencing of Ophiostomatoid genes is currently underway (Bernier et al., 2004). Three representative strains were selected from *O.novo-ulmi*, *O. ulmi* and *O. piceae* for single-pass automated sequencing on cloned cDNA. As a result a large EST library was prepared from yeast like cells of *O. novo-ulmi* with over 3,400 readable sequences that can serve as a general reference library for Ophiostomatoid fungi. Smaller,
specific EST libraries were made from *O. novo-ulmi* mycelia grown at suboptimal temperatures, perithecia formed in laboratory crosses, as well as from *O.piceae* grown on different carbon sources including starch (Bernier *et al.*, 2004).

Bernier *et al.*, (2004) report over 750 *Ophiostoma* unique ESTs being identified through bioinformatic searches in public databases. These showed significant homology with other fungal genes of known function, although a high proportion of *Ophiostoma* ESTs were either orphans (no match to any known gene) or showed homology to genes of unknown function. Starch-derived ESTs were found to include sequences coding for cellobiohydrolase, cellobiose dehydrogenase, endoglucanase, exochitinase, maltose permease, and cellulose-growth-specific protein. Although this information has not been made fully public, it is encouraging that such databases are being formulated, especially since the conditions relevant to our study (i.e. starch) have been used to generate some of these ESTs.

1.8 Specific Aims of the Study

The purpose of this study was to investigate the role of secreted fungal proteins in enhancing mechanical and water retention properties of starch plastics. The fungus used in this study, *Ophiostoma ulmi* has been previously implicated in starch modification and improvement in plastic properties (Huang et al, 2006). Extracellular proteins from *O. ulmi* cultures were isolated and tested for their effect on starch films. This research is the first step towards development of a novel process for bioplastic production using fungal proteins.
2. Materials and Methods

2.1 Maintenance of Fungal Cultures

The fungal isolate that was used is a non-aggressive strain of *Ophiostoma ulmi* that originated in the UK and was supplied by the Great Lakes Forest Center, Canada Forest Services, Sault Ste. Marie, Ontario. Food grade tapioca starch was used in this study as purchased from the supermarket. The fungus was grown with starch 0.1% (w/v) or without it, in Tchernoff media (Tchernoff, 1965). The media was composed of 2% (w/v) D-glucose, 0.2% (w/v) asparagine, 0.15% (w/v) KH$_2$PO$_4$, 0.1% (w/v) MgSO$_4$7H$_2$O, 0.002% (w/v) ZnSO$_4$, 0.001% (w/v) FeCl$_3$, 0.0001% (w/v) pyridoxine, and 0.00001% (w/v) thiamine. The contents were dissolved in deionized water and the pH was adjusted to 6 using concentrated hydrochloric (HCl) acid. The prepared media was sterilized by heating to 121°C for 20 minutes. 100 mL of fungal stock (approximately 300-400mg on dry matter basis) was inoculated into one liter of media and was grown on a shaker at 180 rpm at 25°C for three days.

2.2 Isolation of Secreted Proteins

2.2.1 Ammonium Sulfate Precipitation

Fungal cultures were centrifuged at 5000rpm and 4°C for 30 minutes to remove fungal cells. The supernatant was vacuum filtered using 0.35µm pore size to remove excess spores and any other unwanted matter that might have accumulated in the sample. Proteins from the sample were precipitated using a 95% saturation of ammonium sulfate (65.9g/100ml) which was added gradually. The sample was spun down at 8000 rpm for 30 min to obtain the precipitate. The pellet was resuspended in a buffer containing 50mM Tris pH 7.5 and
100mM NaCl along with 0.01% protease inhibitor (PI; PMSF and benzamidine cocktail). The protein samples were dialyzed for several hours at 4°C to remove ammonium sulfate (dialysis buffer contained 50mM Tris pH 7.5, 100mM NaCl). The proteins were subsequently concentrated by ultracentrifugation using Millipore membrane with a 10kD cut-off. Sample volume was reduced by about 95% of initial volume. Unused portions of the protein samples were stored at -20°C.

2.2.2 Anion Exchange Chromatography

The pH of fungal culture supernatant was adjusted to 7.5 (from an average of about 4) using HCl. Proteins were isolated using DEAE-Sepharose anion exchange. All steps were carried out at 4°C unless otherwise specified; this was done to reduce protein degradation. Approximately 5mL of the DEAE-Sepharose resin (GE Healthcare) was applied to a 50mL column and equilibrated with 5 column volumes of 50mM TRIS-HCl buffer pH 7.5. The culture supernatant was run through the columns. 10 Volumes of the 50mM Tris-HCl buffer pH 7.5 were used to wash the columns. Proteins were eluted from the column with about 15 mL of 800mM KCl in 50mM Tris-HCl pH 7.5 containing 0.01% protease inhibitor. Samples were collected on ice in 1.5 ml fractions; the fractions with the most protein were pooled and stored at -20°C until further analysis.

2.3 Protein Quantification

Protein quantification was carried out using a method adapted from Bradford (1976). The concentration of isolated protein samples was estimated using a BSA standard curve. The curve was made using absorbance data from different concentrations of Bovine Serum
Albumin (BSA) ranging from 5 to 30µg. Bradford Assay Reagent (Biorad) was used as per manufacturer instructions. Absorbance readings were taken at a 595nm wavelength, using the Cary UV-Vis spectrophotometer (Varian) and the Cary WinUV Simple Reads application. The spectrophotometer was blanked with buffer before the proteins.

2.4 SDS-PAGE - Visualization of Isolated Proteins

Proteins were separated and visualized by one dimensional polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Mini-PROTEAN 3 system. 12.5% Tris-HCl gels were run at 200V for about 45 min, using a slightly modified discontinuous SDS buffer system from Laemmli, 1970. Samples were mixed with loading dye and boiled for a few minutes before loading. The gels were stained with 0.1% Coomassie Brilliant Blue G-250 (Bio-Rad) while shaking gently for at least a few hours and de-stained using 10% acetic acid, 40% ethanol till bands became visible. Gel images were captured using a UV gel box.

2.5 Identification of Isolated Proteins

2.5.1 Tryptic Digestion

The trypsin digest protocol was adapted from Shevchenko et al. (1996). Protein bands from de-stained gels were cut into the 1mm pieces and were incubated with 100mM NH₄CO₃ for 10 minutes. NH₄CO₃ was removed and acetonitrile (ACN) was added to the gel plugs for 10 minutes. ACN was removed and 10mM DTT in 100mM NH₄CO₃ was added and incubated at 56°C for one hour. The solution was removed and 50mM IAA in 100mM NH₄CO₃ was added and incubated at room temperature (RT) for 45 minutes in the dark with occasional vortexing. This solution was removed and the gel plugs were washed with 100mM NH₄CO₃.
for 10 minutes. The solution was removed and of ACN was added to dehydrate the gel plugs and was incubated at RT for 10 minutes. The last two washing and drying steps were repeated once. The gel plugs were swelled in 10μL of a trypsin buffer (4ng/μL in 50mM NH₄CO₃) on ice for 10 minutes. The trypsin buffer was removed and was incubated with 50mM NH₄CO₃ at 37°C for a minimum of 4 hours. The gel plugs were spun down and the supernatant was collected. 20mM NH₄CO₃ was added to the gel plugs, vortexed for 2 minutes and the supernatant was transferred to the same tube. 50% ACN with 5% formic acid was added to the gel plugs, vortexed for 2 minutes and the supernatant was saved in the same tube. The supernatant was spun in the speed-vac until dryness and was stored at -20°C until analysis.

2.5.2 Mass Spectrometry

For mass spectrometric (MS) analysis, the sample was re-suspended in 50% CAN with 0.1% TFA. It was mixed in a 1:1 ratio with a saturated solution of α-cyano-4-hydrocinnamic acid and 1μL was spotted on an Opti-TOF 384 well MALDI plate and air dried. An Applied Biosystems 4800 MALDI TOF/TOF™ Analyzer (Framingham, MA, USA) mass spectrometer was used in this study to acquire MALDI and MS/MS spectra. This TOF/TOF instrument is equipped with a Nd:YAG laser with 355-nm wavelength of <500 ps pulse and 200 Hz repetition rate in both MS and MS/MS modes. The precursor ion selector of the analyzer has a mass resolution of about 400. All measurements were performed in automatic mode. For MS/MS experiments, the potential difference between the source acceleration voltage and the collision cell was set at 1 kV. MALDI-TOF/TOF-MS and MALDI-TOF/TOF-MS/MS spectra were recorded in reflector positive ion mode. MS and MS/MS
data were processed using Data Explorer 4.4 (Applied Biosystems). The MS/MS spectra were subjected to a database search via the Mascot (Matrixscience, UK) database search engine. The search parameters were: tryptic enzyme specificity, 100 ppm mass tolerance for the parent mass and 0.8 Da for the fragment masses. S-carbamidomethyl was selected as the fixed modification and no variable modifications were selected. The NCBInr database was used for the search, using fungi as the taxonomic restriction.

2.6 Treatment of Starch with Isolated Protein

For testing the effect of 300µg of protein, solutions were prepared by using distilled water, at room temperature, to disperse 5g of starch and then heating in the microwave for about 2 min. The starch was visibly gelatinized and turned viscous and translucent. Water was added to make up a 100ml sample volume and was mixed thoroughly. For the later experiments where 4mg protein was used, solutions were prepared similarly using 10g starch and 1L Tris-HCl buffer at pH 7.5.

Dialyzed proteins were added to cooled starch solutions in the desired amounts. The samples were kept overnight (unless otherwise specified) at 25°C while shaking at 150rpm. After treatment, starch was precipitated from the treated samples by adding 1:1 (v/v) 95% ethanol. The precipitate formed was strained and centrifuged at low speed to remove the excess liquid and then frozen, and dried to constant mass under a vacuum.
2.7 Scanning Electron Microscopy (SEM) of Starch Precipitate

Untreated starch precipitate as well as that obtained after fungus or protein treatment was thoroughly dried under a vacuum and ground to a fine powder. For each sample, a few milligrams of powder was applied to sticky tape and mounted onto an SEM stub. Each sample was gold coated for 25 seconds with two stub rotations, and a voltage of 15 kV was used for imaging.

2.8 Solution Casting of Starch Film

The starch precipitates were freeze-dried, ground to a fine powder with a mortar and pestle and used to cast films. 4 grams of ground starch powder was first dispersed in a small volume of distilled water and mixed with 1.8g of glycerol (45% w/w of starch). Distilled water was added to make up to 100ml and the mixture heated at 80-90°C in a water bath. Throughout the heating process, the mixture was stirred with a mechanical stirrer and the heating continued until the volume was reduced by 25% (about 45 min). A polytron machine was used to homogenize the mixture prior to heating, in order to remove clumps that formed once ethanol-precipitated starch comes into contact with water. The solutions were poured into 15cm Petri plates and placed in a 50°C oven to dry till films could be peeled off without feeling sticky (usually 2 days).

2.9 Tensile Testing of Plastic Films

Strips (ASTM D638, type I) were cut from the starch-films using a standard die and mechanical press. The strips were kept at 50°C for at least a few hours or till the test was performed. The gage length was fixed at 25.4mm. Each strip had a width of 3mm and the
minimum thickness of the specimens was measured with a digital caliper gauge. The strips were inserted into the Instron Universal Testing Machine to measure tensile properties of the films. The crosshead was manually returned to initial position after each test.

2.10 Water Holding Capacity Measurement of Starch

The water holding capacity (WHC) of treated starch was determined by the method of Mishra and Rai (2006) with minor modifications. Starch was obtained from fungal culture, treated with 300 µg of protein or buffer as described previously. 1% starch (on dry matter basis) was added to 10ml deionized water and vortexed intermittently for an hour. The mixture was centrifuged at 15,000rpm at 25°C and the free water was decanted and the wet starch was weighed. The WHC was calculated as follows:

\[
\text{WHC (g H}_2\text{O g}^{-1} \text{starch)} = \frac{\text{mass of wet starch} - \text{mass of dry starch}}{\text{mass of dry starch}}
\]

2.11 Fourier Transform- Infrared Spectroscopy

This involved preparing a pellet of the starch sample in a potassium bromide (KBr) matrix using a pelletizing device and mechanical press. The pellet was kept in a dessicator till it was ready to be inserted in the FT-IR machine resulting in a spectrum of absorbance peaks indicating the presence or absence of functional groups.

2.12 X-ray Photoelectron Spectroscopy – Surface analysis of treated starch

XPS was performed at the Surface Interface Ontario facility, using the Thermo Scientific K-Alpha spectroscope and an Al monochromatic source (1486.6 eV). Untreated, fungus-treated
and protein treated starch samples were used for the analysis after being thoroughly dried and ground. A 400 µm area was scanned. The following parameters were used: Survey: Scanned – 150 eV pass energy, LR:Snap-mode – 150 eV pass energy and C1s HR: Scanned – 20 eV pass energy. Peaks were shifted to place main C1s peak at 286.5 eV (ie. C-O bonding). Charge compensation was applied.

2.13 Statistical Analysis

Statistical significance was calculated by a 2-tailed, unpaired t-test using Microsoft Excel. A 95% confidence level was used; differences with p values less than 0.05 were considered statistically significant.

2.14 Expression and Purification of non-specific proteins

Glycerol stocks of His-tagged constructs transformed into E.coli strain BL21 were obtained from different members of the lab. HopF3 and HopZ (effector proteins from Pseudomonas syringae pv phaseolicola and syringae respectively) were provided by Van Quach and Shikimate dehydrogenase (SDH) (shikimate pathway enzyme from Arabidopsis thaliana) was obtained from James Peek. His-SDH was used in all assays involving His-tagged proteins unless otherwise specified.

The glycerol stocks were used to grow overnight starter cultures in 50 ml Luria-Bertani (LB) media supplemented with appropriate antibiotics. The starters were used to inoculate 1L LB media (with suitable antibiotics) that was incubated in a 37°C shaker until optical density at 600nm (OD_{600}) reached between 0.6 and 0.8. To induce protein expression,
isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4mM. The cultures continued to grow at 37°C for 4 hours, after which the temperature was reduced to 16°C overnight. The bacterial cells were pelleted at 3500 rpm for 10 minutes. The cells were resuspended in binding buffer (50mM Tris-HCl pH7.5, 5mM imidazole, 5% glycerol and 500mM NaCl) and then sonicated on ice. A duty cycle of 90, output of 5 and 30 sec on/30 sec off cycle was used for 10 min. 0.01% PI was added to the lysate which was spun down at 14000 rpm at 4°C for 30 min.

Ni-NTA resin (Qiagen) (about 4ml) was applied to a glass chromatography column, equilibrated using 2 column volumes of binding buffer and cell lysate was passed through. The column was washed and eluted with buffer that had the same composition as binding buffer except 30mM imidazole and 100mM imidazole respectively. 1mM EDTA was added to the elution fraction, followed by 0.33mM DTT. The eluted protein was then dialyzed at 4°C against buffer containing 10mM Tris-HCl pH7.5, 500mM NaCl, 5% glycerol and β-mercaptoethanol. TEV was added to the protein sample during dialysis if the His-tag was to be cleaved. Upon cleavage of the His-tag, the protein sample was run through another Ni-NTA column as previously described. The flow-through was collected which contained the cleaved protein.

Pure BSA (Bio-Rad) was dissolved in Tris-HCl buffer pH 7.5 to make a 1mg/ml stock. Appropriate volumes of the stock were used to obtain desired quantity of protein for starch treatment.
3. Results

3.1 Establishing the Effect of *O. ulmi* on Starch

The first step in the study was to establish that *O. ulmi* could improve properties of thermoplastic starch such as tensile strength (TS) and water absorption, under the conditions used in our study. This involved culturing the fungus in presence of starch (10g/L) for 3 days after which the starch was precipitated out to generate plastic films. Standard tensile tests were performed on strips cut from the plastic films, using an Instron machine. The film thickness was relatively uniform; in addition to ensure consistencies in our analysis, strips of similar thickness +/- 0.03 were used. Repeated analysis at ambient conditions revealed that the fungus significantly increase the maximum tensile strength of plastic, with values ranging from 8MPa to 36MPa. This represented an increase of about 400% (figures 2, 6 and 11), relative to the control, which was usually from 1.76MPa to 8.43MPa (tables 3, 5 and 10).

Water absorption of treated starch precipitates was assessed by a commonly used method involving measurement of water holding capacity (WHC). The samples were soaked in water and the increase in mass was determined after the excess water had been removed. WHC was calculated as mass of water per unit mass of starch (g H₂O/g starch). At ambient conditions, the WHC of the untreated starch was found to be between 20.1 to 25.1g H₂O/g of starch whereas after fungus treatment, the WHC was between 0.7 to 1.3g H₂O/g of starch (table 11). The fungal treatment therefore resulted in a striking decrease in WHC of about 96% relative to the control (figures 12 and 13).
3.2 Effect of *O. ulmi* Secreted Molecules on Starch Properties

In order to test the hypothesis that the extracellular proteins of *O. ulmi* are mediating the modifying effects on starch, the first consideration was whether the fungus itself was required to produce the changes in starch or if the secreted matter was sufficient for the observed effects. For this purpose, the fungal culture was separated into a pellet that consisted of fungal debris (including cells and spores) and supernatant, which contained all extracellular molecules secreted by the fungus. The pellet and supernatant were tested separately for their effect on tensile properties of thermoplastic starch.

The untreated and fungal treated samples had a tensile strength (TS) that ranged from 1.76 to 8.43 MPa and 7 to 36 MPa respectively (table 3). The pellet and supernatant treatment resulted in a range of TS from 4.84 to 10.24 MPa and from 24.2 to 33.17 MPa respectively (table 3). As shown in Figure 2, the fungal culture and supernatant treatments both resulted in a comparable increase in maximum tensile strength relative to the untreated control, about 376 and 350% respectively (P > 0.05). By comparison, pellet treatment resulted in less than 50% increase as compared to the control. The pellet-treated starch had significantly inferior tensile strength (P < 0.05) relative to the fungal culture treatment.
Table 3. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control), fungal culture (fungus), fungal supernatant and fungal pellet. Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>TS1</th>
<th>TS2</th>
<th>TS3</th>
<th>mean TS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.96</td>
<td>5.8</td>
<td>6.4</td>
<td>6 ± 0.1794</td>
</tr>
<tr>
<td>Fungus</td>
<td>23.4</td>
<td>35.7</td>
<td>25.5</td>
<td>28.2 ± 3.799</td>
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<tr>
<td>Fungal Supernatant</td>
<td>31.06</td>
<td>24.2</td>
<td>24.3</td>
<td>26.52 ± 2.270</td>
</tr>
<tr>
<td>Fungal Pellet</td>
<td>4.84</td>
<td>9.8</td>
<td>6.76</td>
<td>7.2 ± 1.444</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Trial 2</th>
<th>TS1</th>
<th>TS2</th>
<th>TS3</th>
<th>mean TS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>5.8</td>
<td>7.6</td>
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<tr>
<td>Fungus</td>
<td>31.38</td>
<td>31.1</td>
<td>35.5</td>
<td>32.66 ± 1.422</td>
</tr>
<tr>
<td>Fungal Supernatant</td>
<td>33.17</td>
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<td>31.7</td>
<td>30.96 ± 1.538</td>
</tr>
<tr>
<td>Fungal Pellet</td>
<td>8.942</td>
<td>10.24</td>
<td>7.23</td>
<td>8.80 ± 0.8716</td>
</tr>
</tbody>
</table>
Figure 2. Maximum tensile strength (TS) of plastic films made with starch treated with buffer (control), fungal culture (fungus), fungal supernatant and fungal pellet respectively. Each bar represents the mean TS in MPa (n = 3); results from two independent trials are shown.
3.3 Isolation of Secreted Proteins from *O. ulmi*

A key step in the study was to obtain proteins from the liquid culture and to determine whether there was any difference in protein composition obtained from cultures grown with starch compared to without it. Total protein precipitation was achieved using ammonium sulfate precipitation. Secreted proteins isolated in the presence and absence of starch had a different profile as visualized on SDS-PAGE (figure 3). The presence of starch in the culture seemed to cause a few proteins to be expressed and/or secreted that were not seen in its absence. Bands that were approximately 20kDa (F23), 32kDa (F20) and 80kDa (F13) in size were only seen in the presence of starch. Also, the abundance of certain proteins was seen to increase. The SDS-PAGE suggested that starch could induce protein production and/or secretion in *O. ulmi* cultures.

Proteins eluted from the column were quantified and it was found that the culture without starch yielded about 10 times more protein than the starch-containing culture. The proteins from the starch-containing culture were concentrated to normalize the amount loaded onto the SDS-PAGE (figure 3). It was seen that all the proteins that were obtained by (NH$_4$)$_2$SO$_4$ precipitation were not present in elution samples from anion-exchange columns (figures 3 and 4). The profile of eluted proteins from cultures grown with or without starch was very similar as opposed to what was observed with (NH$_4$)$_2$SO$_4$ precipitation.

3.4 Identification of Secreted Proteins

The protein bands visualized by SDS-PAGE were subjected to MALDI-TOF MS/MS. The analysis did not generate high confidence matches to entries in the MS database; however,
some of the higher scoring matches are included in the table 4. The highest scoring matches were mostly hypothetical proteins; therefore, no insight could be obtained into the function associated with each protein. DeNovo sequencing was also performed following MS/MS, but sequence tags generated were too short to generate any identification hits.
Figure 3. Comparative analysis of proteins secreted by *O. ulmi* in the presence (+) or absence (-) of starch in Tchernoff medium. The bands labeled F3 to 25 were subjected to MS, the “**” indicates the bands for which MS data is included in table 4.
Figure 4. Comparative analysis of proteins secreted by *O. ulmi* in the presence (+) or absence (-) of starch as eluted from anion exchange columns. The protein concentration was equalized before loading onto the gel.
Table 4. Proteins identified with high confidence, C.I score >50%, using MASCOT after MS/MS analysis of specified bands as shown in figure 4.

<table>
<thead>
<tr>
<th>Band</th>
<th>Primary Match</th>
<th>Organism</th>
<th>Accession #</th>
<th>Protein Score*</th>
<th>C.I. %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>conserved hypothetical protein</td>
<td>Cryptococcus neoformans</td>
<td>gi</td>
<td>57223881</td>
<td>63</td>
</tr>
<tr>
<td>F12</td>
<td>hypothetical protein</td>
<td>Aspergillus nidulans</td>
<td>gi</td>
<td>40740903</td>
<td>61</td>
</tr>
<tr>
<td>F24</td>
<td>hypothetical protein</td>
<td>Neurospora crassa</td>
<td>gi</td>
<td>28881200</td>
<td>60</td>
</tr>
<tr>
<td>F11</td>
<td>Translocase Inner Membrane</td>
<td></td>
<td>gi</td>
<td>3242407</td>
<td>59</td>
</tr>
<tr>
<td>F3</td>
<td>hypothetical protein</td>
<td>Gibberella zeae PH-1</td>
<td>gi</td>
<td>42550508</td>
<td>56</td>
</tr>
<tr>
<td>F4</td>
<td>unnamed protein product</td>
<td>Kluyveromyces lactis</td>
<td>gi</td>
<td>50307715</td>
<td>56</td>
</tr>
<tr>
<td>F20</td>
<td>predicted protein</td>
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<td>32414693</td>
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<td>F18</td>
<td>hypothetical protein</td>
<td>Neurospora crassa</td>
<td>gi</td>
<td>32403532</td>
<td>55</td>
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<td>F21</td>
<td>hypothetical protein</td>
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<td>gi</td>
<td>40740705</td>
<td>55</td>
</tr>
</tbody>
</table>

*MASCOT scores; >60 is considered significant. **significance in comparison to other database searches; >95% is considered significant.
3.5 Scanning Electron Microscopy of Treated Starch Granules

Scanning electron microscopy was used to observe the morphology of the starch precipitate collected after various treatments. Due to the time-consuming nature of the tensile tests and the inability to control for certain parameters such as humidity, we also wanted to determine whether this method could be used as an assay for starch modification.

The native or uncooked/untreated starch appeared to be smooth, spherical granules whereas all the cooked starch samples, both treated and untreated, were seen to have a rough, porous surface and an irregular shape. As shown in figure 5, after the various treatments (including control) the granule diameter was roughly 60 to 85 µm as compared to that of the native starch granules which was estimated to be between 10 to 25 µm. However, there were no noticeable differences between the cooked starch samples whether they were treated with fungus, protein or buffer without protein.

Additional techniques like Fourier Transform Infrared (FT-IR) and X-ray Photoelectron Spectroscopy (XPS) spectroscopy were also used, to determine whether these could provide a means to distinguish native starch (control) from starch modified by fungus. FT-IR showed no discernable differences between the chemical structures of fungal treated starch compared to the control (appendix III). Also, XPS did not show any major differences in surface composition of starch after the fungal treatments. The nitrogen content of both samples was negligible (appendix III). This suggested the lack of protein adsorption on the granule surface.
Figure 5. Scanning electron micrographs of different types of starch granules; native (a) or treated with buffer (control) (b) fungus (c) fungal protein (d) His-SDH (non-specific protein) (e) and BSA (non-specific protein) (f).
3.6 Effect of Proteins on Tensile Strength of Thermoplastic Starch

3.6.1 Effect of *O. ulmi* Secreted Fungal Proteins

It was further tested whether the proteins isolated from the supernatant had any effect on the tensile properties of starch plastics (table 5 and figure 6). This was done by treating a precooked starch sample (5g starch/100ml buffer) overnight, with a known amount of protein isolated from starch-containing fungal cultures. Repeated trials carried out with fungal extract containing 300 µg of fungal protein (0.06mg protein/g starch) resulted in TS values ranging from 2.33 to 11.89MPa, this translated to a consistent increase of about 110% relative to the control, which ranged from 1.76 to 4.5MPa. This was repeated with proteins isolated from cultures without starch. The resulting tensile strength measurements ranged from 3.24 to 10.62MPa, an increase of about 130% compared to the untreated control. The increase caused by 0.06 mg protein/g starch treatment was intermediate to the effect of the fungus which produced a TS between 6.54 to 18MPa and a relative increase of about 370%.
Table 5. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control), fungus and fungal proteins isolated from cultures grown with or without starch. Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>TS1</th>
<th>TS2</th>
<th>TS3</th>
<th>mean TS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>1.76</td>
<td>1.9</td>
<td>1.89 ± 0.0696</td>
</tr>
<tr>
<td>Fungus</td>
<td>6.54</td>
<td>10.26</td>
<td>9.8</td>
<td>8.87 ± 1.171</td>
</tr>
<tr>
<td>Fungal Protein Extract (Starch Culture)</td>
<td>5.06</td>
<td>4.5</td>
<td>2.33</td>
<td>3.96 ± 0.8338</td>
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<tr>
<td>Fungal Protein Extract Protein (Non-starch Culture)</td>
<td>4.68</td>
<td>5.1</td>
<td>3.24</td>
<td>4.34 ± 0.5639</td>
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</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Trial 2</th>
<th>TS1</th>
<th>TS2</th>
<th>TS3</th>
<th>mean TS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7</td>
<td>2.6</td>
<td>2.89</td>
<td>3.06 ± 0.3292</td>
</tr>
<tr>
<td>Fungus</td>
<td>13.2</td>
<td>18</td>
<td>13.83</td>
<td>15.01 ± 1.506</td>
</tr>
<tr>
<td>Fungal Protein Extract (Starch Culture)</td>
<td>6.4</td>
<td>8.98</td>
<td>6.22</td>
<td>7.2 ± 0.8922</td>
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<tr>
<td>Fungal Protein Extract Protein (Non-starch Culture)</td>
<td>9</td>
<td>7.8</td>
<td>6.18</td>
<td>7.66 ± 0.8186</td>
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</table>

(c)

<table>
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<th>Trial 3</th>
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<th>TS3</th>
<th>mean TS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.5</td>
<td>4</td>
<td>2.3</td>
<td>3.6 ± 0.6658</td>
</tr>
<tr>
<td>Fungus</td>
<td>17.8</td>
<td>13</td>
<td>14.59</td>
<td>15.13 ± 1.412</td>
</tr>
<tr>
<td>Fungal Protein Extract (Starch Culture)</td>
<td>7</td>
<td>6.7</td>
<td>11.89</td>
<td>8.53 ± 1.682</td>
</tr>
<tr>
<td>Fungal Protein Extract Protein (Non-starch Culture)</td>
<td>8.1</td>
<td>7.8</td>
<td>10.62</td>
<td>8.84 ± 0.8942</td>
</tr>
</tbody>
</table>
Figure 6. Maximum tensile strength (TS) of plastic films made with starch treated with buffer (control), fungal protein isolated from cultures grown with or without starch and the fungal culture. 0.06mg protein/g starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from three independent trials are shown.
3.6.2 Effect of Non-specific Proteins

Since fungal proteins had a favorable effect on properties of thermoplastic starch, we wanted to test the effect of randomly selected proteins to establish whether these changes could be a result of non-specific interaction between starch and protein. For this purpose, BSA and a histidine-tagged protein, His-SDH, previously expressed recombinantly in our lab, were tested. A precooked starch sample (5g starch/100ml buffer) was treated with 300µg of protein (i.e. 0.06 mg protein/g starch). Following overnight treatment, starch was precipitated and subjected to tensile tests. BSA caused a TS of 6.89 to 11.95 MPa whereas the His-tagged protein resulted in TS ranging from 8.4 to 14.16 MPa (table 5). This was a respective increase of about 65 and 100%, relative to the untreated sample (figure 7).

3.6.2.1 Effect of Hexahistidine Tag

It was subsequently tested whether the effect of the His-tagged proteins was due to the imidazole ring contained in the hexahistidine tag. To determine this, starch was treated with the intact His-tagged protein and the same protein with the His-tag cleaved off. The subsequently made films were tested. The TS measurements for treatments with buffer, with His-tag and without His-tag were in the ranges 5.3 to 6.3 MPa, 11.8 to 14.73 MPa and 9.7 to 16.45, respectively (table 6). Therefore, both the proteins caused a comparable relative increase in TS of about 120% and 100% respectively (P > 0.05) (figure 7). It should be noted that the His-tag alone could not be tested separately as there was no means to verify whether the His-tag had been successfully isolated.
Table 6. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control) and non-specific proteins BSA and His-SDH. Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th></th>
<th></th>
<th></th>
<th>mean T ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.2</td>
<td>4.8</td>
<td>4.59</td>
<td>4.53 ± 0.1758</td>
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<tr>
<td>BSA</td>
<td>6.97</td>
<td>7.89</td>
<td>9.33</td>
<td>8.06 ± 0.6868</td>
</tr>
<tr>
<td>His-tagged Protein</td>
<td>8.4</td>
<td>10.23</td>
<td>11.68</td>
<td>10.10 ± 0.9478</td>
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</tbody>
</table>

(b)

<table>
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<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.2</td>
<td>5.46</td>
<td>5.34</td>
<td>5.67 ± 0.2689</td>
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<tr>
<td>BSA</td>
<td>8.76</td>
<td>10.2</td>
<td>9.26</td>
<td>9.41 ± 0.4221</td>
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<tr>
<td>His-tagged Protein</td>
<td>9.54</td>
<td>12</td>
<td>14.16</td>
<td>11.9 ± 1.335</td>
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</tbody>
</table>

(c)

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<td>T2</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.87</td>
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<td>5.44</td>
<td>5.27 ± 0.20</td>
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<tr>
<td>BSA</td>
<td>7.65</td>
<td>6.89</td>
<td>11.55</td>
<td>8.7 ± 1.44</td>
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<tr>
<td>His-tagged Protein</td>
<td>13</td>
<td>9.45</td>
<td>9.17</td>
<td>10.54 ± 1.23</td>
</tr>
</tbody>
</table>
Figure 7. Maximum tensile strength of plastic films made with starch treated with buffer (control) and non-specific proteins, BSA and His-SDH. 0.06mg protein/g starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from three independent trials are shown.
Table 7. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control) and His-HopF3 before and after cleavage of the hexahistidine tag. Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>mean T ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.9</td>
<td>5.23</td>
<td>6.27</td>
<td>5.8 ± 0.3044</td>
</tr>
<tr>
<td>With His-tag</td>
<td>11.8</td>
<td>13</td>
<td>13.6</td>
<td>12.8 ± 0.5292</td>
</tr>
<tr>
<td>Without His-tag</td>
<td>9.7</td>
<td>13.4</td>
<td>11.67</td>
<td>11.59 ± 1.069</td>
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</table>

(b)

<table>
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<th>T3</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.3</td>
<td>5.6</td>
<td>6.1</td>
<td>6 ± 0.2082</td>
</tr>
<tr>
<td>With His-tag</td>
<td>12.2</td>
<td>14.73</td>
<td>13.27</td>
<td>13.4 ± 0.7332</td>
</tr>
<tr>
<td>Without His-tag</td>
<td>11.8</td>
<td>10.45</td>
<td>16.45</td>
<td>12.9 ± 1.817</td>
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</tbody>
</table>
Figure 8. Maximum tensile strength of plastic films made with starch treated with his-tagged protein (His-HopF3) before and after cleavage of the hexahistidine tag. 0.06mg protein/g starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from 2 independent trials are shown.
3.6.2.2 Role of Protein Concentration in Non-Specific Protein-Mediated Effect

It was further tested if the amount of protein used in this assay had any effect on the results obtained. Different quantities of His-SDH protein were used in the assay; 0.3mg, 2mg and 4mg (0.03mg, 0.2mg and 0.4mg protein per g starch) were used to treat 10g starch/L. The TS values lay in the following ranges: 10.8 to 12.34MPa, 10.54 to 11.86MPa and 10.45 to 12.3MPa respectively while the control had a TS between 8 to 8.43MPa (table 8). 0.06mg protein produced an increase of about 38% in tensile strength compared to the control while 0.2mg and 0.4mg produced an increase of about 34% and 39% respectively (figure 9). Therefore the treatments were statistically equivalent (P > 0.05).

3.6.2.3 Role of Protein Size on Non-specific Protein-Mediated Effect

It is conceivable that proteins with a different molecular weight would have a different effect on the mechanical properties of starch. We therefore investigated this dependency as well. For this purpose, three proteins with a difference of about 15-20kDa were selected to treat starch. These were His-SDH (25kDa), His-HopZ (42kDa) and BSA (66kDa). Each of these protein treatments led to TS values of 7.97 to 8.9MPa, 7.98 to 9MPa and 7.9 to 8.6MPa (table 9). While all protein treatments caused an increase in tensile strength of about 40%, 38% and 35% respectively (figure 10), all treatments were statistically equivalent (P > 0.05). This indicated that the treatment of starch with any non-specific protein might produce a small increase in maximum tensile strength.
**Table 8.** Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control) and different amounts (mg/g of starch) of His-SDH as indicated. Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>mean T ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.156</td>
<td>8.32</td>
<td>8.43</td>
<td>8.302 ± 0.0761</td>
</tr>
<tr>
<td>0.03 mg</td>
<td>12.2</td>
<td>11.33</td>
<td>10.84</td>
<td>11.46 ± 0.3977</td>
</tr>
<tr>
<td>0.2 mg</td>
<td>11.48</td>
<td>11</td>
<td>10.89</td>
<td>11.12 ± 0.1811</td>
</tr>
<tr>
<td>0.4 mg</td>
<td>11.8</td>
<td>10.45</td>
<td>12.3</td>
<td>11.52 ± 0.5525</td>
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(b)

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<th>T3</th>
<th>mean T ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.1</td>
<td>8.02</td>
<td>8.35</td>
<td>8.16 ± 0.0994</td>
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<tr>
<td>0.03 mg</td>
<td>10.9</td>
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<td>11.75 ± 0.4338</td>
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<tr>
<td>0.2 mg</td>
<td>10.536</td>
<td>11.86</td>
<td>10.85</td>
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</tr>
<tr>
<td>0.4 mg</td>
<td>12</td>
<td>11.364</td>
<td>11.38</td>
<td>11.58 ± 0.2088</td>
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Figure 9. Maximum tensile strength (TS) of plastic films made with starch treated with different amounts (/10g starch) of His-SDH as indicated on the horizontal axis. Each bar represents a mean TS measurement in MPa (n = 3); results from 2 independent trials are shown.
Table 9. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control) and non-specific proteins of different sizes as indicated. Results from independent trials are shown.

(a)

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<td>Buffer</td>
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<td>25kDa</td>
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<td>8.56</td>
<td>8.64</td>
<td>8.43 ± 0.1691</td>
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<tr>
<td>40kDa</td>
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<td>8.78</td>
<td>9</td>
<td>8.93 ± 0.0739</td>
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<td>60kDa</td>
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<td>8.6</td>
<td>7.99</td>
<td>8.31 ± 0.1754</td>
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(b)

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<td>Buffer</td>
<td>6.2</td>
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<td>5.9</td>
<td>6 ± 0.1000</td>
</tr>
<tr>
<td>25kDa</td>
<td>8.9</td>
<td>7.97</td>
<td>8.33</td>
<td>8.4 ± 0.2707</td>
</tr>
<tr>
<td>40kDa</td>
<td>8.76</td>
<td>8.1</td>
<td>7.98</td>
<td>8.28 ± 0.2425</td>
</tr>
<tr>
<td>60kDa</td>
<td>8.54</td>
<td>7.8</td>
<td>7.96</td>
<td>8.1 ± 0.2248</td>
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</table>
Figure 10. Maximum tensile strength (TS) of plastic films made with starch treated with proteins of different sizes as indicated on the horizontal axis. 0.4mg protein/g starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from 2 independent trials are shown.
3.7 Comparative Effect of Fungal Protein Extract and Non-Specific Proteins

To allow for a better comparison between the effect of proteins and fungus, a consolidated experiment was carried out where 10g/L starch was treated with either buffer, 0.4 mg of protein/g starch or fungus. This was especially desirable since the fungal treatment was carried out over a period of 3 days in a sample volume of 1L with 10g starch/L whereas protein treatment were carried out overnight with 5g starch in 100ml sample volume.

Tensile strength values from 4.3 to 7.4MPa were obtained for the control. BSA and His-protein treatments resulted in TS values of 5.92 to 10.04MPa and 5.81 to 11.17MPa (table 10). Fungal protein and fungus caused TS values that ranged from 19.65 to 33.44MPa and 18.74 to 30MPa. The tensile strength measurements were plotted as percentages that were normalized against the buffer-treated sample (control). Figure 12 is representative of the effects of the fungus and various protein treatments on the tensile properties of starch plastic. The fungal extract treatment resulted in about 350% increase in maximum tensile strength and the starch from the fungal culture showed an approximately 310% increase compared to control. Both BSA and the His-tagged protein resulted in a respective modest increase of about 35% and 45% relative to the buffer-treated control.
Table 10. Tensile strength (TS) measurements in MPa, for plastic films made with starch after treatment with buffer (control), fungal culture (fungus) and fungal proteins or non-specific proteins, BSA and His-SDH (0.4 mg protein/g starch). Results from independent trials are shown.

(a)

<table>
<thead>
<tr>
<th>Trial 1</th>
<th></th>
<th></th>
<th></th>
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<td>T3</td>
<td></td>
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<tr>
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<td>6.17 ± 0.0219</td>
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<td>8.1</td>
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<tr>
<td>His-tagged protein</td>
<td>8.198</td>
<td>9.3</td>
<td>8.97</td>
<td>8.82 ± 0.3366</td>
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<tr>
<td>Fungal protein</td>
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<td>27.83</td>
<td>26.78 ± 0.8213</td>
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<tr>
<td>Fungus</td>
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<td>23.722</td>
<td>26.03</td>
<td>24.99 ± 0.6764</td>
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(b)

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<td>9.77</td>
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<tr>
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<td>32.2</td>
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<td>Fungus</td>
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(c)

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<td>T1</td>
<td>T2</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.3</td>
<td>4.58</td>
<td>4.92</td>
<td>4.6 ± 0.1793</td>
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<tr>
<td>BSA</td>
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<td>7.3</td>
<td>5.92</td>
<td>6.44 ± 0.4331</td>
</tr>
<tr>
<td>His-tagged protein</td>
<td>7.21</td>
<td>5.81</td>
<td>6.99</td>
<td>6.67 ± 0.4347</td>
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<td>19.65</td>
<td>21.07 ± 1.1979</td>
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<td>Fungus</td>
<td>21.2</td>
<td>18.74</td>
<td>22.16</td>
<td>20.7 ± 1.0184</td>
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Figure 11. Maximum tensile strength (TS) of plastic films made with starch treated as indicated on the horizontal axis. 0.4mg protein/g of starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from three independent trials are shown.
3.8 Temporal Effect of Fungal Protein Extract

In order to see if the effect of fungal proteins was enzymatic, we wanted to determine if the effect of the fungal proteins on tensile properties of thermoplastic starch was time-dependent. For this purpose, an assay was performed by treating a large volume of cooked starch with 4mg of protein/10g of starch. Starch was extracted at different time points. The resulting starch precipitate was used to make films for tensile tests.

After 5 hours of treatment, average TS measurement was 17.84MPa. After 1, 2, 3 and 4 days of treatment, mean TS was found to be 10.71, 5.875, 17.94 and 14.1MPa respectively while the buffer-treated control had mean TS of 3MPa. The TS measurements for all treatments were variable nonetheless all durations caused a significant increase (P < 0.05) in TS compared to the control. The relative increase in TS was 494, 350, 95, 498 and 370% respectively (appendix II). It should be noted that only preliminary data for the time-course was collected and reported here and further verification is necessary by independent replication of the assay. However, 5 hours of treatment had the same outcome as 3 days of treatment, which suggests that the effect of the fungal protein extract on mechanical properties of starch plastic, might be independent of the time duration. It may also suggest that the change in mechanical properties of starch plastic is not enzymatic. Nonetheless, this experiment needs to be repeated in order to obtain more conclusive results.

3.9 Effect of Proteins on Water Holding Capacity of Starch

We wanted to determine if protein treatment could change water barrier properties of starch to decrease water absorption. The water holding capacity was measured as the mass of water
absorbed as a function of the dry mass of starch. Taking the WHC of the untreated sample as the maximum water that can be absorbed by starch (i.e. 100% WHC), relative percentage WHC was calculated. Three independent trials were carried out with 300 µg of fungal protein as well as non-specific proteins. The fungal protein treatment resulted in a WHC of about 58% with absolute values ranging from 11.6 to 14.4 g H₂O/g of starch. However this was similar to the outcome of the non-specific protein treatments. BSA-treated starch had a WHC between 14.3 and 15.6 g H₂O/g starch (table 11), which translated to almost 69% relative WHC. His-tagged protein (His-SDH) treatment resulted in a 67% WHC with values ranging from 12.9 to 16.5 g H₂O/g starch (figure 12).

The assay was repeated with starch samples (10g/L) that had been treated with 4 mg of each protein (0.4 mg protein/ g starch). Treatment with fungal extract, BSA and His-tagged protein resulted in WHC values that ranged from 3.1 to 4.6, 8.1 to 9.2 and 10.5 to 12.3 g H₂O/g starch, respectively (table 10). A 17% WHC was seen after treatment with fungal extract. Both BSA and His-tagged protein caused much higher water absorption, with a WHC of approximately 40-50% relative to the control (figure 13).
Table 11. Water holding capacity (WHC) of starch, precipitated from fungal culture (fungus), treated with buffer (control) and specified amounts of fungal protein, BSA and His-SDH.

<table>
<thead>
<tr>
<th></th>
<th>WHC1</th>
<th>WHC2</th>
<th>WHC3</th>
<th>mean WHC ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.1</td>
<td>25.1</td>
<td>22.1</td>
<td>22.43 ± 1.453</td>
</tr>
<tr>
<td>Fungus</td>
<td>0.7</td>
<td>1.1</td>
<td>1.3</td>
<td>1.03 ± 0.1764</td>
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<tr>
<td>Fungal Protein (0.06mg/g starch)</td>
<td>11.6</td>
<td>14.4</td>
<td>13.4</td>
<td>13.33 ± 0.8192</td>
</tr>
<tr>
<td>Fungal Protein (0.4mg/g starch)</td>
<td>3.1</td>
<td>4.6</td>
<td>3.4</td>
<td>3.7 ± 0.4583</td>
</tr>
<tr>
<td>BSA (0.06mg/g starch)</td>
<td>14.3</td>
<td>16.4</td>
<td>15.6</td>
<td>15.43 ± 0.6119</td>
</tr>
<tr>
<td>BSA (0.4mg/g starch)</td>
<td>8.1</td>
<td>9.2</td>
<td>8.9</td>
<td>8.73 ± 0.3283</td>
</tr>
<tr>
<td>His-tagged Protein (0.06mg/g starch)</td>
<td>12.9</td>
<td>16.5</td>
<td>15.6</td>
<td>15 ± 1.082</td>
</tr>
<tr>
<td>His-tagged Protein (0.4mg/g starch)</td>
<td>10.5</td>
<td>11.5</td>
<td>12.3</td>
<td>11.43 ± 0.5207</td>
</tr>
</tbody>
</table>

WHC 1, 2 and 3 represent measurements from three independent trials.
Figure 12. Water holding capacity (WHC) of starch treated with buffer (control), fungal culture, fungal protein extract and non-specific proteins. 0.06mg protein/g starch was used. Each bar represents a mean of three independent WHC measurements in g of H₂O/g starch +/- SE (n = 3).
Figure 13. Water holding capacity (WHC) of starch treated with buffer (control), fungal culture, fungal protein extract and non-specific proteins. 0.4mg protein/g starch was used. Each bar represents a mean of three independent WHC measurements in g of H₂O/g starch +/- SE (n = 3).
4. Discussion

Our hypothesis was that starch modification by *O. ulmi*, is mediated by secreted proteins. Therefore the goal of this study was to investigate the effect of secreted proteins on starch as well as to identify them. We observed and compared the tensile properties and water holding capacity of untreated starch, fungus treated starch and starch treated with fungal extract with proteins. The protein-treated sample consistently produced an improvement in plastic properties. Even though positive identification of proteins could not be achieved, our findings supported our hypothesis and led us to conclude that the *O. ulmi* extracellular proteins can modify starch and improve plastic properties. However, our findings have also potentiated investigation into the role of extracellular polysaccharide (EPS) in modification of starch properties.

4.1 Establishing Effect of *O. ulmi* and Secreted Molecules on Starch

This study established that under our experimental conditions, *O. ulmi* is able to alter starch properties such that plastic films with a significantly higher tensile strength and water barrier properties can be obtained, relative to native starch. Once the effect of the fungus was determined, it was investigated whether the starch modification effect, and thereby improvement in tensile properties, could be achieved by exposing starch to the culture supernatant (after the fungus was removed). It was seen that the culture supernatant, and therefore, the secreted molecules, had the same effect on starch tensile properties as the whole culture whereas the fungal pellet did not have much of an effect (figure 5). This confirmed that the factors that mediate the modification are extracellular. Fungal exudates have not been previously shown to improve tensile properties of thermoplastic starch as
demonstrated in this study. However, as described previously, fungi secrete polysaccharides as well as lipids in culture, in addition to proteins (Wu, 2007). Therefore, it remained to be tested whether the modifying factors were fungal proteins.

4.2 Protein Purification

To confirm the involvement of secreted proteins in the modification process, the proteins had to first be purified from the fungal culture. Using neutral salts in a process known as “salting out” is a well established method for protein purification. The most commonly used salt for this is ammonium sulfate as it is cheap and sufficiently soluble. Different degrees of salt saturation may be required to purify different proteins, depending on their surface hydrophobicity. The propensity for aggregation and hence precipitation is directly proportional to the degree of surface hydrophobicity (Roe, 2001). Since complete protein precipitation was desired, 95% saturation was used. When the precipitated proteins were dialyzed and run on an SDS-PAGE, the bands appeared diffuse. This may be ascribed to protein size heterogeneity due to glycosylation. The majority of the secreted proteins from filamentous fungi are known to be highly glycosylated (Oda et al., 2006).

It was suspected that starch modification may be specific to factors induced by the starch in cultures. Proteins were therefore collected and compared from cultures grown in the presence and absence of starch. It was interesting to see that the secretion profile was slightly different in both cases, as seen by bands F13, 20, 21, 22 and 23 in figure 4. Attempts were made to identify all the proteins visualized on the SDS-PAGE by mass spectrometry. Differentially expressed proteins (figure 4) could be of particular interest since the presence
of starch might induce specific proteins that modify starch. However, the presence of starch in the fungal cultures is known to induce production of starch hydrolyzing enzymes (Ventura et al., 1995). Thus, some of the additional or more intense bands in figure 4 might be starch hydrolyzing enzymes that could confound results.

A concern was that using ammonium sulfate precipitation was extremely time-consuming due to the large sample volume and the high saturation of ammonium sulfate (95%) used could cause functional denaturation of the proteins. This is especially undesirable if the starch modification occurs through enzymatic action of these proteins. There was also a frequent problem of starch precipitating out of solution with the protein, making the samples viscous and therefore difficult to run on the SDS-PAGE. Ion exchange chromatography (IEC) is another straightforward, non-denaturing method for protein purification. Most proteins are positively charged at physiological pH; therefore, anion exchange is more useful for isolating a wider range of proteins (Simpson, 2003). Thus IEC, using DEAE-Sepharose (positive resin), became the method of choice for protein isolation. This reduced the risk of functional denaturation as well as starch precipitation.

Some bands visible in the SDS-PAGE after (NH₄)₂SO₄ precipitation (figure 3) were missing from ion exchange elution fractions (figure 4). This was not surprising as 95% saturation of ammonium sulfate would most likely cause total protein precipitation whereas the ion-exchange conditions used would be more suitable for some proteins over others. The profile of eluted proteins from cultures grown with or without starch was very similar as opposed to what was observed with ammonium sulfate precipitation. A possible explanation could be
that certain proteins formed associations with starch which were then unable to pass through the column. The elution conditions used may not have been suitable for breaking those interactions, thus preventing some proteins from passing through the column. Nevertheless, this may not be a major problem since the eluted proteins were sufficient to cause a significant increase in maximum tensile strength of thermoplastic starch. In fact, this may suggest that the missing proteins are not essential for the desired effect on starch and they could potentially be ruled out.

4.3. Protein Identification

Upon repeated mass spectrometric analysis by MALDI-TOF MS/MS, only a few of the proteins were matched to existing proteins in the database with some confidence. Furthermore, these matches were only made with hypothetical protein with unknown functions, from various organisms. The identification process was therefore largely unsuccessful. The major obstacle was the absence of \textit{O. ulmi} proteomic information in the present databases. Though several Expressed Sequence Tags (ESTs) for Ophiostomatoid fungi exist in the NCBI database including 561 from \textit{O.novo-ulmi} and 9589 from \textit{O.piliferum} (redundant sequences included), none of the proteins analyzed by MS were matched to them. All hits obtained were from non-Ophiostomatoid organisms. This sort of cross-species protein identification can be accomplished by partially aligning the analyzed protein from an unsequenced organism to a homologous sequence of a related organism present in the database. The previously described study by Medina et al., (2005) successfully used this approach to identify \textit{A. flavus} proteins because complete genomes sequences from other \textit{Aspergilli} were available. However, there are no Ophiostomatoid fungi with complete
genomic sequences and as organisms become phylogenetically distant, their homologous gene sequences and corresponding proteins are unlikely to retain a high percent identity (Shevchenko, 1996). Thus it is imperative to such studies that sequence information is available for the specific organism of interest.

4.4 Non-specific Effect of Proteins on Starch

During the course of this research, we tested some random proteins to investigate whether the effects of the fungal proteins is specific in nature. Upon testing BSA, it became apparent that non-specific interactions might play some role in causing the effects of fungal protein. This opened up the exciting possibility of developing an alternate method to improve starch plastics without the complexity of O. ulmi protein identification.

The phenomenon of non-specific interaction was further tested by using histidine-tagged proteins. The rationale was that a hexahistidine tag containing the charged imidazole ring might further enhance physical interaction between starch and protein. Preliminary findings showed that these proteins had a similar effect to fungal protein which was higher than the BSA-mediated effect. It was further investigated whether the effects of the histidine-tagged proteins were in fact dependent upon the hexahistidine residues. However, the relative increase in tensile strength using the his-tagged protein was about the same, with or without the his-tag (figure 8). Thus, the His-tag or imidazole ring was not responsible for the improved tensile properties.
This led the investigation towards testing if the effects were dependent on the size of protein used in the assay. This was warranted by the fact that in initial testing, two individually tested His-tagged proteins of similar size (25-28kD) had a similar effect on starch whereas BSA (66kD) had a lesser effect (figure 11). It was thought that perhaps a smaller sized protein could form a more effective association with starch molecules and may act as a crosslink between two starch molecules. The smaller size of the protein may allow the starch molecules to remain in closer proximity, thus enabling more chances for bonding between neighboring molecules. Furthermore, different amounts of the His-tagged protein had a similar effect on tensile properties (figure 10). Therefore, it seems that non-specific protein-mediated improvement observed in starch films may not be easily enhanced in an amount or size-dependent fashion.

4.5 Specific Effect of Secreted Fungal Proteins on Starch

Although using an increasing amount of non-specific protein did not have an incremental effect on the tensile strength of the plastic (figure 9), the maximum improvement in tensile strength of about 450% was observed when the highest amount of fungal protein (4 mg/g of starch) was used (figure 11). However, these 4 mg protein treatments were carried out over a period of 3 days instead of overnight as is the case for the previous experiments with lesser protein. Therefore an experiment was done to test the temporal effect of 0.4mg of fungal protein/g of starch and investigate whether treatment duration was a factor in the observed increase in tensile strength (figure 16). Due to time constraints only preliminary data could be collected. The data showed that the tensile strength after 5 hours was the same as that after
3 days of treatment. This suggested that the modification effect was not time-dependent and therefore may not be enzymatic. However, this experiment needs to be repeated in order to confirm the findings. Therefore, a non-enzymatic, physical interaction between starch and protein may occur to improve starch tensile properties. This interaction may be amount-dependent and this needs to be investigated.

The 4 mg treatment involved the maximum volume of fungal extract used in this study and it should be noted that the volume used in the experiments varied with the amount of protein that was desired for each treatment. Therefore, any non-protein component and its concentration were unaccounted for in the treatments. Interestingly, the fungus is known to produce an extracellular polysaccharide (Jeng et al., 2006) and lack of enzymatic modification could point to a potential role of the EPS in enhancing starch properties.

Fractions eluted off the anion exchange columns always had a yellow or brownish coloration. A darker color of the eluent coincided with more protein, as detected by Bradford reagent. Interestingly, Binz and Canevascini (1997) describe a yellow sample being obtained upon laccase purification from *O.novo-ulmi*, using ion exchange chromatography. They associate the yellow coloration with heavy contamination with EPS. The presence of EPS in the eluted protein sample and its presence during fungal protein treatment could explain the variability between treatment outcomes. It would also explain the huge increase in maximum tensile strength upon addition of a larger volume of fungal eluted sample (hence a larger amount of EPS) as compared to treatment with an equivalent amount of non-fungal protein. Furthermore, Jeng et al. (2007) observed that *O. ulmi* strain W9, which is the strain used in
our study, produces the highest amount of EPS compared to *O. novo-ulmi* as well as other strains of *O. ulmi*. It is therefore reasonable to postulate that EPS could be a key factor in starch modification and its effects on tensile properties.

The individual effect of the EPS and the protein can be determined by separating the two from the eluted sample after ion-exchange chromatography. The molecular weight of EPS is estimated to be around 2-8MDa (Jeng *et al.*, 2006), whereas most of the visualized proteins are between 10kDa to 200kDa (around 10 orders of magnitude smaller than the EPS), therefore their separation can be done by size exclusion. Gel filtration could be used; however, since larger volumes might be involved, a concentrating process using a filter membrane with a MWCO of around 300kD may be used. This would allow the two fractions to be individually rich in either protein or EPS. The effect of these fractions can then be tested on starch to determine which component can be used more effectively to produce the desired properties in starch plastics.

### 4.6 Surface Properties of Starch

Native starch granules had a smooth surface while the buffer-treated as well as protein-treated starch samples were swelled up, rough and porous. The porosity and increase in granule size is therefore an effect of the gelatinization process and not the protein treatment. Since the fungus or protein treatment did not result in any noticeable morphological differences compared to the control, (figure 8), SEM could not be used as a means to evaluate the modification effect.
Proteins were detected on SDS-PAGE after the starch precipitate from fungal culture was washed with 10% SDS (appendix I). However, based on XPS results, it seems that neither fungal proteins nor the other proteins tested are adsorbed onto the starch. One reason could be that 0.06mg of protein/g of starch is an insufficient amount to be detected by XPS even though the same amount is able to bring about a change in the physical properties of the material. It may also be that all the starch granules are not able to form interactions with the protein and since only a miniscule amount of the sample is scanned, the proteins remain undetected. Another reason for not being able to detect protein on the treated starch may be that the XPS only provides surface analysis with a 10nm depth whereas from the SEM micrographs it is evident that the starch granules are quite rough and porous. It is possible that the proteins used in the treatment get lodged inside the crevices of the granules and cannot be detected. This is supported by the fact that proteins were detected using Bradford reagent on fungus or protein-treated starch granules that were not precooked (results not shown).

Hydrophobin assembly could occur on the surface of starch as the fungal culture grow as well as upon treatment with fungal protein. This would explain the decrease in water absorption (figures 12 and 13). Future investigation will be needed to confirm the hydrophobin-mediated effect on starch. This could include isolating hydrophobins from Ophiostoma cultures, based on their small size (about 10kD). Also, known hydrophobins from other filamentous fungi, for example Trichoderma or Aspergillus, can be expressed and tested for their effect on the water absorption capacity of starch. Furthermore, the fungal
extract should be investigated for the presence of lipids, which may cause the increase in hydrophobicity.

One method of determining hydrophobicity is measurement of the contact angle. This is the angle that is formed at the interface between a liquid and a solid surface. A water droplet can be placed onto the film and the contact angle can be measured using a goniometer. The contact angle depends on the attractive forces between the solid and the droplet, if the film is hydrophilic the water droplet will spread out and the contact angle would be close to zero whereas, on a hydrophobic surface, the droplet will bead up and the contact angle will be higher. In this way the hydrophobicity of the films can be determined quantitatively.

**4.7 Water Absorption**

The fungus was seen to reduce water absorption by almost 96%. A small amount of protein (0.06 mg/g starch) led to a more modest but consistent decrease in water holding capacity of about 40% and non-specific proteins led to a decrease of around 20-30%. Increasing the amount of protein used for the treatment caused a further decrease in water absorption, even for non-specific proteins; this suggested that part of the effect of fungal proteins may also be non-specific. The fact that 83% reduction in water holding capacity occurred 0.4mg protein/g of starch suggested that this reduction in hydrophilicity was dependent on amount of protein used to treat starch. Presumably, as more protein molecules are available to interact with starch, less hydroxyl groups might be available for interaction with water.
4.8 Limitations

The major challenge faced in this study was the lack of background information on the subject and the lack of established protocols. The lack of sequence information has already been discussed; moreover, very few studies have been published on the subject of proteins secreted by *Ophiostoma*; these include Binz and Canevascini (1997) and Wu et al (2007). However, these studies involved isolation and characterization of specific proteins like laccasses and serine proteases respectively and did not take a holistic proteomic approach. Also, we have no current knowledge on the manner in which *O. ulmi* modifies starch, and therefore, no reasonable predictions can be made regarding the nature of proteins that may be involved.

It is curious that despite its ability to produce hydrolyzing enzymes, *Ophiostoma ulmi* is able to improve certain properties of starch instead of breaking it down completely. One possibility is that the growth medium contained glucose, which would presumably be more readily absorbed than starch, thereby reducing the need for starch degradation. Another reason may be that *O. ulmi* is like certain other filamentous fungi that retain some major hydrolyzing enzymes like α-amylases and β-glucosidases in their cell wall and do not secrete them into liquid culture (Oda et al., 2006).

4.8.1 Protein Yield

Small amounts of fungal proteins were used for the treatments; this was mainly due to low protein recovery from purification procedures. This was not a major concern for MS analysis as only minute quantities of protein were required. However for the starch treatment, larger
quantities of protein were needed. Initial protein concentration was also low and the proteins in the cultures could not be detected with Bradford reagent or spectrometry ($\lambda = 280\text{nm}$). Concentrating the culture supernatant repeatedly enabled a rough estimation of the total protein content in the 1 liter culture to be about 20-30mg on average. The purification methods used resulted in further reduced amounts of protein. The ammonium sulfate method involves several different steps and each subsequent step can lead to protein loss, while the anion exchange method is not specific and therefore is likely to have a diminished yield. In fact, the highest amount of total protein obtained from a 1L culture off the column was about 8 mg. For future studies and large-scale application, initial protein yield or protein secretion capacity of the fungus should be improved (discussed later in 4.10.2).

### 4.8.2 Sources of Variance in Tensile Test Data

Several problems were faced when trying to optimize the solution casting and testing procedure for the plastic films. These included air bubbles entrapped within the films, shrinkage and cracking as well as brittleness while cutting specimens for testing. Many of these were resolved over the course of the study for example using shear to reduce viscosity allowed air bubbles to be removed from the casting mixture completely while adjusting drying time and temperature (reducing temperature to 50C and drying for longer periods) reduced issues with cracking and brittleness. This reduced in-sample variance nonetheless some issues still remained.

Atmospheric conditions like relative humidity (RH) could not be regulated at the instron facility or during the drying process. In order to effectively minimize variance both
within replicates of one sample and between different samples, the RH must be controlled. This is especially true for thin plastic films because even small fluctuations in RH could upset the moisture equilibrium within the film. While the data collected in this study are a good means for preliminary evaluation, any further research done in this area, particularly for industrial application, should take this into consideration. This may be achieved by storing films in a controlled humidity chamber prior to testing and ideally performing the tests at a facility that also has controlled humidity. Furthermore, the RH conditions used should be in accordance with specific application intended for the material.

A phenomenon known as anisotropy may also lead to variance in tensile strength measurements. Anisotropy refers to the lack of homogenous directionality or orientation of starch molecules within the film. If the films are anisotropic, or if test specimens are cut in different directions, the mechanical properties and measurements may not be accurate (Wang, J., Lu, Y., Yuan, H and Dou, P., 2008). It is difficult to control anisotropy in manual casting procedures therefore, mechanical methods like extrusion casting or compression molding would allow for more homogenous, isotropic films. Such methods can also eliminate residual starch granules that are sometimes left insoluble and can affect tensile measurements (van Soest and Vliegenthart, 1997). Furthermore, high glycerol content was used in this study (45%) to prevent brittleness and facilitate film handling and mechanical casting methods may also reduce the amount of glycerol needed. Low plasticizer content leads to higher tensile strength (Thunwall, Boldizar and Rigdahl, 2006)
Phase separation, which entails formation of separate amylose and amylopectin-rich regions within a film, can also result in inconsistencies in strength and barrier properties (Paes, Yakimets and Mitchell, 2008). Linear amylose chains can intertwine more effectively by hydrogen-bonding and crystallization to form a cohesive matrix with high strength and stiffness whereas, amylopectin cannot form such strong bonds due to its branched nature and leads to more flexible structures (van Soest and Vliegenthart, 1997). Therefore, use of either pure amylose or amylopectin would result in more consistent properties. Starches with a high amylopectin such as that from tapioca result in lower strength properties while amylose rich starches are reported to have higher tensile strength (Paes, Yakimets and Mitchell, 2008). The use of the polytron to homogenize starch mixtures, may also explain the large standard deviation in data since high shear rates have been shown to cause data scattering and reduced reproducibility (Paes, Yakimets and Mitchell, 2008).

4.9 Suggestions for Improved Protein Identification

This study has implicated O. ulmi proteins in starch modification and has further necessitated their identification. MALDI-TOF MS is reported as being most effective when the genome sequence of the organism is known (Medina, et al, 2005). The proteins in the complex fungal extracts might be more successfully identified using ion-trap MS/MS. This is a useful method for microorganisms that do not have a completely sequenced genome (Oda et al., 2006). It has also been demonstrated that LC-MS/MS is efficient in high-throughput protein identification in samples where limited genomic sequence data are present and identifications are based on databases containing homologous protein sequences (Medina et al., 2005).
Alternatively, fractionation of isolated protein samples would allow for less complex protein mixtures to be used for starch treatment. A comparative analysis of the effects of different fractions could simplify the identification process if only one or few fractions can bring about the desired effects. Reducing the complexity of sample could increase the success of positive identification of proteins. Also, the individual proteins in such fractions can be subjected to N-terminal sequencing using degenerate primers. This was the approach used by Wu et al. (2006) to clone an α-amylase gene from *O. floccosum*. The sequences obtained could be used to individually express the proteins which could then be tested for their effect on starch. This could help bypass the major issue of *O. ulmi* being unsequenced. However, this may not be very easy given that preliminary attempts to fractionate proteins by using different ionic strengths for elution failed. Even when different saturation levels of (NH₄)₂SO₄ were used, there was significant overlap between the proteins found in each fraction (appendix I). Gel filtration could be considered as it would enable separation of proteins based on their size and will allow for fractions with more or less individual proteins to be collected. The samples would have to be concentrated beforehand given that only small volumes can be processed by this method.

4.10 Future Directions and Considerations for Large-Scale Application

Once the gene sequence is determined, proteins of interest can be expressed in appropriate hosts, in order to investigate function and activity through kinetic and structural studies. Furthermore, the biochemical properties of the amino acid residues that make up these proteins will give insight into the type of interaction and/or bonding that may occur between
them and starch. Ultimately, the proteins may be engineered to produce more robust forms, or new proteins can be designed such that they are more suitable for industrial processes.

### 4.10.1 Overexpression of Proteins

Protein identification strategies have been discussed earlier but another foreseeable challenge in accomplishing the above is finding a suitable host to express the *O. ulmi* proteins. *E.coli* is the most commonly used host for recombinant overexpression of proteins; however, fungal proteins are reported to have best yields when recombinantly expressed in fungal hosts. For example, secretion of *Rhizomucor miehei* aspartic proteinase was up to 3g/l in *A. oryzae* in a controlled fermentation (Christensen *et al.*, 1988). Given that fungal proteins will have several unique post-translational modifications (PTMs), *E.coli* may not be a viable option. In this event, yeast could be considered, as it is a well-established industrial microorganism and can be used conveniently and cost-effectively for industrial production of enzymes (Ahmed *et al.*, 2009). In light of recent literature, filamentous fungi themselves or even *Ophiostoma* can be explored (Wu *et al.*, 2006). *T. reesei*, *A. niger* and *A. oryzae* are already being used as fungal expression systems while *Ophiostoma* is in its earlier stages of being developed as a recombinant host (Nevalainen *et al.*, 2005 and Wu *et al.*, 2006). Ahmed *et al.* (2009) reviewed various studies on recombinant expression of xylanases in different expression systems; *E.coli* did not give optimal expression of the enzyme while yeast and filamentous fungi were most effective. As mentioned previously, dominant enzymes secreted by *Ophiostoma* spp include proteases, lipases and amylases (Brush *et al.*, 1999; Gao and Breuil, 1998; Wu *et al.*, 2006). Genes encoding these efficiently secreted proteins provide a potential source for strong promoters for high level gene expression which is an essential requirement.
for an efficient expression system. Furthermore, *Ophiostoma* can be cultivated on cheap medium, thus reducing the cost for large-scale production of commercially important proteins (Wu *et al.*, 2006).

### 4.10.2 Improving Protein Secretion

While efforts for protein identification are ongoing, strategies to improve protein secretion would be helpful especially for large-scale use. It has been found that filamentous fungi primarily secrete proteins through their hyphae at actively growing tips (Moukha *et al.*, 1993). Lee *et al.* (1998) reported an increase in the amount of extracellular proteins in response to a mutation in *N. crassa* that caused increased growth surface area. Furthermore, filamentous fungi have been found to secrete quantities of enzymes in solid-state that far exceed submerged cultures, for example *A. oryzae* (Oda *et al.*, 2006). This is probably due to the fact that dimorphic fungi grow exclusively with mycelia on solid media and are therefore able to secrete more protein through their hyphae (Wu, 2007). Therefore, if mycelial growth of the fungus is encouraged, protein secretion could be improved. One method to improve protein secretion could be to grow the fungus on solid media instead of liquid culture. Another way is to alter the contents of the growth medium; for example use of nitrogen sources like ammonium, asparagine and arginine promotes mycelial growth whereas, proline is known to lead to yeast-like growth (Wu, 2007). The presence of calcium has also been shown to be required for hyphal growth (Gadd and Brunton, 1992).

### 4.11 Conclusion

The results from this study show that extracellular proteins from *O. ulmi* can be used to improve tensile strength and water barrier properties. The fungal protein extract appeared to
act in a specific, time-independent manner in order to mediate these effects and did not require starch for induction. These findings are novel in that (to the best of our knowledge) fungal exudates have not been used for improvement of tensile strength of thermoplastic starch. This study is crucial to our understanding of how *O. ulmi* can be used in a cost-effective and efficient method for the production of starch plastics with enhanced properties. The secreted proteins and their effect on starch should be further investigated and other molecules secreted by *O ulmi* should also be studied.
Appendix I

Figure 14. Coomassie Blue stained SDS-PAGE of proteins removed from starch, using 10% SDS, after fungal treatment.

Figure 15. Silver-stained SDS-PAGE of proteins from fungal cultures grown with starch, fractionated using 30%, 60% and 95% saturation of ammonium sulfate saturation.
Appendix II

Figure 16. Tensile strength measurement of plastic films made with starch treated with fungal protein extract for various time durations as indicated on the horizontal axis. 0.4mg protein/g of starch was used. Each bar represents a mean TS measurement in MPa (n = 3); results from a single trial are shown.

The measurements used to generate the above graph are as follows:

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<th>TS2</th>
<th>TS3</th>
<th>mean TS ± SE</th>
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<tr>
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<td>13.16</td>
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Appendix III

**FT-IR**

![FT-IR spectra](image)

**Figure 17.** FT-IR spectra of native starch and fungus-treated starch showing only one additional peak in the native starch spectrum indicating CO₂.

**XPS**

XPS: Thermo Scientific K-Alpha  
Al monochromatic source (1486.6 eV)  
Area: 400 µm  
Survey: Scanned – 150 eV pass energy  
LR: Snap-mode – 150 eV pass energy  
C1s HR: Scanned – 20 eV pass energy

Peaks shifted to place main C1s peak at 286.5 eV (ie. C-O bonding “C2”)

Charge Compensation applied

The spectra shown are in the following order:  
1. Starch treated with buffer (control)  
2. Starch treated with fungus  
3. Starch treated with protein
Overall Elemental Spectrum of Control Treated with Buffer

Survey
1 Scan, 1 m 3.1 s, 400µm, CAE 150.0, 1.00 eV

Counts / s
Binding Energy (eV)

O1s
C1s
N1s
Individual Elements for Control Starch Treated with Buffer

**Elemental ID and Quantification**

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Overall Elemental Composition of Starch Treated with Fungus

Survey
1 Scan, 1 m 3.1 s, 400µm, CAE 150.0, 1.00 eV

Counts / s

Binding Energy (eV)

C1s
N1s
O1s
C1s
N1s
O1s
Individual Elemental Composition of Starch Treated with Fungus

C1s Snap

N1s Snap

O1s Snap

Elemental ID and Quantification

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Overall Elemental Composition of Starch Treated with Fungal Protein Extract

Survey
1 Scan, 1 m 3.1 s, 400µm, CAE 150.0, 1.00 eV

Counts / s

Binding Energy (eV)

C1s
N1s
O1s
Individual Elemental Composition of Starch Treated with Fungal Proteins

Elemental ID and Quantification

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References


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