[UCI]I804:24011-200000263301

August 2012

Ph.D. Dissertation

Biological and Functional Properties of Proteins Isolated from a Brown-Rot Fungi, *Sparassis crispa*

Graduate School of Chosun University

Department of Chemical Engineering

Gayathri Chandrasekaran

Biological and Functional Properties of Proteins Isolated from a Brown-Rot Fungi, *Sparassis crispa*

24th August 2012

Graduate School of Chosun University

Department of Chemical Engineering

Gayathri Chandrasekaran

Biological and Functional Properties of Proteins Isolated from a Brown-Rot Fungi, *Sparassis crispa*

Advisor: Prof. Hyun-Jae Shin

This dissertation is submitted to the Graduate School of the Chosun Univeristy in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

April 2012

Graduate School of Chosun University

Department of Chemical Engineering

Gayathri Chandrasekaran

This is to certify that the Ph.D. dissertation of Gayathri Chandrasekaran has successfully met the dissertation requirements of Chosun University.

Chairman	**: Prof. Jung-Heon Lee**	.....
Committee Member	**: Prof. Young Jin Jeon**	.....
Committee Member	**: Prof. Hyun-Jae Shin**	.....
Committee Member	**: Prof. Geun-Joong Kim**	.....
Committee Member	**: Dr. Deuk-Sil Oh**	.....

June 2012

Graduate School of Chosun University

Table of Contents

LIST OF TABLES

CHAPTER 4

CHAPTER 5

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

초 록

갈색부후균 *Sparassis crispa* 유래 단백질의 생물학적 특성 및 기능연구

가야트리 챤드라세카란

지도교수: 신현재

생명화학공과

조선대학교 대학원

갈색부후균의 일종인 꽃송이 버섯 (*Sparassis crispa*)은 식용이 가능한 약용버섯으로 최근 그 의학적 효용성이 주목을 받고 있다. 본 연구의 목적은 꽃송이 버섯의 자실체에 유래한 다양한 단백질을 분리하고 그 특성을 규명하며 그 단백질의 응용을 살펴보는 것이다. 환경처리, 식품산업, 의약용 성분으로의 활용 가능성에 대하여 검토하였다.

2 장에는 그간 진행된 꽃송이 버섯의 영양성분과 의학적 특성에 관한 연구를 요약 기술하고 본 연구를 통해 달성하고자 하는 목적과 논문의 구성에 대하여 기술하였다.

3 장에서는 식용버섯이며, 의학적 특성이 우수한 꽃송이 버섯의 자실체로부터 리그닌 과산화효소(Lignin peroxidase, LiP)를 정제 분리하였다. 단일벽 탄소나노튜브 (Single-walled carbon nanotubes, SWNTs)와 열처리와 화학적으로 처리하여 카르복실화된 SWNT 의 LiP 에 의한 분해특성을 조사하였다. 탄소나노튜브와 LiP 의 분해상호작용과 효소분해 생성물은 여러 가지 물리 화학적 분석장치를 이용하여 조사하였다. LiP 효소의 촉매 작용에 의한

탄소나노튜브의 분해 결과를 증명하였으며, 퍼옥시다아제에 의해 생성된 라디칼이 탄소나노튜브의 분해에 큰 역할을 함을 알 수 있었다. 뿐만 아니라 효소분해 생성물인 이산화탄소(CO_2)의 농도가 증가함을 관찰하였다. 꽃송이 버섯으로부터 활성효소를 완전히 순수하게 추출하지 않은 상태에서도 이를 탄소나노튜브의 분해에 이용할 수 있다는 것은 환경 복원(environmental bioremediation) 분야에 버섯유래 효소가 적용 가능하다는 반증이다.

4장에는, 꽃송이 버섯 자실체로부터 *p*-nitrophenyl acetate 기질에 기질특이성을 갖는 호알칼리성 에스테라제 (Alkaliphilic esterase)를 분리 정제하였다. 분리된 효소는 SDS-PAGE로 분석한 결과 약 60 kDa의 분자량을 가지고 있으며, tributyrin을 이용한 zymogram 분석에서 이 효소는 에스터 가수분해 효소임을 확인하였다. 이 효소의 활성도가 최적인 산성도는 pH 8, 온도는 50℃이다. 효소의 활성도가 안정적인 pH는 알칼리성이며, 30~40℃ 온도에서 이 효소는 더 안정함을 보였다. 본 에스터라제는 식품산업 전반에 활용범위가 무척 클 것으로 사료된다.

5장에서는 꽃송이버섯으로부터 추출한 탄수화물 결합단백질인 렉틴을 정제하였고, 정제된 렉틴은 SDS-PAGE 와 응결적혈구 반응에서 1개의 단백질 띠를 나타내었다. 꽃송이버섯으로부터 추출한 렉틴은 그람 양성균, 그람 음성균, 그리고 약에 대한 내성을 가진 박테리아의 성장을 저해하는 특성을 보였다. 또한 렉틴은 곰팡이균인 *Fusarium oxysporum*와 *Fusarium solani* 균사체의 성장을 억제하는 항진균 특성을 보였다. 원형편광법으로 렉틴은 완충용액에서 α-헬릭스 구조를 가지고 있음을 밝혔으며, 당지질, 만난, 라미나란 등의 성분을 세포벽에 가지고 있고, 트립토판 청색이동 측정법 (tryptophan blue shift assay) 에 의하면 렉틴은 세포벽에 존재하는 당지질, 만난, 라미나란 등과 상호작용하고 있음을 알 수 있었다. 특히 균의 세포벽 성분에 렉틴이 항균작용을 함을 알 수 있었다. 결과적으로 렉틴은 여러 균주에 대한 항균특성을 가지고 있으며, 꽃송이 버섯은 생화학적 활성을 가진 천연 자원이라 말할 수 있다.

결론적으로 꽃송이 버섯 자실체는 다양한 단백질과 효소의 공급원으로서 그 가치가 무척 크며 식품, 환경, 의약 분야로의 활용가능성이 무궁무진 하다고 판단된다.

Chapter 1. Research background and thesis structure

1.1 Research background

Sparassis crispa is an edible and medicinal mushroom, which commonly called the cauliflower mushroom (Figure 1.1). This mushroom has recently become popular in Asian countries like Korea and Japan. *S. crispa* is a good source of food and nutraceuticals due to their rich flavor compounds and β-glucan content. This mushroom has immense potential in many diverse applications (Figure 1.2).

Figure 1.1 Morphology of *Sparassis crispa* mushroom.

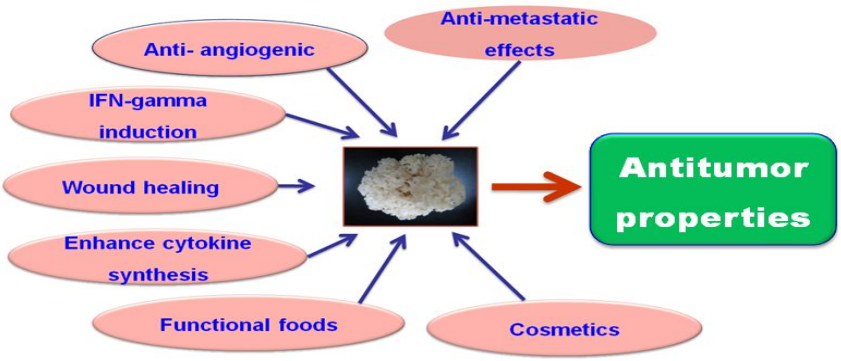


Figure 1.2 Applications of *S. crispa*.

In general white-rot fungi (WRF) can degrade a wide range of organopollutants; the degradative property is due to the lignin-degrading systems of these fungi. Specifically, this fungus produces two types of extracellular peroxidases, lignin peroxidases (LiP) (EC.1.11.1.14) and manganese dependent peroxidase (MnP) (EC.1.11.1.13), while others secrete laccase and versatile peroxidase. LiP consists of a single polypeptide chain, with an iron protoporphyrin prosthetic group. It has a unique ability to degrade lignin polymer through an oxidative electron transfer mechanism (Figure 1.3). They also find application in various fields such as the paper and pulp industries and in the textile industry for the decolorization of dyes and the transformation of polyaromatic hydrocarbons. The fungal peroxidases exhibit special characteristics to detoxify xenobiotic compounds by virtually complete degradation or complete mineralization. Several fungi are able to degrade various pollutants (Baldrian et al., 2000; Mehboob et al., 2011).

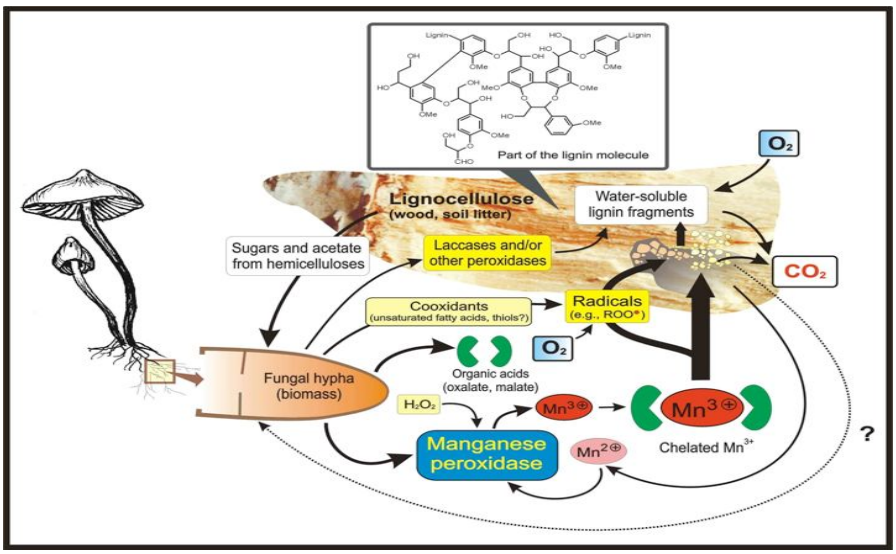


Figure 1.3 Schematic representation of lignocelluloses degradation by basidomycetous fungi.

Biodegradation is the use of living organisms such as bacteria, fungi, and plants to degrade chemical compounds. Bioremediation is the process of cleaning up environmental sites contaminated with chemical pollutants by using living organisms to degrade hazardous materials into less toxic substances. In general bioremediation approaches convert harmful pollutants into relatively harmless materials such as carbon dioxide, chloride, water and simple organic molecules. There are chemicals in the environment which causes carcinogens, mutagens, cause skin rashes, birth defects, poison plant and animal life. Indigenous microbes those found naturally at a polluted site are bacteria, algae and fungi. There are two ways to stimulate the bioremediation one is nutrient enrichment and bioaugumentation. Nutrient enrichment is defined as the fertilizers are added to a contaminate environment to stimulate the growth of indigenous microorganisms that can degrade pollutants. Bioaugmentation is defined as bacteria are added to the contaminated environment to assist indigenous microbes with biodegradative processes (Figure 1.4).

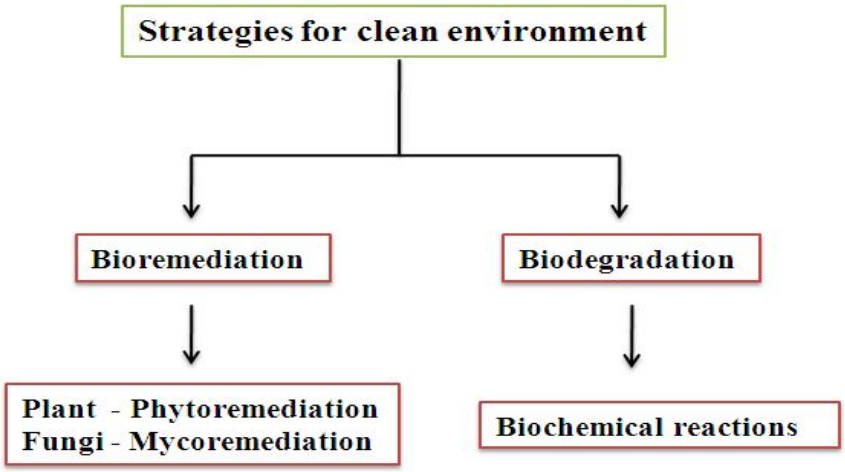


Figure 1.4 Scheme showing the strategies for clean environment.

Nanotechnology has become one of the most important and exciting aspects of research in various fields and especially in engineering and biology. Presently, the environmental and health concerns over nanomaterials such as single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) are increasing, an important issue to be taken into consideration. Moreover, it was stated that carbon nanotubes affect the environment as much as they affect humans, and the levels of risk are proportional to the amount of engineered nanomaterial produced globally. Concerning the environmental hazards of carbon nanotubes, there are only a few reports available and a small amount of research performed thus far. Furthermore, there is a report that SWNTs and MWNTs can be degraded by enzyme-catalyzed reactions within a few weeks or days (Allen et al., 2009).

Various reports have stated that the carbon nanotubes cause skin cancer, oxidative stress, granuloma formation, fibrosis, lung cancer, genotoxicity and mutagenicity, and mesothelioma. Separately, there is a recently published study, performed *in vivo* and *in vitro*, concerning the health risks of exposure to carbon nanotubes. Moreover, the dermal toxicity of the SWNTs causes oxidative stress to the skin. Athough they contain lower amounts of catalyst and iron, the overall research has revealed that CNTs tend to cause epithelioid granulomas and interstitial inflammation in animals (Warheit, 2006). Recently, it has been demonstrated that CNTs can trigger some biological responses similar to those of asbestos. Notably, the multiwalled CNTs are short (less than 20 μm) and soft, which can cause serious health problems in the same manner as asbestos (Sanchez et al., 2009). Enzymes are a new alternative method that provides a new strategy to remove organic pollutants from wastewater and the soil. The focus of this study is to similarly eradicate carbon nanotube pollution by an enzyme catalyzed reaction. Recently, a first report on the enzymatic degradation of carbon nanotubes

using horseradish peroxidase (HRP) was published. Here, this study demonstrates that *S. crispa* mushroom lignin peroxidase could be an option choice for the large-scale economic and ecofriendly remediation of nanomaterials (Li et al., 2010).

Currently, an increasing number of chiral drugs and organophosphorus compounds have been produced through esterase-catalyzed reactions (Bornscheuer, 2002). Different esterases are produced from various sources, and researchers are looking for novel esterases for suitable production strategies. Considering the biocatalytic applications, it is of great interest to study the specificity and selectivity of novel enzymes that lead to potential applications in the food, fat, oil and pharmaceutical industries. Owing to the interest of mushroom esterases, a comprehensive study was made to find them in various *S. crispa* strain stocks from the Jeonnam area of South Korea and Japan. To our knowledge, the *S. crispa* esterase has not been reported previously, so this is the first report on the esterase from *S. crispa* fruiting bodies.

In mushrooms, lectins probably play an important role in dormancy, growth and morphogenesis, morphological changes consequently on parasitic infection and molecular recognition during the early stage of mycorrhization. The fungal lectins play different roles in different circumstances, for example, the explosive growth of sporomes is accompanied by depolymerisation of stored material and movement of sugars that is directly controlled by lectins via their specific binding sites or indirectly via an enzyme system. For glycoprotein enzymes, lectins can bind to their carbohydrate moiety to repress or activate the enzymes (Guillot and Konska, 1997). Since the amounts of lectin present in *Pleurotus cornucorpiae* increases gradually from the primordium to the mature state (Kaneko et al., 1993; Oguri et al., 1996), the lectin may be involved in the cohesion of hyphae during the development stage of the basidiome.

Moreover, it has been observed that the level of lectins in the sporomes varied with the time of harvest that may be connected with the lifting of dormancy of the mycelium. Many studies of plant lectins have assumed that they are implicated in host defense mechanism as antifungal proteins. However, to date only a small number of lectins have been reported to have antifungal activity. Eventually, the antifungal activity assay was carried out in the higher fungi; *Agrocybe cylindracea*, *Agaricus bisporus* (Wang et al., 2001) and *Lyophyllum shimeiji* lectins (Lam and Ng, 2001). Here, we describe the isolation and characterization of a lectin from fruiting body of the *S. crispa* mushroom.

1.2 Structure of thesis

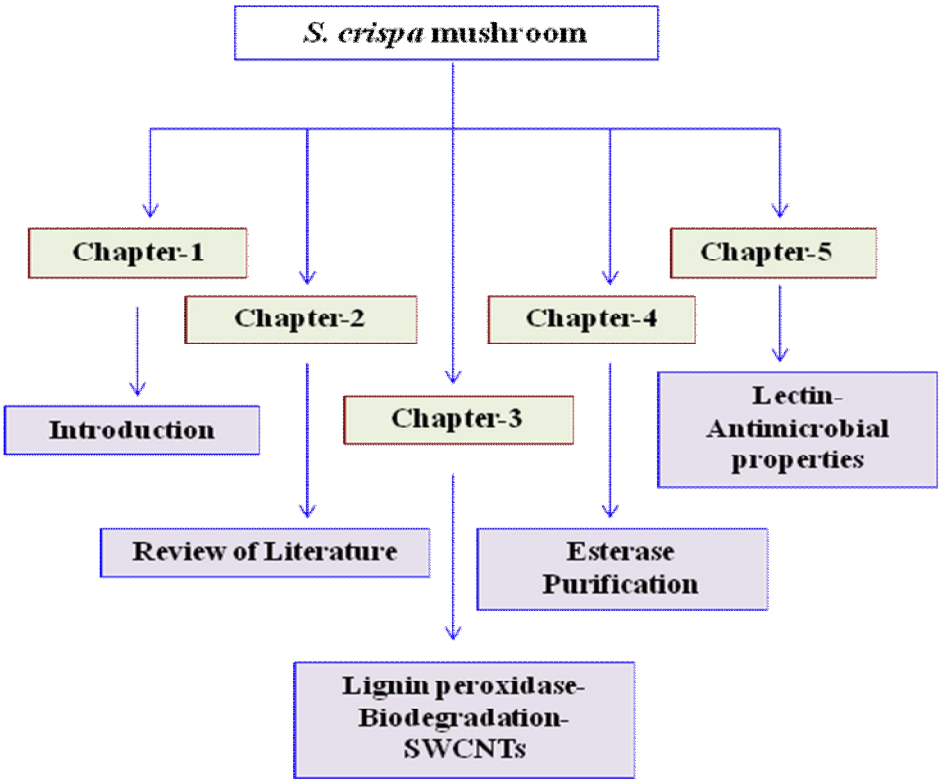


Figure 1.2.1 Outline of the thesis.

This thesis consists of five chapters. The first chapter comprises of research background and outline of the thesis structure. The second chapter is the review of literature about the properties and potential applications of the culinary-medicinal cauliflower mushroom *S. crispa*. A comprehensive view of general constituents, functional ingredients, and medicinal applications are summarized in this part.

The third chapter of the thesis is about the purification of lignin peroxidase from *S. crispa* mushroom and their biodegradation aspects on single-walled carbon nanotubes. Lignin peroxidase (LiP) was isolated and partially purified from the fruiting bodies of the medicinal and edible mushroom *Sparassis crispa*. The degradation of both raw grade and thermally treated carboxylated single-walled carbon nanotubes (SWNTs) by the LiP from *S. crispa* was investigated.

The fourth chapter of the thesis describes about the fruiting body of the medicinal and edible mushroom *Sparassis crispa* produces an alkaliphilic esterase. The substrate specificity of this esterase was high for a *p*-nitrophenyl acetate substrate. The *S. crispa* esterase was purified using ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The recovery and purification yields of the enzyme were 15-17% and folds 70-73 from six different strains of *S. crispa*, respectively.

Fifth chapter describes about the lectin which has been isolated from the fruiting bodies of *S. crispa*. The purified lectin showed one protein band in SDS-PAGE and agglutinated human erythrocytes. The lectin inhibited the growth of Gram-negative and Gram-positive bacteria, including drug-resistant strains. The lectin also displayed antifungal activity against various fungal strains. Furthermore, it is indicated that cell

wall components is having essential role in antimicrobial activity of the lectin. Overall, these results indicated that lectin could be used as antibiotics against microbial strains.

Chapter 2. Review of literature

2.1 Introduction

Mushrooms have been used in forestry, industry, biotechnology, bioremediation, restoration, and reforestation. They have been consumed as food and medicine since ancient times due to their enormous health benefits. It is estimated that there are 140,000 mushroom species on the earth and nearly 10% are known (Wasser, 2002). Mushrooms can improve one's health significantly because they are low in calories, sodium, fat and cholesterol; they also contain a large amount of protein, carbohydrate, fiber, vitamins and minerals. All together, these nutritional properties make mushrooms part of a well-balanced diet (Manzi et al., 1999). More than 3,000 species are consumed around the world, and more than 100 have shown promising clinical activity against cancer and other chronic diseases. In the food and pharmaceutical industries, some useful mushrooms are in demand (Garibay-Orijel et al., 2009). *Sparassis crispa* is also commonly called the cauliflower mushroom in English, Hanabiratake in Japanese and Ggoksongee (meaning a blossom) in Korean. The scientific name is *S. crispa* Wulfen: Fries, which was derived from their curly or crisped branches. *S. crispa* is a good source of bioactive natural products that have antiviral and anticancer properties and also has a rich flavor.

2.2 Distribution and Taxonomy

2.2.1 Morphology

Fungi are not photosynthetic. Some of the edible forest fungi are saprobic, decomposing organic matter to set up their life span, and they are therefore eco-friendly (Garibay- Orijel et al., 2009). The physical appearance of *S. crispa* is a cluster of flattened, leaf-like branches, which are wrinkled and usually cream to pale yellow in

color; the older leaves are darker at their edges. Generally, they have branches which arise from a thick (2-5 cm), deep root (5-14 cm) and usually have a dark brown to black stalk. Their fruiting bodies are 5-20 cm tall, 6-30 mm in diameter and are waxy and flexible with a pleasant odor. The spores of *S. crispa* are colorless and oval shaped with dimensions of 4.5-6 µm × 3.5-4.5 µm. The mycelium is 5-8 µm with a diameter reaching 2-4 µm and is smooth with frequent clamp connections.

2.2.2 Taxonomy and phylogeny analysis

These mushrooms typically grow in autumn near the roots of coniferous trees (*Pseudotsuga* sp. and *Pinus* sp.). *S. crispa* is the best example of the edible saprobic mushrooms, utilizing wood debris as their substrate (Pilz and Molina, 2002; Oh, 2009). *S. crispa* is a unique species of *Sparassis* found in the Pacific Northwest and is distinguished by their sporocarps (Jones and Lynch, 2007). Generally, the eastern North American *S. crispa* is morphologically similar to the European *S. crispa,* and their distribution patterns are closely related to eastern North American *S. spathulata* and European *S.* brevipes; Western North American *S. radicate* is similar to Asian *S. crispa*. Also the *Sparassis* species showed morphological variations in eastern Asia and Australia when compared to North America and Europe. There are additional reports that the *S. crispa* species is similar to *S. radicata* (Wang et al., 2004).

To find the phylogenetic relationships between the *Sparassis* genus and species, the nuclear and mitochondrial ribosomal DNA and RNA polymerase sequences were examined. Various nuclear gene phylogeny analyses (nuclear large subunit rDNA, ITS, and *rpb2*) suggest morphological differences, geographic distribution, and host shift, which produce the clamp connection among the *S. crispa* species. It was also observed that *S. crispa* and *S. radicata* produce clamp connections mainly with conifers. This analysis also proved that the *S. crispa* is distributed in Europe and eastern North

America, whereas *S. radicata* is found in western North America. Therefore, this finding suggests that host shifts must have occurred during the evolution of *Sparassis* species (Wang et al., 2004; Dai et al., 2006).

2.3 Screening, cultivation and harvest

2.3.1 Habitats, screening and preservation

According to the investigations of the ecological condition of the *S.crispa* productive sites, the isolation sites showed a very wide altitudinal distribution, ranging from 240 m to 1,100 m, and were found especially in very high altitudes (>1,000 m). In general, the mushrooms showed distinct features of heart-rot fungus, as it was found on soils around the stems of larches (*Larix kaempferi*) and Korean white pines (*Pinus koraiensis*). However, some of the mushrooms could be found on the stem or stump of dead trees, which indicated that the fungus might have several invasion routes and capabilities to grow on various ecological conditions (Oh, 2009). Matsumoto et al., (2001) suggested that hybrid glycoprotein and neoproteoglycan probes can be used for the screening of a unique lectin from various mushrooms, including *S. crispa*. Interestingly, *S. crispa* manifests the reactivity in both hybrid glycoprotein and neoproteoglucan probes, indicating the presence of multispecific or multiple lectins.

Different storage protocols have been acquired, such as liquid nitrogen for the mycological research (Smith, 1998). Homolka et al., (2003) reported the viability of 250 Basidiomycete strains, including *S. crispa,* after cryopreservation. Two freezing protocols were used for the comparative study: An OP protocol (original cryopreservation protocol using the agar plugs) (Butterfield et al., 1978) and a CP protocol (modified cryopreservation protocol) (Hoffmann, 1991). They reported that the OP procedure failed for the storage of *S. crispa,* whereas a 100% success rate was achieved in the CP procedure. Recently, an easy CP protocol using perlite was

developed and was also one of the suitable methods for the survival of strains like *S. crispa* (Homolka et al., 2006).

2.3.2 Cultivation and harvest

S. crispa's mycelium and fruit body can be cultured using solid and liquid substrates by three methods: bottle, bag and log cultivation. In the bottle cultivation method, neither the sawdust nor the liquid as an inoculum showed significant differences for the growth of mycelium. The treatment of low temperature in 4°C for a day was the most effective way to induce primordium of *S. crispa*. The composition of fermented sawdust of *Pseudotsuga menziesii*, wheat flour, corn chips, cottonseed meal, and 10% molasses (starch syrup) was selected as the most suitable medium, and it showed 41% of fruit body production rate. When the molasses replaced by water, it resulted in 27% of fruit body production rate. As the weight reduction rate of medium increases, the fruiting body production rate increased (Shim et al., 1998; Lee et al., 2004).

The results of the bottle cultivation method were applied to the plastic bag cultivation method. The mycelial growth was best when utilizing the same media composition (fermented sawdust of *Pseudotsuga menziesii*, wheat flour, corn chip, cotton seed meal, and 10% molasses). Mushroom production was also the largest with this media, and the fruiting body production rate reached 47%. However, the medium with raw sawdust of *Pinus thunbergii*, *Callicarpa japonica* and *P. koraiensis* showed relatively low production rates of 28-30%. The additives like molasses played significant roles in the production rates of the mushroom. By the same mechanism, the addition of hot-water extracts from the black pine for 5 h on PDA (potato dextrose agar) media showed a lower mycelium growth rate compared to the rate grown on control media. When comparing the mushroom production in a bottle to plastic bag

cultivation, the production with the media composed of fermented sawdust of larch, wheat flour, corn chips, cotton seed meal, and 10% molasses in plastic bags showed higher production rates of 38%, whereas those in bottles showed 22%. In addition, the bag cultivation showed a higher portion of pileus (82%) than the bottle cultivation (45%) (Shim et al., 1998; Lee et al., 2004).

Park et al. (2006) developed an efficient cultivation method for *S.crispa* with steam-treated sawdust media of *Larix leptolepis*, *Pinus densiflora* and *Pinus koraiensis*. By this method, the mycelial growth was stimulated, and the mushroom productivity was increased by the steam treatment. This method also minimizes the culturing period and provides an effective way of utilizing coniferous saw dust for the cultivation of *S. crispa*.

A survey of the Pacific Northwest harvest data showed that there are 35 commercial species of edible mushrooms which are harvested every year, including *Sparassis*. Mushroom harvesters can improve the production and develop small scale management to sustain their harvesting (Jones and Lynch, 2007). Biodiversity management in the Pacific Northwest was improved by the non-timber forest products (NTFP) like mushroom cultivation. In the case of *S. crispa,* their cultivation needs scale experimentation to adapt the culture technologies to local conditions.

2.4 General constituents

2.4.1 Nutrient analysis

Mushrooms are a sufficient source of protein, ranging from 5.27 to 9.62% of their dry weight (Crisan and Sands, 1978). The free amino acids and carbohydrates in Korean edible and medicinal mushrooms were reported in 2009 (Kim et al., 2009). The nutritional analysis indicated that dried *S. crispa* grown in Korea comprises 10.1% moisture, 30.0% protein, 2.7% lipid, 36.4% fiber, 7.0% ash and 13.5% of mineral and

vitamins. They reported the presence of monosaccharides and disaccharides in the *S. crispa* such as xylose and fructose, mannose, glucose, and trehalose and also some essential amino acids. Potassium, phosphorus, sodium, and magnesium were the major mineral components of cauliflower mushrooms; potassium was the most abundant (1,299 mg/100g of mushroom dry weight). In the case of total amino acids, glutamine and glutamic acid was the largest (1,960 mg/100 g). Among the free amino acids, glutamic acid, tryptophan, glutamine and aspartic acid were found abundant. The Vitamin E was detected as the highest in amount in the *S.crispa* mushroom (409 mg/100 g). The content of vitamin D_3, which helps calcium absorption, was 0.17 mg, which is higher than in other mushrooms (Shin et al., 2007).

2.4.2 Flavor compounds

Mushrooms produce a unique flavor and are therefore used in food preparations. Mau et al., (1994) reported that of the C8 compounds that are typically common to most mushroom, 1-octen-3-ol is one of the essential aroma compounds. They also reported that these compounds differ in their specific genera, species or strains (Mau et al., 1997). Moreover, there is a report on the mushroom texture, nutritive values, and volatile contents. The results clearly show that mushrooms are also rich sources of vitamins, minerals and the aroma volatile components, which are typically esters and their hydrocarbon, fatty acid derivates as analyzed by gas chromatography and mass chromatography (Caglarirmak, 2007). Another report manifests that mushrooms have a distinctive umami taste due to the presence of aspartic and glutamic acids (e.g., monosodium glutamate) (Yamaguchi, 1979).

Kavishree et al., (2008) examined the total fat and fatty acid content (dry weight basis) in the various mushrooms fruit bodies collected in the forest areas of India. They proposed that unsaturated fatty acids such as linoleic acid, which are precursors of the

mushroom alcohols, along with their association with the C8 ketones, are the responsible factors for the mushroom flavor. They noticed that the pentadecanoic acids were found in the *S. crispa* species. In the group of monounsaturated fatty acids, palmitoleic acid is found in the *S. crispa* species, whereas in the case of the polysaturated fatty acids, linoleic acid was present. In particular, the linoleic:oleic ratio is very important to differentiate the species of the same genus; the *S. crispa* the ratio was below one. Therefore, they esteemed the wild edible mushroom species as a healthy food and they also determined the high content of essential fatty acids, which regulates the high blood cholesterol in human.

2.4.3 Enzymes

Mushrooms are recognized to be a good enzyme source. According to the biocatalytic applications view, it is very interesting to study the specificity and selectivity of novel enzymes that leads to potential applications in the food, fat, oil and pharmaceutical industry. For example, esterases are employed in food processing, beverages and cosmetics. Considering their habits and physiology, many enzymes such as cellulases, peroxidases, and esterases could be present in mycelium and fruiting bodies of *S. crispa*. However, there is no report of that yet because the research is in their early stages. Based on our research on this medicinal mushroom, we have found that the fruit bodies of this *S. crispa* possess esterase and cellulase activities. Recently our lab succeeded in isolating esterase in *S. crispa* (data not shown). Concerning the mushroom esterases, the characterization of activity from only three mushroom species, *Amanita vaginata* var. *vaginata*, *Tricholoma terreum,* and *Lycoperdon perlatum* was reported (Colak et al., 2009; Ertunga et al., 2009). This research could be accelerated by the proteomics analysis of *S. crispa* (Horie et al., 2008). The authors reported proteomics analysis of *S. crispa* fruit bodies using one and two dimensional gel

electrophoresis (1-DGE and 2-DGE) based complementary proteomics approaches (http://foodfunc.agr.ibaraki.ac.jp/mushprot.html). This study provides evidence for the presence of a large number of functionally diverse proteins, expressed in the fruit body of *S. crispa*.

2.5 Nutraceutical, medicinal and cosmetic applications

2.5.1 Extraction and extracts

The hot water extracts of the fruiting bodies of *S. crispa* are specifically useful for the application of the traditional natural remedies. Ohna et al. (2000) reported 52.0% of β-glucan content by the hot water, cold alkaline and hot alkaline extraction in *S. crispa*. The larger molecular compounds were precipitated from the crude water extracts by methanol. Until now, approximately 20 different major diverse classes of natural products from *S. crispa* mushroom were noted, which can be put into practice in medicinal research (Politi et al., 2007). Recently, the tungsten carbide (WC) nanoparticle (so-called nanoknife technology) was used to reduce the particle size of the β-glucan extraction to enhance the biological activity (Park et al., 2009). The molecular weight of the *S. crispa* polysaccharides from the extraction was 510 kDa, and the average particle size diameter was 150±14.1 nm. These fine particles are useful for industrial applications and drug delivery because they are easily absorbed by the human intestine. They also reported that the alkaline extraction and hot water extraction method gave a β-glucan content of 70.2%.

2.5.2 Pre- and probiotics

Foods containing prebiotics have been commonly consumed by humans. Currently, various products are sold in the market, like probiotic-containing breads, cereals, confectioneries, sauces, infant milk products, beverages and health drinks. Mushrooms can also serve as the prebiotics because they have rich sources like

carbohydrates. Digestive enzymes secreted by the pancreas of mammals are unable to hydrolyze specific β-1,3-glucosidic bonds. This makes them resistant to acid hydrolysis in the stomach and remains non-digestible by human enzymes (Aida et al., 2009). This non-digestible property of mushroom carbohydrate makes them a potential source of prebiotics. Prebiotics are reported to be particularly suited to the growth and activities of probiotics, bifidobacteria and lactobacilli that suppress the growth of clostridia and bacteroides (Wang, 2009). In the future, the production of a new potential prebiotic from mushrooms are good sources for the food industry which can be helpful for human health.

2.5.3 Medicinal applications

Focusing on different literature, several authors summarized the pharmacologic potential of mushrooms (Wasser and Wasser, 2002). They reported that medicinal data on mushroom polysaccharides have been gathered from 651 species and 7 infraspecific taxa from 182 genera of higher hetero- and homo-basidiomycetes. Many researchers also reported that the fungi from Basidiomycota yield a variety of biologically active compounds such as polysaccharides, glycoprotein, terpenes and antibiotics (Mahajna et al., 2009). Basidiomycetes mushrooms hold the biologically active polysaccharides in fruit bodies, cultured mycelium, and culture broth. Many properties of polysaccharides are amenable for the antitumor activities such as water solubility, molecular size and their branched form. The antitumor activity was examined mainly in the β-glucan (1-3) branched moiety. However, the antitumor activity can be enhanced by chemical modifications like smith degradation, formolysis and carboxymethylation. The clinical evidence for antitumor activity derived from the commercial polysaccharides lentinan, PSK (krestin), and schizophyllan have been reported in several medicinal mushroom species (Wasser, 2002). Tada et al., (2002) elucidated the structure of β-glucans from

the fruit bodies of *S. crispa* by NMR. The researchers suggested that the primary structure of purified β-glucan from *S. crispa* possesses the backbone structural units as β-(1→3)-D- glucan with single β-(1→6)-D-glucosyl side branching units at every three residues. Polysaccharides derived from *S. crispa* play a significant role in the antitumor activity (Lindequist et al., 2005). Ohno et al., (2000) noticed that the polysaccharides fractions of 1,3-β- glucan in *S. crispa* possess strong antitumor activity against the solid form of sarcoma in mice. Harada et al., (2002) suggested that β-glucan is an eminent biological response modifier (BRM), which possesses the immunomodulative effects and are the substances that stimulate the body's response to infection and disease. The *in-vitro* studies demonstrated that *S. crispa* enhanced the hematopoietic response in cyclophosphosphamide-induced leucopenic mice, stimulated the large amounts of interferon-γ in splenocytes and induced interleukin-12 p70 (IL-12p70). They reported that a 43.6% β-glucan content exists in *S. crispa* and identified it as the source for the antitumor activity. Harada et al., (2004) reported that the cytokines induced by *S. crispa* β-glucan were IFN-γ, tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-12 (IL-12p70). In this study, they examined the mechanism of IFN- γ production by *S. crispa in vitro* in splenocytes from DBA/2 mice to determine the cause for sensitivity in leukocytes from DBA/2 mice. They suggested that receptors such as dectin-1 are responsible for the *S. crispa* induced cytokine production. In addition, Hasegawa et al., (2004) reported that cancerous mice (sarcoma 180) treated with *S. crispa* dry powder for approximately 5 weeks showed a reduction in tumor size and prolonged survival. Additionally, the polysaccharide fractions extracted from the *S. crispa* showed strong antitumor activity against the solid form of Sarcoma 180 in ICR mice and the hematopoietic response in cyclophosphamide-induced leucopenic mice (Harada et al., 2006). The author also

demonstrated that *S. crispa* may possess the suppressing symptoms of allergic rhinitis through its immunomodulating activities (Yao et al., 2008). Yamamoto et al. (2009) first reported that the oral administration of *S. crispa* β-glucan suppressed angiogenesis and metastasis. The researchers also suggest that these effects are not a result of direct action on the endothelial cells because cell growth, migration and capillary-like tube formation were not affected in the human umbilical vein endothelial cells by *S. crispa* β-glucan administration.

The antioxidant properties of the water and methanol extracts of the fruiting bodies of *S. crispa* were studied. The *S. crispa* water extract shows the presence of gallic acids, protocatechuic acid, gentisic, and coumaric acid, and they also possess antioxidant properties. Therefore, antioxidant properties of this mushroom are effective for nutraceuticals (Puttaraju et al., 2006).

Hyun et al., (2006) studied the extraction and characterization of platelet aggregation inhibitory peptides from the mushroom, including *S. crispa*. The ethanol extract from the *S. crispa* mycelia showed a platelet aggregation inhibitory activity of 52.8%. Therefore, they can be utilized as antithrombotic drug candidates.

Large investments and plenty of research are directed towards the studies on wounds and wound care systems. In the case of diabetic patients impaired wound healing is a major clinical problem. Oral administration of *S. crispa* extracts exhibits the wound healing properties in streptozotocin-induced diabetic rats. It was found that the macrophage and fibroblast migration, collagen regeneration and epithelial formation were increased in the diabetic induced rats. The type I collagen level in the dermal fibroblast is greater in *S. crispa* β-glucan and a significant concentration was observed at more than 100 μg/ml (Kwon et al., 2009).

The first report on the separation and characterization of a microbial antibiotic was from *S. crispa.* The compound sparassol, was shown to be methyl-2-hydroxy-4-methoxy-6-methylbenzoate (Wedekind & Fleischer, 1923 and 1924*)*. Additionally, *S. crispa* shows the antibiotic production (Wilkins & Harris, 1944; Robbins et al*.,* 1945; Hervey, 1947) and suppression of the growth of *Bacillus subtilis* on agar media are known to be due to the sparassol (Siepmann, 1987). Woodward et al., (1993) reported that the basidiomycete fungus *S. crispa* produced three antifungal compounds while it was submerged in culture in a 2% malt broth. The result showed that one compound, sparassol, and two more antifungal compounds, ScI and ScII, which showed higher antifungal activity than sparassol against the *Cladosporium cucumerinum*. These two compounds (ScI and ScII) are found naturally in wood decayed trees where the *S. crispa* sufficiently inhibits the growth of the other fungi.

The reverse transcriptase is key enzyme for the HIV replication. The natural products extracted from edible medicinal fungi possess lower toxicity and fewer side effects than chemical drugs. Screening inhibitors for this enzyme derived from various edible medicinal fungi, including *S. crispa,* have been reported. The hot-water of the *S. crispa* fruiting bodies extract inhibited HIV-1 reverse transcriptase over 70.3% at a concentration of 1 mg/ml (Wang et al., 2007).

There is a clinical trial report on the *S. crispa* powder in cancer patients. *S. crispa* (Wulfen): Fr. (300 mg per day) was administrated orally to patients with various types of cancer, including lung, stomach, colon, breast, ovarian, uterine, prostate, pancreas, and liver. The results suggest that *S. crispa* is a good source for cancer immunotherapy because after one course of lymphocyte transfer immunotherapy, 9 of 14 patients showed improvement after after several months of monitoring (Ohno et al., 2003, Petrova et al., 2005). Because their polysaccharide exhibits immunomodulating

properties, antitumor activities and anticancer activities, there are benefits from incorporating mushroom extracts in food.

2.5.4 Cosmetic ingredients

The dried fruit bodies of the *S. crispa* yielded a novel compound which prevents melanin biosynthesis and methicillin-resistant *Staphylococcus aureus* (MRSA) growth. The IC_{50} value for the melanin production inhibition assay was 12 μM and the MIC value against MRSA was 1.0 mM (Kawagishi et al., 2007).Therefore, this novel compound can be a constituent for cosmetic products, although the structure has not been elucidated yet. A new terpene of sesquiterpenoid has been isolated from dried fruit bodies of the *S. crispa.* The NMR and ESI-MS analyses revealed the newly found compound structure as (3R*, 3aS*, 4S*, 8a*)-3-(1'-hydroxy-1'-methylethyl)-5,8 -dimethyldecahydroazulen-4-ol. This is the first isolation of an isodaucane-type sesquiterpenoid from mushrooms and fungi (Kodani et al., 2009).

2.6 Summary

Many different aspects of this cauliflower mushroom, *S. crispa*, paved an interest for the clinical research and industrial applications. This mushroom's significant role helped the scientists and researchers to isolate novel components. In particular, *S. crispa* is regarded as a functional food due to the remarkable β-glucan content and other medicinal ingredients. For the expansion of the mushroom industry, mutual research between Asian, European, and American countries would be helpful (Berch et al., 2007). In the future, efforts should be taken to screen other novel components for potential contributions in research and food processing in the food industry.

Chapter 3. The enrichment of lignin peroxidase from the *Sparassis crispa* mushroom and its capacity to biodegrade single-walled carbon nanotubes

3.1 Introduction

Recently, a proteomic analysis of the fruiting bodies of *S. crispa* mushroom and the economic importance of these structures was reported (Hori et al., 2008). However, the microbial degradation by lignolytic fungi has been intensively studied during the past few years due to the irregular structure of lignin; the lignolytic fungi produce extracellular enzymes with low substrate specificity that allow the degradation of various compounds. Specifically, white rot fungi and brown rot fungi are able to degrade a polyaromatic hydrocarbon (PAH) found in contaminated soil (Bamforth and Singleton, 2005).

Various types of carbon nanotube products, including raw grade and thermally treated grade, are available; they are composed of iron, nickel and yttrium, which exhibit proven pulmonary toxicity. In this study, the degradation of two different types of carboxylated SWNTs including those thermally treated and those of a raw grade using LiP partially purified from the fruiting bodies of the mushroom *S. crispa* was carried out. The results showed that LiP could play an important role in the oxidation of carboxylated SWNTs and also emphasize that cost-effectiveness and environmental friendliness is very important in the fields of bioremediation or biodegradation.

3.2 Materials and Methods

3.2.1 Materials

SWNTs purchased from Hanwha Nanotech Corporation (Incheon, South Korea), were used in this study. ASA-100 F is a raw grade type of SWNTs prepared by

the arc-discharge process; it is 20-30% pure, AST-100 F is a SWNT product created by thermal treatment. Both these products consist of carbon nanoparticles, graphite, and catalyst metal. The *S. crispa* mushroom fruiting bodies were collected in Jeonnam, South Korea and Japan. Lyophilized HRP type VI and 30% H_2O_2 was obtained from Sigma, Aldrich. The reagents used were of analytical grade. All the substrates, pyrogallol, 2,6-dimethoxy phenol, veratryl alcohol, 2,2 ' -azino-bis (3-ethyl benzo thiazoline-6-sulphonic acid (ABTS)) and guaiacol, were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE–Sepharose Fast Flow and PD-10 desalting columns were acquired from Amersham Biosciences (Sweden).

3.2.2 Media composition

To study the media compositions and their effect on enzyme production, five different sawdust media compositions with controlled percentages of media moisture were used such as: a. Larix leptolepis (100%) + distilled water, b. Larix leptolepis (100%) + 10% corn syrup solution, c. Larix leptolepis (90%) + 10% wheat flour + 10% corn syrup solution, d. Larix leptolepis (80%) + wheat flour(20 %) + 10% corn syrup solution, and e. Larix leptolepis (80%) + wheat flour (10%) + corn pellet (10%) + 10% corn syrup solution. *S. crispa* fruiting bodies samples were prepared using these media compositions, and their LiP activity was tested.

3.2.3 Enzyme extraction

The fresh fruiting body of the mushroom was isolated and freeze-dried. Each frozen fresh fruiting body (50 g) of the mushroom was ground to a fine powder in liquid nitrogen using a pre-chilled ceramic mortar pestle. Next, the mushroom samples were separately extracted in acetate buffer (pH 5.0), phosphate buffer (pH 7.0) and Tris–HCl buffer (pH 9.0) containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. The concentration of all the buffers was

10 mM. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. The resulting supernatants were used as crude enzyme extracts (Chandrasekaran et al., 2011).

3.2.4 Protein purification

The enzyme was purified from the crude supernatant by the following steps: 1) ammonium sulfate precipitation and 2) DEAE–Sepharose anion exchange chromatography. The crude extracts were precipitated by the addition of ammonium sulfate to 65% saturation. The solution was then centrifuged at 8,000 rpm for 15 min at 4°C. The precipitate was dissolved in a Tris–HCl buffer (10 mM, pH 9.0) and desalted over a PD-10 desalting column in the same buffer. The filtered enzyme solution was applied to a DEAE–anion exchange column (5 ml) that had been equilibrated with a Tris–HCl buffer (100 mM, pH 9.0) containing 0.15 M NaCl and then eluted with a linear salt gradient at a flow rate of 0.5 ml/min. The fractions with a high LiP specific activity were collected, concentrated and used in the following study.

3.2.5 Enzymatic activity assay

The mixture consists of 50 mM pyrogallol (2.5 ml), 100 mM sodium acetate buffer pH 5.4 (16 ml) and 50 mM hydrogen peroxide (1.5 ml). Approximately 250 µl of the assay mixture was aliquotted to each well of a 96-well microtiter plate; 10 µl of the enzyme sample was added and incubated at 37°C for few minutes. The appearance of a dark color indicated the presence of peroxidase activity (Johri et al., 2005; Periasamy et al., 2004). To screen for the various types of peroxidase, the following protocols were used. The activity of LiP was assayed by the reported method (Tien and Kirk, 1984) using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde spectrophotometrically at 310 nm. The reaction solution (1 ml) consisted of 2 mM veratryl alcohol and 0.4 mM of H_2O_2 in 50 nM sodium tartarate buffer, pH 3, at 25°C. The reaction was begun by adding 50 µl of the enzyme solution.

Manganese peroxidase and laccase were then assayed by the reported methods (Paszczynski et al., 1985; Mansur et al., 2003)

3.2.6 Electrophoresis and zymographic analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 10% polyacrylamide gel (Laemmli, 1970). A high-range molecular weight marker (EBM-1031, Elpis Biotech Inc.) was used as a standard. In each step, the proteins were quantified by the BCA method using BSA as a standard (Smith et al., 1985). To assay peroxidase activity, a zymogram analysis was performed in the polyacrylamide gels; the protein samples were dissolved in loading buffer without SDS or thiol-reducing agents and separated on a 7.5% Native-PAGE gel. After the protein separation, the gels were equilibrated for 30 min in 50 mM sodium acetate buffer, pH 5.4 prior to their incubation with 80 mM pyrogallol and 80 mM H_2O_2 in fresh sodium acetate buffer.

3.2.7 The carboxylation and examination of SWNTs by scanning electron microscopy (SEM)

SWNTs were carboxylated as described previously. About 25 mg ASA or AST (SWNTs) was sonicated in H_2SO_4 /H_2O_2 (30%) at a ratio of 3:1 for 24 hr at 0°C. After 10-15 hr, 2.0 ml H_2O_2 was added to the reaction to replace the spent H_2O_2. The final dispersion was then heated at 70°C for 10 min, and subsequently diluted 10-fold and filtered through a 0.22 μm Teflon membrane filter; the sample was washed with copious amounts of water to neutralize the pH (Wei et al., 2006). The carboxylated SWNTs were subjected to an SEM (Hitachi S-2400N, Japan) analysis. To remove the metal catalyst and other impurities, the ASA and AST (SWNTs) were treated with H_2SO_4/H_2O_2; this step also improves their solubility in aqueous media. This reaction is known as carboxylation, and it was confirmed through the SEM studies.

3.2.8 The treatment of the SWNTs with LiP and H_2O_2

About 1 mg of each types carboxylated SWNTs was added to 4.0 ml phosphate buffered saline (PBS) and sonicated for 1 min. As a positive control, lyophilized HRP type VI was solubilized in PBS at 0.385 mg/ml. The negative control was buffer alone. The experimental samples consisted of the LiP purified from *S. crispa* and solubilized in PBS at the same concentration as that of positive control. Four ml of each enzyme solution was added to the vials containing the carboxylated SWNTs and brought to a total volume of 8 ml. All the vials were then sealed with a septum and wrapped with parafilm. Beginning after a one day incubation, 8 ml 40 μM H_2O_2 was added through 20 daily additions of 250 μl by needle to all the vials; during the 20 days, the vials were kept at 25°C (Allen et al., 2009). Also crude LiP was incubated under identical condition except that the incubation temperature was lowered to 4°C.

3.2.9 Transmission electron microscopy (TEM)

The samples in PBS solution were centrifuged at 3,400 rpm for 3 hr to remove salts from the buffer (Allen et al., 2009). The supernatant was removed and the pellet was resuspended in DMF and sonicated for 1 min.The sample was dropped on a lacey carbon grid (pacific-Grid Tech) and allowed to dry for 1 hr and the TEM image (FEI Morgagni, 80 keV or JEOL 2100F, 200 keV) was then processed.

3.2.10 Visible near infrared and Raman spectroscopy

For the measurement, 150 μl of the aqueous samples of both types of carboxylated (SWNTs) were analyzed in 2 ml glass cuvettes using a Lambda 900 spectrophotometer (Perkin-Elmer). The SWNTs were scanned from 600 to 1,300 nm. Six samples (previously mentioned in section 2.9) were subjected to the vis-NIR spectroscopy analysis. All the samples were centrifuged at 3,400 rpm for 3 hr to remove salts from the buffer. The precipitated samples were then treated with MeOH

following a 2 min sonication. Approximately 20 µl of the samples were placed on the microscope slide and dried. All the spectra were collected on a Renishaw in Via Raman microscope using an excitation wavelength of 633 nm. The samples were scanned from 1,000 to 1,800 cm^{-1} to visualize the changes in the D and G band intensities resulting from the degradation process. All the spectra were collected using a 15 s exposure period and averaged 5 scans per sample (Allen et al., 2009).

3.2.11 Electron spin resonance

ESR spectra were recorded on a JES-FA ESR spectrometer (JEOL, Tokyo Japan). The measurements were performed at room temperature in gas permeable Teflon tubing. The tubing was filled with 10 µl of sample, folded over, and placed in an open 3.0 mm internal diameter EPR quartz tube. The EPR solutions were prepared by incubating LiP with 0.02 mg/ml nanotubes in PBS for 1 min at room temperature; pyrogallol was then added and the peroxidase reaction was initiated by adding H_2O_2 (80 µM). As a control, HRP (0.35 mkM) was incubated with nanotubes (0.02 mg/ml) in PBS for 1 min at room temperature, and ascorbate (100 mkM) was then added. The EPR spectra of the radicals were recorded 1 min after the addition of H_2O_2. The spectra of these radicals were recorded using the following conditions: 3,270 G, center field; 10 G, sweep width; 10 mW, microwave power; 0.4, field modulation; 10^3, receiver gain; 0.1 s, time constant; and 1 min, scan time (Allen et al., 2008).

3.2.12 Liquid chromatography-mass spectrometry (LC-MS)

Three milliliters of the aqueous samples of both the ASA and AST (SWCTs) were acidified by the addition of 500 µl 0.1 M HCl and extracted with dichloromethane (3 ml). The dichloromethane was then removed and the products were then redispersed in pure MeOH (500 µl). Approximately 5 µl of the concentrated sample was injected onto a C18 column (100 x 2.1 mm, 1.7 µm, 40°C in a mobile phase of 20:80 formic

acid and water: formic acid and acetonitrile. The samples were then analyzed for positive ions using electrospray mass spectrometry. An accurate mass measurement was performed with a Synapt high-definition mass spectrometry system (HDMS; Waters Co.). Leucine enkephalin was used as an independent reference lock-mass via the LockSpray to ensure mass accuracy and reproducibility. The LC-MS profiling experiments were performed on an Acquity UPLC system (Waters Co., Milford, MA) equipped with a binary solvent delivery system and an autosampler. The chromatographic separation was performed on an Acquity UPLC BEH (Allen et al., 2009).

3.2.13 Gas chromatography (GC)

About 2 µl of both the ASA and AST (SWCTs) samples headspace (total headspace volume: 5 ml) was taken by sampling through the septum of the vials and injected into a Shimadzu QP5050A GC-MS unit with an XFI-F capillary column. The temperature program was set to hold at 100°C for 1 min, followed by temperature ramping at a rate of 10°C/min until a maximum temperature of 325°C was achieved and held for an additional 10 min.

3.3 Results and discussion

3.3.1 The identification and Purification of LiP

The crude *S. crispa* mushroom fruiting bodies exhibited peroxidase activity towards the substrate pyrogallol in a 96-well-plate assay. The appearance of a dark reddish brown color in the 96-well-plate indicated the presence of the peroxidase activity. Generally, there are three types of oxido reductase enzymes in the fungal sources; therefore, we sought to screen for the enzymes types including LiP, manganese peroxidase and laccase using the spectrophotometric method. The screening results indicated that veratrylaldehyde was formed by LiP activity. Other enzymes such as

manganese peroxidase and laccase were shown absent. Therefore, the results conclude that LiP was present and manganese peroxidase and laccase were not. Furthermore, the relative specificity of LiP towards the substrates was as ranked: pyrogallol > veratryl alcohol > ABTS > guaiacol.

S. crispa LiP was purified by ammonium sulfate precipitation followed by DEAE column chromatography. In regard to the ammonium sulfate precipitation, enzymatic activity present after 65% saturation was higher than after 45% saturation (Figure 3.1A). Furthermore, the peroxidase protein of the 65% fraction was purified by anion exchange chromatography from the other proteins present. The unbound fraction (Figure 3.1B lane 1) demonstrates the absence of the LiP band, but two major peaks were observed within the bound fractions. The first peak did not exhibit LiP activity (Figure 3.1B lane 2), while the second peak showed the presence of LiP that was confirmed by the enzyme assay (Figure 3.1B lane 3). Therefore, the DEAE fractions were used in the characterization and application studies without further purification. This study was to demonstrate that the enzyme only partially purified could promote the degradation of the carbon nanotubes.

The LiP of *S. crispa* was purified from its fruiting bodies. SDS–PAGE revealed that the molecular mass of the LiP was approximately 45 kDa (Figure 3.1B). Some fungal LiP, including LiP isoenzyme 41, a 44 kDa enzyme from *Irpex lacteus* and *Phanerochaete sordida* YK-624, 45 kDa, have been reported with a molecular mass similar to that of the *S. crispa* LiP (Rothschild et al., 2002; Hirai et al., 2005).

To locate the target protein, the results of a zymogram assay was analyzed in which the development of a brown color in a native gel indicates the presence of peroxidase (Figure 3.1C). The zymographic analysis strongly suggested that the protein retained its peroxidase activity. Furthermore, the lower molecular weight protein at 26 kDa was

purified and subjected it to the enzyme activity assay; there was no LiP enzyme activity present. Overall, the zymographic results indicated that the partially purified protein retained its peroxidase activity, and the results were similar to those of another study (Johri et al., 2005).

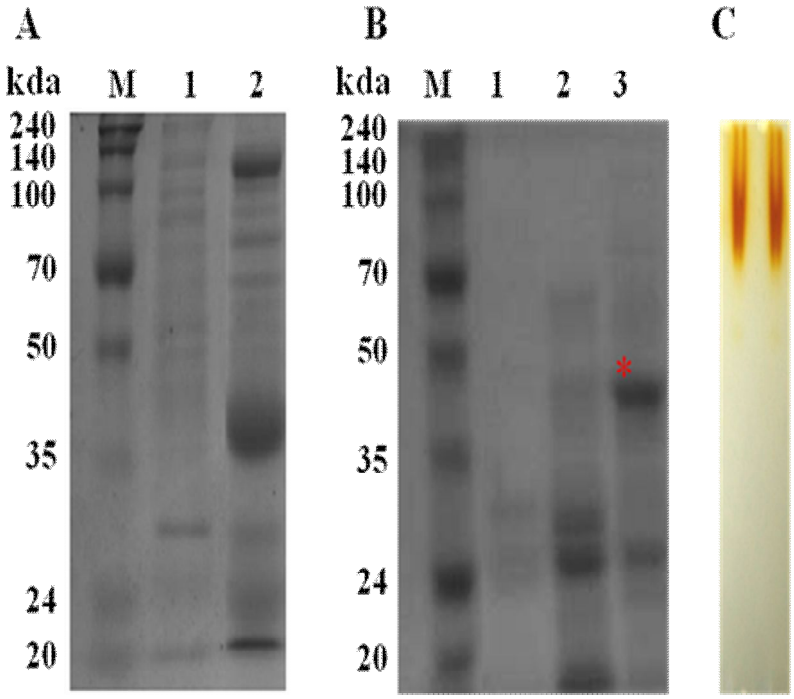


Figure 3.1 The identification and purification of lignin peroxidase (A) SDS-PAGE: M, standard protein marker; lane 1, 40% ammonium sulfate precipitate, lane 2, 65% ammonium sulfate precipitate. (B) SDS-PAGE: M, standard protein marker; lane 1, DEAE unbound fraction; lane 2, DEAE 1st bound fraction; lane 3, DEAE 2nd bound fraction. (C) Native PAGE (7.5 %) zymography of peroxidase from *S. crispa*.

3.3.2 The enzymatic degradation of CNTs observed by Microscopy

The carboxylated, single-walled carbon nanotubes were prepared by acid treatment. This treatment imparts carboxylic acid groups and improves the CNTs dispersion in aqueous solution (Wei et al., 2006). Furthermore, this process removes the residual metal catalyst; the SEM analysis (Figure 3.2) clearly shows both the ASA and AST (SWCNTs) were carboxylated, and observed the metal impurities are significantly reduced after the acid treatment compared with the pristine CNTs. After the acid treatment, the tubes are not only cut into short pipes but also purified because the acid mixture is known to intercalate and exfoliate graphite. Short, carboxylated, single-walled carbon nanotubes were used throughout the study.

The photography demonstrating the enzyme degradation is shown in the (Figure 3A). The vial 1 contained the initial sample of the carboxylated nanotubes without degradation at day 1, while vial 2 is a positive control containing HRP and vial 3 contains the SWNTs with *S. crispa* LiP. The vials 2 and 3 exhibited the noticeable color change that indicates degradation after 20 days incubation. This result confirmed that both enzymes degrade the carbon nanotubes. In regard to the degradation of the pristine SWNTs, the visual observation indicated that complete oxidation did not occur. Therefore, the degradation studies of that material have not further pursued. Moreover, as proposed in the previous study, the degradation rate was considerably lower than that of the carboxylated SWNTs.

Furthermore, TEM analyses were performed to compare the degradation of the sample prior to and after a 20 day incubation with LiP and H_2O_2. (Figure 3B) shows the non-degraded nanotubes on the initial day, but after 10 days, the degradation of the carboxylated nanotubes by LiP with H_2O_2 is observed; this degradation is indicated by the reduced lengths of the carbon nanotubes. When compared with the initial stage, the

10 day and 20 day samples showed the presence of non-tubular structures, indicating the degradation of the carbon nanotubes; however, it was unable to discern complete oxidation at day 20, which would be indicated by the presence of the intermediates of carbonaceous products.

To observe the longevity of the enzyme, a microtiter plate assay was performed using pyrogallol as a substrate for the LiP. This assay was performed for each sample after the 20 day incubation, and the results showed that enzyme activity was retained.

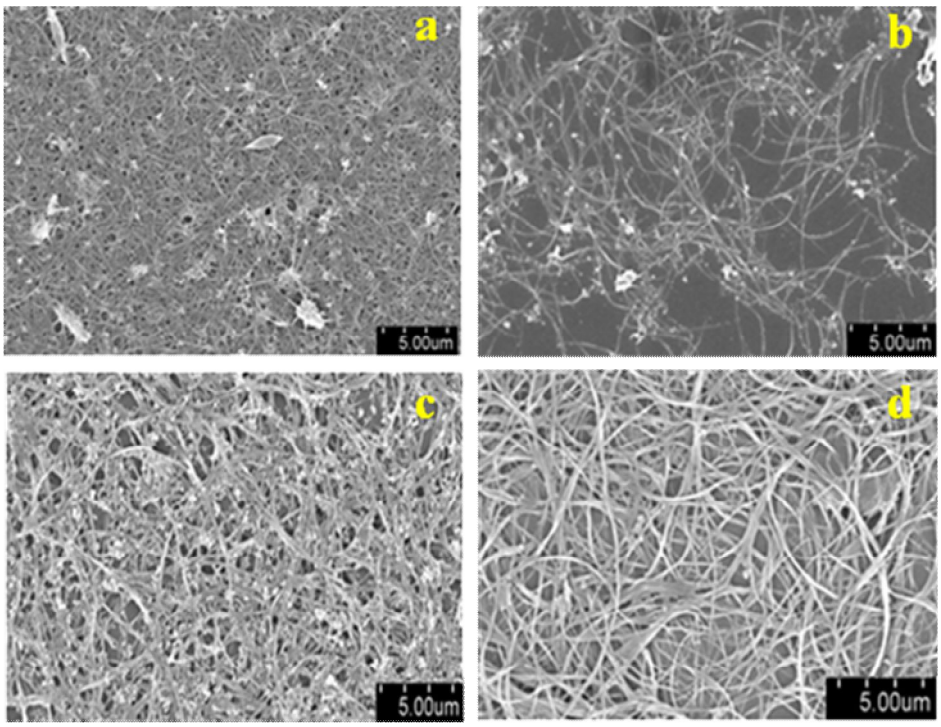


Figure 3.2 SEM micrographs for the carboxylation of SWNTs. (A) ASA SWNTs pristine (B) ASA SWNTs carboxylated which shows the gradual decrease in the metal catalyst and impurities; (C) AST SWNTs pristine (D) Thermally treated AST purified SWNTs carboxylated which shows the gradual decrease in the metal catalyst and impurities.

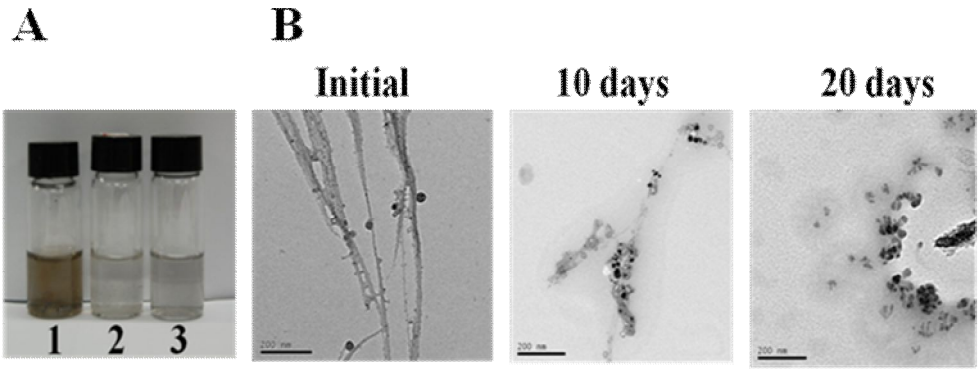


Figure 3.3 Photography and TEM analysis of enzymatic degradation (A) The photographs represent the enzymatic degradation of the carboxylated SWNTs; vial 1, carboxylated; vial 2, after 10 days of incubation with HRP; vial 3, after 10 days of incubation with *S. crispa* LiP. (B) TEM micrographs confirming the degradation of the carboxylated SWNTs.

3.3.3 Spectroscopic analysis

The visible near infrared (vis-NIR) and Raman spectroscopy methods can be used to monitor the degradation of carbon nanotubes (Allen et al., 2009). The degradation of SWCNTs composed of both ASA and AST (SWNTs) were monitored using a vis-NIR spectroscopy method after a 5 day incubation with the partially purified LiP and 40 μm H_2O_2 (aq). Non-degraded carboxylated carbon nanotubes are mixtures of various diameters and helicities, exhibiting metallic and semiconducting electronic properties. (Figure 3.4) shows the spectral range of the M_1 metallic band between 650 and 750 nm, as well as the broad S_2 semiconducting band of the carbon nanotubes absorbing between 1,000 and 1,100 nm. By monitoring these bands over the 5 day incubation period with partially purified LiP and H_2O_2 at 25°C, the M_1 and S_2 band of the carbon nanotubes decreased. The graphitic structure of the carbon nanotubes diminished and completely disappeared as a result of the enzymatic degradation during the 10 day incubation period (Figure 3.4 A,B). Also, observed these results on the 20th day to confirm this phenomenon, indicating similar results for both types of SWNTs. Furthermore, when the incubation of the carbon nanotubes with crude lignin peroxidase and H_2O_2 took place at 4°C, the degradation process slowed and required 60 days. This result indicated that at 25°C, the degradation was faster than at 4°C. The results are similar to those of the horseradish peroxidase reaction that serves as a control. Furthermore, the degradation was also confirmed by Raman spectroscopy, and the results showed that at 4°C, the carboxylated carbon nanotubes display both a G and a D band. After 60 days incubation with crude lignin peroxidase, these bands were decreased, indicating the degradation of the carbon nanotubes (Figure 3.5).

Temperature has a considerable effect on the ability of the HRP to degrade carbon nano tubes (Allen et al., 2009). Because approximately 60 days at a lower

temperature are required to achieve the same level of degradation, this demonstrates that carbon nanotubes degradation was slower compared with the degradation at 25°C. This result is also evident in the observation that the rates of degradation generally increase with increasing temperature. Furthermore, Raman spectroscopy showed that the D and G bands disappeared after the incubation with HRP. When the samples were incubated with LiP, however, the bands did not completely disappear; due to variations in the samples, some fluctuations in the D:G ratios were observed that were consistent with another study (Kotchey et al., 2011). However, this result indicated the LiP reduced the D and G bands. The result indicated that degradation does occur but that the rate is low compared with the partially purified samples (data not shown). Clearly, the crude LiP was sufficient to initiate and promote the degradation process. The results are consistent with other reported studies of the enzymatic degradation by crude manganese peroxidase of polyaromatic hydrocarbons (Kwon et al., 2008). Therefore, the results suggest that the crude or partially purified LiP from *S. crispa* fruiting bodies is able to catalyze the oxidation of both carboxylated raw grade and thermally treated single-walled carbon nanotubes. Therefore, the crude fruiting bodies of *S. crispa* can potentially be used in most applications of nanomaterial bioremediation. *S. crispa* crude extracts could be a highly efficient and low cost source of the materials for environmental remediation. Furthermore, the results are consistent with other studies including those that show that crude purification of the enzyme is sufficient to degrade organic pollutants. Crude MnP was sufficient to initiate and promote the degradation of anthracene and pyrene (Eibes et al., 2006). Similarly, another study reports that crude LiP from white rot fungus *P. chrysosporium* can degrade pharmaceutically active compounds such as carbamazepine and diclofenac (Zhang and Geiβen, 2010).

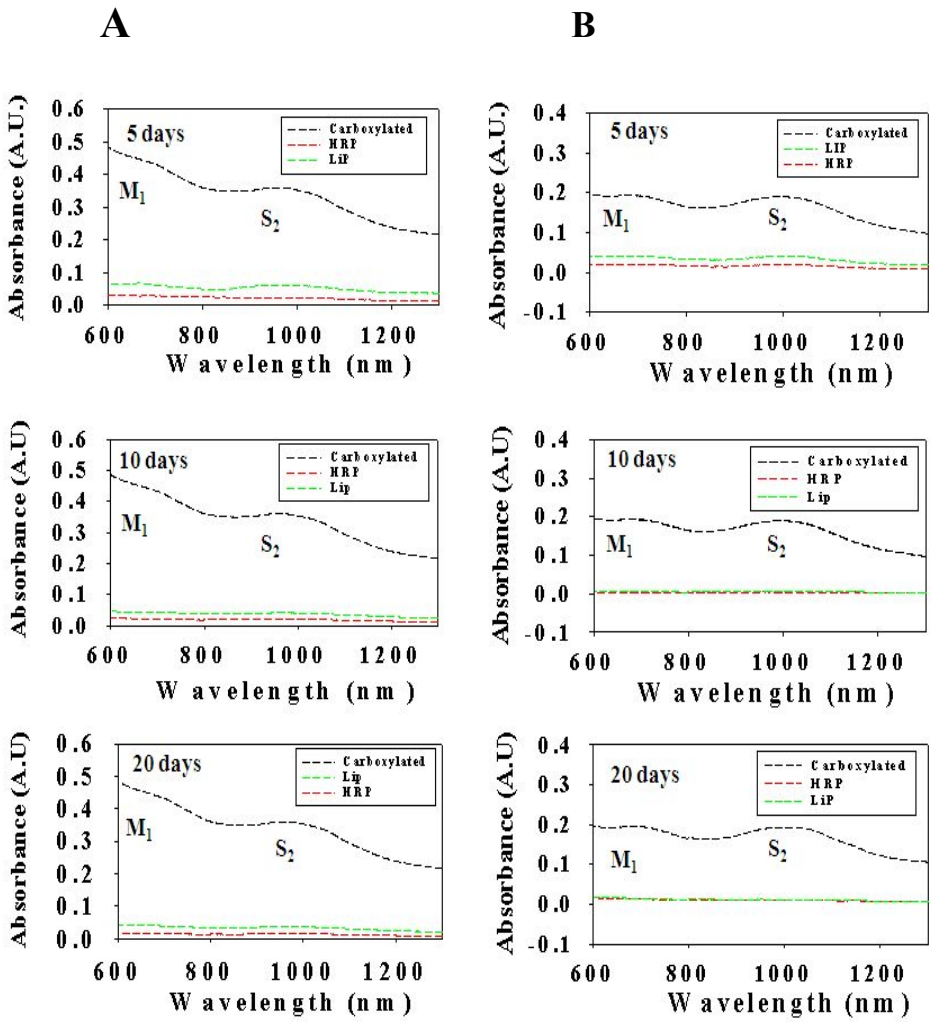


Figure 3.4 Vis-NIR spectroscopic analysis (A). Carboxylated ASA (SWNTs) degraded by HRP and *S. crispa* lignin peroxidase after 5, 10 and 20 days at 25°C. (B). Carboxylated AST (SWNTs) degraded by HRP and *S. crispa* LiP after 5, 10 and 20 days at 25°C.

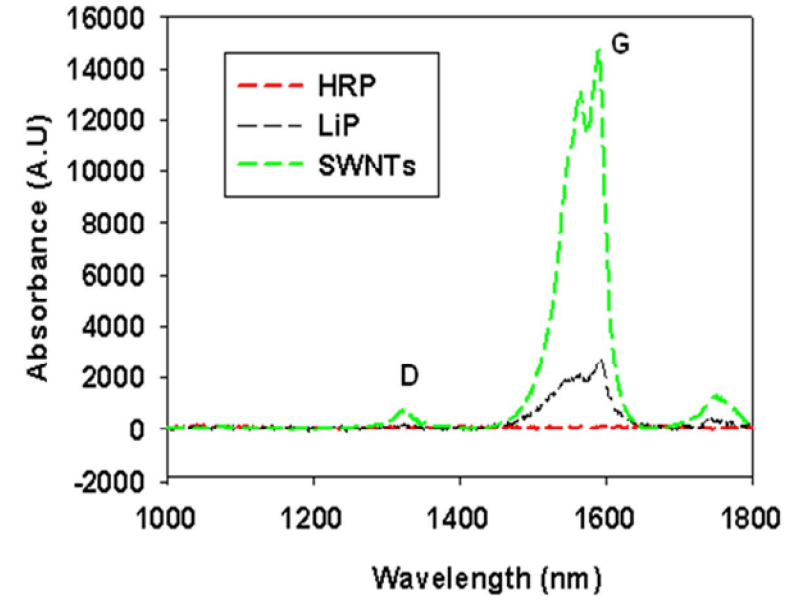


Figure 3.5 Raman spectra of the degraded carboxylated AST (SWNTs) after 60 days incubation at 25°C with (black) *S. crispa* crude LiP, (red) HRP, and (green) buffer alone.

3.3.4 Electron spin spectroscopy

Electron spin–resonance measurements of the LiP and HRP activity in the presence of the carbon nanotubes was used to detect the radicals formed during the one-electron oxidation of pyrogallol and ascorbate compounds by LiP and HRP, respectively. The addition of H_2O_2 to LiP in the presence of pyrogallol produced the characteristic EPR signal of a pyrogallol oxygen radical (pyrogallol-O*). The free radical spectrum is characterized by a signal at g=2.00 (Figure 3.6A, 1, 2). The signal of the ascorbate radical was detected upon the incubation of HRP with ascorbate in the presence of H_2O_2 (Figure 3.6B, 1, 2). The addition of the nanotubes to the incubation mixture did not change the EPR signals of the ascorbate and pyrogallol radical (Figure 3.6A and B, 2). In the absence of LiP and H_2O_2 or HRP and H_2O_2, the free radical signal was several-fold lower, thus confirming that the oxidation of pyrogallol and ascorbate occurred mainly via the peroxidase reaction (Figure 3.6A and B, 3, 4). The spectra were identical in the presence and in the absence of nanotubes.

In this study, LiP produces free radical signals from the substrate in the presence of H_2O_2 similarly to that produced by HRP. Therefore, both enzymes produced strong signals from the respective peroxidase substrates, thus demonstrating that carbon nanotubes did not inactivate the enzymes. Other studies reported that ESR spectroscopy signals showed that cation radicals were produced during the degradation of organopollutants, which proves the cation radical oxidizes these compounds (Hammel et al., 1986).

The catalytic cycle of LiP is similar to that of other peroxidase wherein ferric enzyme is first oxidized by H_2O_2 to generate the two-electron oxidized intermediate, Compound 1 (Koduri and Tien, 1995). The degradation mechanism occurs through a classical compound I spectrum, similar to that described for other peroxidases and

indicative of the formation of a porphyrin π cation radical. (Doyle et al., 1998). Generally, Compound I contains two oxidizing equivalents; one is stored in the enzyme as an oxoferryl moiety [Fe (IV)=0]$^{2+}$ and the other (OH) as a radical. It is interesting that in almost all the peroxidases and other heme-containing proteins that react with H_2O_2, the radical is a π-orbital delocalized porphyrin radical. Moreover, there is a report that porphyrins physisorb onto SWNTs providing close proximal contact with the iron site, which further promotes degradation (Allen et al., 2009). The enzymatic breakdown of the raw grade and thermally treated SWCNTs is proposed to occur through a mechanism of action that includes the generation of an aryl cation radical by reaction with H_2O_2. It has been proven that the biodegradation of carbon nanotubes is induced by free radicals which aid in the oxidation of single-walled nanotubes (Vlasova et al., 2011)

Alternatively, there is a report on the induction of extracellular hydroxyl radical production in white rot fungi through quinone redox cycling. This mechanism can be explained by the production of OH radicals from the generation of Fe^{2+} and H_2O_2 (the Fenton reaction). Like the white rot fungi, brown rot fungi can also able to produce OH radicals during the deploymerization of cellulose. *S. crispa* is a brown rot fungi (Woodward et al., 1993). However, the production of reactive oxygen species in the extracellular environment in both white and brown rot fungi is evidence of quinine redox cycling. This was explained as a simple strategy for the white rot fungi to induce extracellular hydroxyl radical production (Cromex-Toribio et al., 2009; Gómez-Toribio et al., 2009).

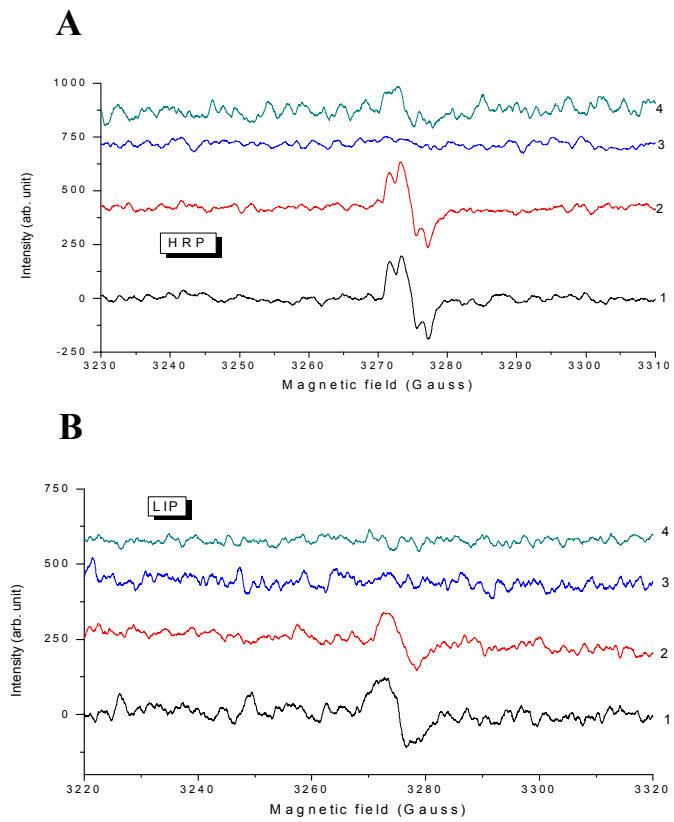


Figure 3.6 The ESR spectra characterizing the peroxidase activity of *S. crispa* LiP and HRP in the presence and absence of single-walled carbon nanotubes. (A). The radicals produced by the LiP with ascorbate as the substrate. (1) HRP and H_2O_2, (2) Nanotubes, HRP and H_2O_2, (3) Nanotubes and H_2O_2, and (4) Nanotubes and HRP. (B). The radicals produced by the *S. crispa* LiP with pyrogallol as the substrate. (1) LiP and H_2O_2, (2) Nanotubes, lignin peroxidase and H_2O_2, (3) Nanotubes and H_2O_2, and (4) Nanotubes and lignin peroxidase.

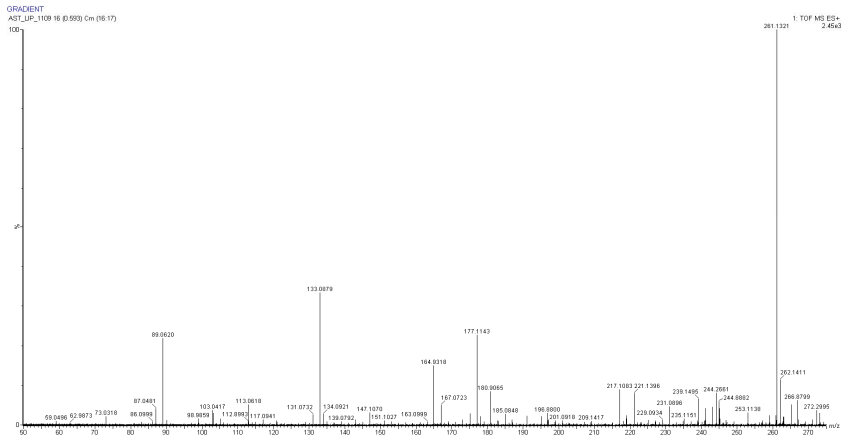
3.3.5 Liquid-chromatography-mass spectrometry

To identify the final products of the degradation of the ASA and AST carbon nanotubes by LiP and HRP, LC-MS was performed, with monitoring for negative ions with a positive detector (Figure 3.7, A, B). The results revealed that the major degraded products of both ASA and AST (SWNTs) are acid and aldehydes. For example, in this study we found salicylic acid as the degradation product; this identification was also confirmed later with an authentic standard. Thus, these compounds are within a class of compounds similar to the identified horseradish peroxidase biodegradation products of the SWCNTs. However, the entire spectrum of the products was likely impossible to identify or interpret.

As shown in Figure 3.7 (A), the mass to charge (m/z) value, 136.93, was observed for the LiP-degraded SWNTs, indicative of salicylic acid. Moreover, the product identified was similar to those observed in the studies of the bioremediation of polyaromatic hydrocarbons (PAHs) (Bamforth and Singleton, 2005). The molecular masses of the degraded products were very similar in the studies of the HRP- and LiP-treated carbon nanotubes. This indicated that the enzymes perform a similar cleavage of the carbon nanotubes. However, it is important to note that mass spectrometry cannot distinguish between molecular ions and fragments, and the preparative scale experiments involving product separation still must be performed. Furthermore, the LiP purified from *Gloeophyllum sepiarium* MTCC-1170, which degrades coal humic acid, and the mechanism it uses involves the generation of an aryl cation radical during the reaction with H_2O_2 (Yadav et al., 2009). It has been proven that *P. chrysosporium* oxidizes PAH compounds through a radical mechanism. Horseradish peroxidase also degrades the PAHs through cation radical intermediates. The first enzyme shown to attack lignin-type compounds was the LiP isolated from *P. chrysosporium*. Their

lignolytic capacity makes most taxa of fungi of interest for use in bioremediation. Pollutants such as chlorophenols, nitrophenols and polyaromatic hydrocarbons can be transformed by the lignolytic enzymes because of the free radical reactions (Gadd, 2001).

A



B

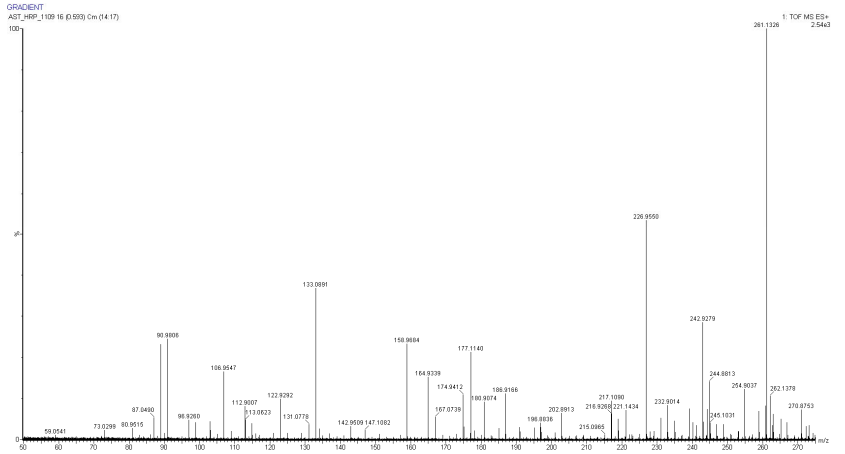


Figure 3.7 The LC-MS spectra of degraded (SWNTs) (A). AST degraded by *S. crispa* LiP. (B) AST degraded by HRP.

3.3.6 Gas chromatography (GC)

GC was used to analyze the final degradation product of the carbon nanotubes. Furthermore, the evolution of CO_2 gas in the sample headspace on the10th day of incubation was monitored and also compared the degradation process of the carboxylated ASA and AST nanotubes incubated with LiP and H_2O_2 or with HRP and H_2O_2 (Figure 3.8). Carbon nanotubes (both AST and ALT) in the presence of H_2O_2 did not produce any significant concentration of CO_2 in the sample headspace over the course of 10 days. In contrast, when carboxylated ASA and AST nanotubes were incubated with LiP and H_2O_2 and HRP and H_2O_2, CO_2 was measured in the sample headspace and was evidence of the degradation of the carbon nanotubes. It was observed that the AST carbon nanotubes produced more CO_2 than the ASA carbon nanotubes. Furthermore, the ASA carbon nanotubes incubated with HRP and H_2O_2 exhibited a higher CO_2 concentration compared to those incubated with the LiP and H_2O_2.

Finally, the product analysis indicates that complete degradation produces CO_2 gas. A previous study proposed that the lignolytic fungus *Pleurotus ostreatus* degrades phenanthrene, producing intermediate products such as 9,10-phenanthrenequinone and 2,2-diphenic acid before finally forming CO_2 (Bezalel et al., 1997). Therefore, the results were agreed with the other findings and proposed that most of the fungal degradation products of the organopollutants are oxidized to carboxylic acids and finally form CO_2 gas (Cajthaml et al., 2002). Furthermore, the degradation of several environmental pollutants to CO_2 by the white rot fungus has been already reported.

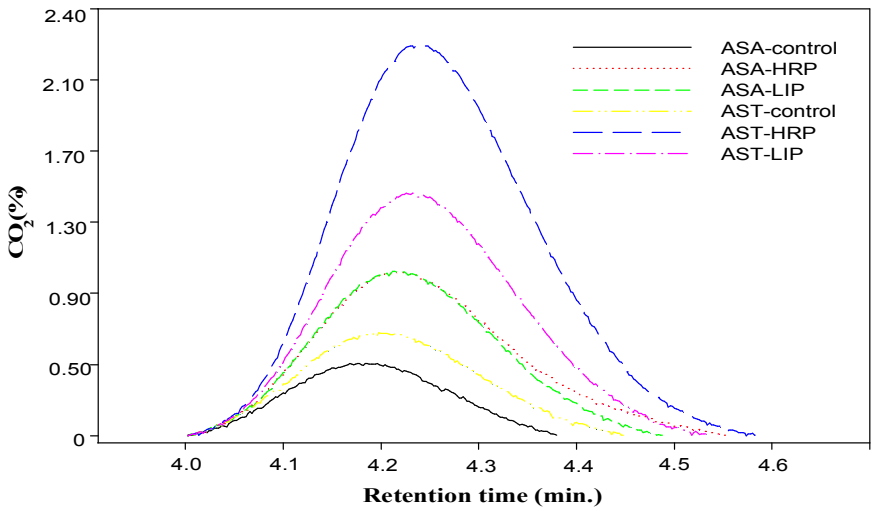


Figure 3.8 GC spectra represents the percentage change of CO_2 in a vial which contains the carboxylated carbon nanotubes as control and after 10 days the percent CO_2 levels was increased both ASA and AST.

3.3.7 Enzyme production under the nitrogen-limited growth of S. crispa mushroom

An attempt has been made for large-scale enzyme production using nitrogen-limited medium compositions. As expected, the higher LiP enzyme activity resulted from the cultivation of *S. crispa* mushrooms in nitrogen-limited growth conditions; the results are similar those observed with various wood-rotting basidiomycetes that produce lignocellulolytic enzymes under the described nutrition conditions.

3.4 Summary

LiP isolated from the *S. crispa* mushroom catalyzed the oxidation of raw grade and thermally treated SWNTs. This study proposes that LiP employs a mechanism based on the formation of cation radicals for its enzymatic action. Therefore, the partially purified enzyme from the *S. crispa* mushroom can economically perform the bioremediation and biodegradation of single walled carbon nanotubes. All the results suggest that low concentrations of H_2O_2 and partially purified enzyme solution facilitate the degradation.

Chapter 4. Purification and characterization of an alkaliphilic esterase from a culinary medicinal mushroom *Sparassis crispa*

4.1 Introduction

Esterases (EC 3.1.1.1) catalyze the hydrolysis and synthesis of ester compounds. They are ubiquitous and usually prefer to hydrolyze the short chain fatty acid, while lipase hydrolyzes triglycerides of long chain acyl groups (Verger, 1997). For instance, esterases were applied to enhance flavor in the cheese making process (Choi and Lee, 2001) and to develop aroma from *Pseudomonas fragi* (Kermash et al., 2000). It has been reported that pentylferulate ester, a flavor precursor in cosmetics, was prepared in water-in-oil emulsions by a fungal esterase (Giuliani et al., 2001). For example, the characterization of esterase activity from three mushroom species, *Amanita vaginata* var. *vaginata*, *Tricholoma terreum*, and *Lycoperdon perlatum*, has been reported (Colak et al., 2009; Ertunga et al., 2009).

We have isolated and partially purified the esterase from six different strains of *S. crispa.* In addition, comparisons of this esterase's physicochemical characteristics with other fungal esterases are discussed in this study.

4.2 Materials and methods

4.2.1 Materials and chemicals

The reagents used were of analytical grade. All the *p*-nitrophenyl ester substrates, tributyrin and β-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was purchased from Biosesang, Inc. (Korea). DEAE–Sepharose Fast Flow was obtained from Amersham biosciences (Sweden). PD-10 desalting column were acquired from Amersham Biosciences (Sweden). *S. crispa*

mushroom species were collected from Jeonnam, South Korea and Japan.

4.2.2 Enzyme extraction

The fresh fruiting body of the mushroom was isolated and freeze-dried. Crude enzyme extracts were prepared as reported previously with some modification (Ertunga et al., 2009). Each frozen fresh fruiting body (50 g) of the mushroom was ground to a fine powder in liquid nitrogen using a pre-chilled ceramic mortar pestle. Then, the mushroom samples were separately extracted in acetate buffer (pH 5.0), phosphate buffer (pH 7.0) and Tris–HCl buffer (pH 9.0) containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. The concentration of all the buffers was 10 mM. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. The resulting supernatant was used as crude enzyme extracts.

4.2.3 Purification procedures

The enzyme was purified from the crude supernatant by three steps: 1) ammonium sulfate precipitation, 2) DEAE–Sepharose anion exchange chromatography, and 3) gel filtration chromatography. The crude extracts were precipitated with (65% saturation) ammonium sulfate. Then the solution was centrifuged at (8000 rpm, 15 min, and 4°C), and the precipitate was dissolved in a Tris–HCl buffer (10 mM, pH 9.0) and dialyzed in a PD-10 desalting column with the same buffer. The filtered enzyme solution was subjected to a DEAE–anion exchange column (5 ml) which was equilibrated with a Tris–HCl buffer (100 mM, pH 9.0) containing 0.15 M NaCl and then eluted with a linear salt gradient at a flow rate of 0.5 ml/min. The fractions with a high esterase specific activity were collected, concentrated and loaded in the gel filtration Sephadex G–75 column for further purification.

4.2.4 Enzyme assay

Esterase activity was assayed by measuring the amount of *p*-nitrophenol released by the method (Lee et al., 1999). Activity was measured using *p*-nitrophenyl esters as substrates. The substrate mixture contained 10 mM *p*-nitrophenyl acetate, ethanol and Tris–HCl buffer (10 mM, pH 9.0) in the ratio of 1:4:95 (v/v/v), respectively. The reaction was monitored spectrophotometrically at 405 nm for the release of *p*-nitrophenol. The non-enzymatic reaction was measured using a substrate solution without enzyme as a control. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μmol of *p*-nitrophenol per min under standard assay conditions.

4.2.5 Electrophoresis and zymographic analysis

In order to determine the molecular mass of the esterase, SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 10% polyacrylamide gel according to the accepted method (Laemmli, 1970). A high range of molecular weight size marker (EBM-1031, Elpis Biotech Inc.) was used as standard. For each step, the proteins were quantified by the BCA method using BSA as standard (Smith et al., 1985). Zymographic analysis using tributyrin agar plates was performed to detect the hydrolytic activity of the protein on the SDS–PAGE gel (Park et al., 2007). After running the gel, it was then washed with a mixture containing Tris–HCl Buffer (10 mM, pH 9.0) and 1% Triton X-100. Subsequently, the gel was placed on the TBN (Tributyrin) agar plate containing (1% TBN, 20 mM NaCl, 1 mM $CaCl_2$ and 0.5% gum arabic) and incubated at 37 °C.

4.2.6 Substrate specificity and kinetic study

The substrate specificity was analyzed by the release of *p*-nitrophenol from various *p*-nitrophenyl esters. A series of different chain length fatty acids nitrophenyl

esters, like *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl hexonate, *p*-nitrophenyl caprylate (*p*NPC), *p*-nitrophenyl palmitate (*p*NPP) and *p*-nitrophenyl stearate, were prepared at 10 mM concentrations. The esterase activity was assayed using the standard method described previously (Lee et al., 2005). The K_m and V_{max} of esterase were assayed according to a Michaelis-Menten equation from Lineweaver-Burk plot.

4.2.7 Effects of pH and temperature on enzyme activity

Optimum pH of the esterase was determined by measuring the enzyme activity at a pH ranging from 3.0 to 10.0. The following buffers all at 10 mM were used for this assay: glycine–HCl buffer (pH 3.0), acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 and 7.0), Tris–HCl buffer (pH 8.0 and 9.0) and glycine–NaOH buffer (pH 10.0). The pH stability was monitored using the same buffers and by incubating the mixture of enzyme and buffer for 24 h at 4°C. All the experiments were performed in triplicate, and the mean was taken for the statistical analysis (Faiz et al., 2007). In order to determine the optimum temperature, the enzyme activity was estimated at various temperatures over the range of 20-70°C, and their thermal stability was monitored at the same range of temperatures for 60 min with 10°C increments. All the temperature experiments were performed in triplicate, and the mean was taken for the statistical analysis (Faiz et al., 2007).

4.2.8 Effects of metal ions

In order to determine effects the metal on the enzyme activity, various metal ions such as Na^+, Fe^{2+}, K^+, Co^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Cd^{2+} and Cu^{2+} at 5mM concentration were added to the EDTA-treated enzyme solution and screened against the *p*-nitrophenyl acetate substrate. The residual activities were determined by the standard assay conditions (Choi et al., 2003).

4.3 Results and discussion

Recently, a report was published on the mycelium culture of edible mushrooms that secreted a ferulic acid esterase, which indicates applications for the functional foods and fermentation industries (Xie et al., 2010). We selected *S. crispa* as our source due to its high nutrition value and the presence of interesting novel components. This wild mushroom is mostly present on wood debris, and our idea is to utilize this mushroom for the production of esterase-based reactions and economical food processing. In order to perform a comparative study of this mushroom species, the six different strains of this mushroom were screened to find their biochemical properties. However, all the strains showed similar biochemical and physical properties. Therefore, in this paper, we have given the results from the one mushroom species. We have focused on the fruiting body of this mushroom due to its medicinal properties, such as antitumor, wound healing, anti-angiogenic properties and novel bioactive compounds which have been reported (Kawagishi et al., 2007; Kwon et al., 2009; Ohno et al., 2000; Park et al., 2009; Yamamoto et al., 2009).

4.3.1 Esterase purification

In the present study, the purified esterase was obtained by ammonium sulfate precipitation and chromatographic techniques. In the case of ammonium sulfate precipitation, the highest enzyme activity was present at 40-65% saturation. Next, the resulting fractions were purified by anion exchange chromatography. The anion exchange chromatography yielded two peaks on the bound fractions. The fraction with high specific activity of esterase was further purified by gel filtration chromatography. Subsequently, all the purification steps were confirmed by the enzyme assay and SDS–PAGE. The overall purification results are summarized in Table 4.1. The *S. crispa* esterase showed 73-fold purification and 17% yield recovery with a specific activity of

175 U/mg. Yield ratios and specific activity were similar in all six mushroom specimens (data not shown). The similar biochemical properties in the different strains prove that this mushroom is a reliable source and can be applicable for general use.

Table 4.1 Purification steps of the esterase from *S. crispa*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purity (fold)
Crude extract	842	2038	2.4	100	1
Ammonium sulfate	162	1035	6.3	50	3
DEAE–Sepharose	20	700	35	34	15
Sephadex G–75	2	350	175	17	73

4.3.2 Properties of the esterase

SDS–PAGE revealed that the molecular mass of the esterase was approximately 60 kDa (Figure 4.1a). Some fungal feruloyl esterases were reported with a molecular mass similar to the *S. crispa* esterase, such as *Fusarium oxysporum* (62 kDa), *Aspergillus niger* (63 kDa) and *Aspergillus oryzae* (61 kDa). The *S. crispa* esterase also was larger than the molecular mass of reported feruloyl esterases from *Sporotrichum thermophile* (23 kDa), *Aspergillus awamori* (35 kDa) and *F. oxysporum* (31 kDa), whereas it was smaller than the molecular mass of *A. awamori* (112 kDa) and *Aureobasidium pullulans* (210 kDa) (Koseki et al., 2009). In addition, the *S. crispa* esterase is similar to the molecular mass of the recombinant cocaine esterase from the *Rhodococcus* sp. MB1 (Bresler et al., 2000).

4.3.3 Zymogram analysis

Zymogram data revealed that the purified esterase could hydrolyze a tributyrin (TBN) substrate (Figure 4.1b). We carried out this experiment with our target protein and the other smaller molecular weight protein in order to find the esterase activity. We were able to see the hydrolyzing activity at 60 kDa on the gel, which was stained by the Coomassie brilliant blue R-250. This result proved that the enzyme is an esterase and revealed that no other hydrolyzing enzyme was present. At the same time, we could not see the hydrolysis activity at 25 kDa protein. To sum up, the zymographic results indicated that the protein retained the esterase activity, and the results were similar to the other study (Park et al., 2007; Kim and Park, 2002).

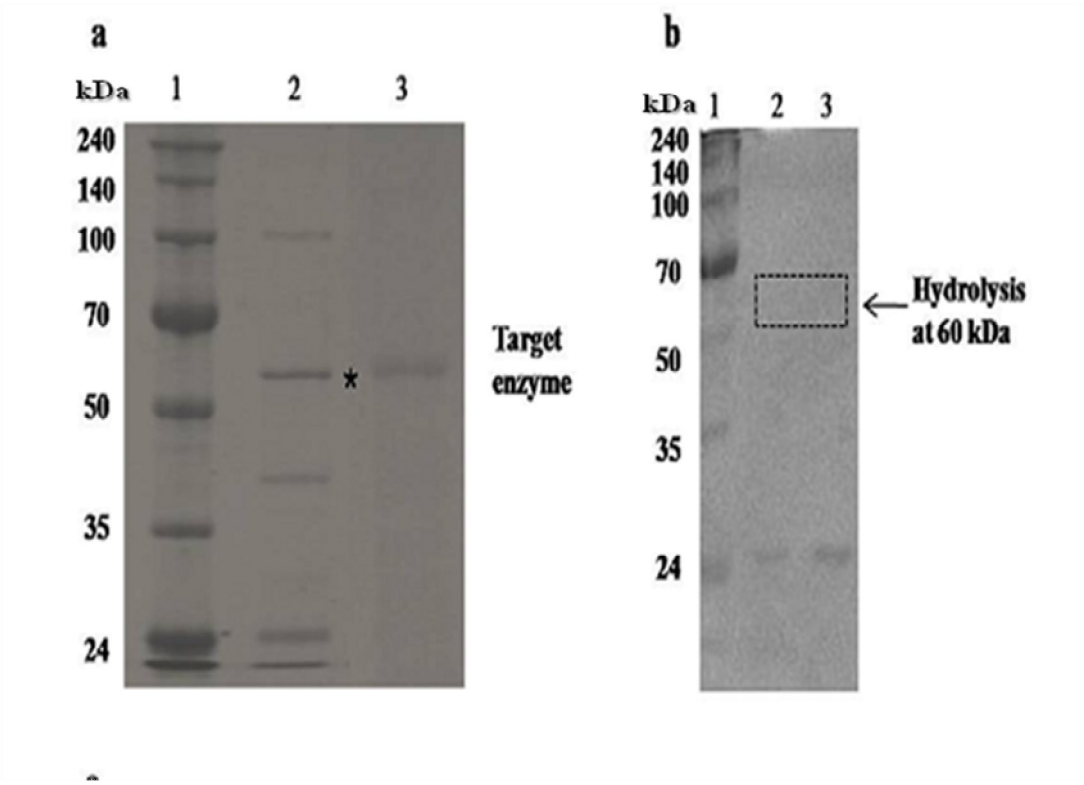


Figure 4.1 SDS–PAGE and Zymogram analysis of the esterase from the mushroom *S. crispa*. (a), Molecular mass of *S. crispa* esterase by SDS–PAGE: Lane 1, Marker; Lane 2, Esterase purified by DEAE–Sepharose column chromatography; Lane 3, Purified esterase by Sephadex G-75 (b), Zymogram-TBN after SDS–PAGE: Lane 1, Marker; Lane 2 and 3, hydrolytic activities at 60 kDa and no hydrolytic activity at 25 kda.

4.3.4 Substrate specificity and kinetics

The purified esterase was tested on various *p*-nitrophenyl esters with different chain lengths. The relative activity was calculated and shown in Table 4.2. The activity of the esterase decreased as the number of carbon atoms increased. The highest activity was observed with *p*-nitrophenyl acetate, whereas low or no considerable activity was observed with the long chain substrates. A similar result was obtained for a *Pseudomonas citronellolis* ATCC 13674 esterase, which shows affinity for *p*-nitrophenyl esters-C4 (Chao et al., 2003). The reported esterase from mushrooms species such as *Amanita vaginata* var. *vaginata*, *Tricholoma terreum* (Ertunga et al., 2009) and *Lycoperdon perlatum* (Colak et al., 2009) showed specificity towards short chain fatty acids such as *p*-nitrophenyl butyrate, *p*-nitrophenyl acetate. Apparently, this specificity study suggests that the enzyme is an esterase not a lipase. The K_m and V_{max} values of the *S. crispa* esterase acting on *p*-nitrophenyl acetate were 200 μM and 0.5 U/mg of protein, respectively (Figure 4.2).

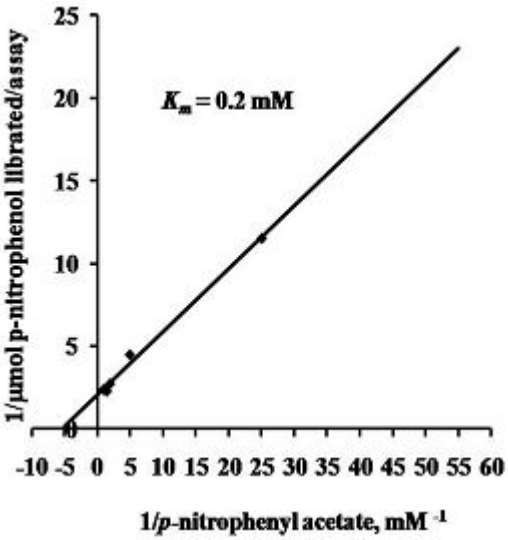


Figure 4.2 Determination of K_m and V_{max} by a Lineweaver–Burk plot for the *S. crispa* esterase enzyme with *p*-nitrophenyl acetate as a substrate.

Table 4.2 Effects on different substrate specificities

Substrate	Relative activity (%)
p-nitrophenyl acetate	100
p-nitrophenyl butyrate	50
p-nitrophenyl hexonate	45
p-nitrophenyl deconate	0
p-nitrophenyl palmitate	0
p-nitrophenyl stearate	0

4.3.5 Optimum pH and stability properties

The optimum pH for the *S. crispa* esterase was 8.0 in Tris–HCl buffer (Figure 4.4a). However, the activity was decreased in acidic pH (4.0-6.0) reflects the catalytic nature of enzyme including active site groups.

The pH stability of *S. crispa* esterase was determined by monitoring the residual activity after 24 h incubation at different pH values. The results showed that the enzyme was more stable over a broad range of pH (7.0-10.0) when compared to pH 6.0 (Figure 4.4b). Our results are similar to the fungal feruloyl esterases from fungi, such as *Sporotrichum thermophile* (pH 6.0-10.0) and *A. oryzae* (pH 7.0-10.0) (Koseki et al., 2009). Our results were compared with other mushroom esterases from *A. vaginata* var. *vaginata*, *T. terreum* (Ertunga et al., 2009) and *L. perlatum* (Colak et al., 2009), as shown in the Table 4.3. Therefore, the enzyme stability in acidic and basic condition may be an advantage in industrial applications. All the experiments were performed in triplicate, and the mean was taken for the statistical analysis.

4.3.6 Optimum temperature and stability properties

The *S. crispa* esterase has an optimum temperature of 50°C (Figure 4.3c). This is similar to other esterases from *A. oryzae, A. niger and Penicillium chrysogenum* with optimum temperatures at 50°C (Koseki et al., 2009). Its optimum temperature is higher than the esterase from *Cucurbita pepo* Elc and EII with an optimum temperature between 30 and 40°C (Fahmy et al., 2008), *Lactobacillus casei* subsp. *casei* (Lee and Lee, 1990) and *Lactobacillus fermentum* (Gobbetti et al., 1997) at 30-35°C, and is lower than the esterase from *A. fatua* (Mohamed et al., 2000) at 65-75°C. According to the temperature stability profile, this esterase was most stable at temperatures between 20-30°C. However, the 50% enzyme activity remained at 40°C for 60 min. Therefore, the enzyme was stable at 30-40°C. The residual activity of the enzyme was up to 30%

for 60 min at 50°C, while the activity sharply decreased as the temperature increased over 70°C (Figure 4.4d). Our results are similar to a fungal feruloyl esterase from *F. oxysporum*, which is stable at 30°C (Koseki et al., 2009). It was also reported that some fungal feruloyl esterases, such as *A. oryzea* and *S. thermophile,* show stability at 60°C (Koseki et al., 2009). If our results are compared with other mushrooms, such as *A. vaginata* var. *vaginata*, *T. terreum,* then the data is consistent: all are stable at 30 and 40°C (Ertunga et al., 2009). This is in contrast with the mushroom *L. perlatum* that shows stability at 20°C (Table 4.3) (Colak et al., 2009).

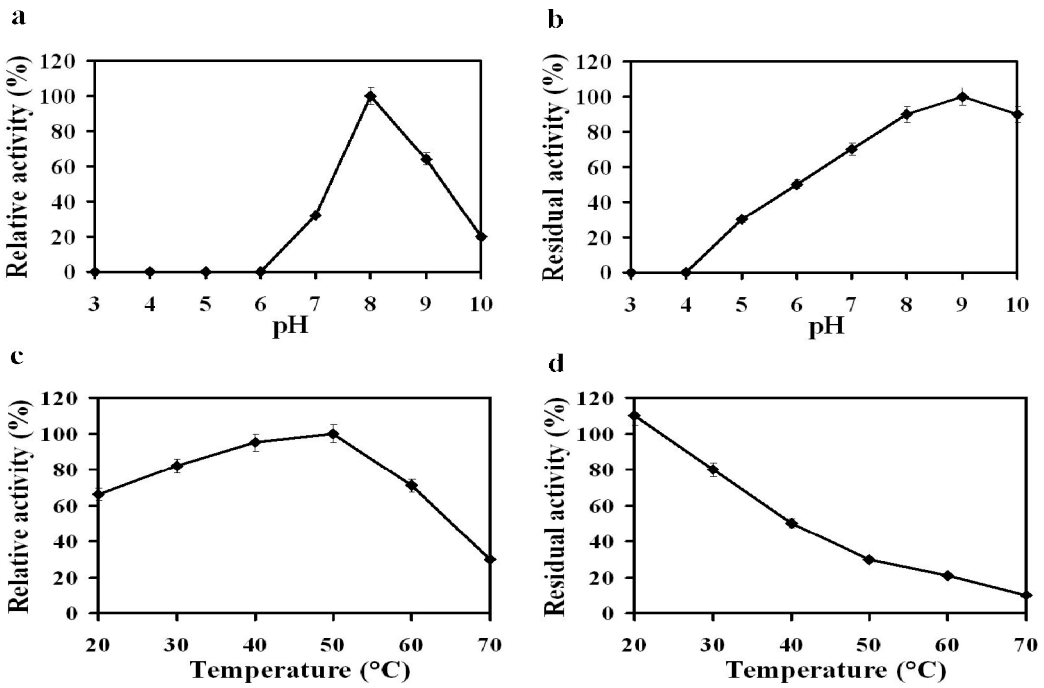


Figure 4.3 Effects of pH and temperature on the *S. crispa* esterase. (a) The activity was measured at various pH values and presented as a percentage of the maximum activity. (b) The pH stability profile of *S. crispa* esterase. The enzyme was incubated in various buffers with a pH range from 3.0-10.0 for 24 h at 4°C. The reaction mixture contained the enzyme and the *p*-nitrophenyl acetate substrate were assayed by standard assay conditions, and the residual activity was calculated. (c) Effects of temperature on esterolytic activity of *S. crispa.* The reaction mixture containing the enzyme and *p*-nitrophenyl acetate was incubated at various temperatures for 10 min. The esterase activity was measured by standard assay conditions. (d) Thermal stability profile for the *S. crispa* esterase. The enzyme solution was incubated in a water bath in a temperature range of 20-70°C with 10°C increments for 60 min. The percentage residual activities were calculated by comparing with unincubated enzyme.

Table 4.3 Comparison of the properties with reported mushroom esterases

Mushroom species	Substrate	Relative activity (%)	pH_{opt}	T_{opt} (°C)	pH_{sta}	T_{sta} (°C)	Reference
Sparassis crispa	*p*-nitrophenyl acetate	100	8.0	30	7.0-10.0	30-40	This study
Lycoperdon perlatum	*p*-nitrophenyl acetate	100	8.0	40	5.0-8.0	20	Colak et al., 2009
Amanita varianta var.*vaginata*	*p*-nitrophenyl butyrate	100	8.0	30	3.0-10.0	30-40	Ertunga et al., 2009
Tricholoma terreum	*p*-nitrophenyl butyrate	100	8.0	30	3.0-10.0	30-40	Ertunga et al., 2009

4.3.7 Effect of metal ions

Various metal ions at 5 mM concentration were tested on *S. crispa* esterase. Among them metal ions such as Zn^{2+}, Co^{2+}, K^{+} reduced the esterase activity more than 40%. The overall metal ions study revealed that esterase activity had inhibitory effect to certain degrees but not completely except Ca^{2+} ions which maintained enzyme activity (Table 4.4). When we compared our results with mushroom esterases in case of *L. perlatum* mushroom the addition of Ca^{2+} ion shows an stimulatory effect (Colak et al., 2009) whereas the Ca^{2+} ion inhibits the esterase activity in the mushroom *A. vaginata* var. *vaginata*, *T. terreum* (Ertunga et al., 2009). So, enzyme enhancing or decreasing activity may be attributed on the metal ions property towards protein as ligand (Colak et al., 2009).

Table 4.4 Effects of various metal ions on the *S. crispa* esterase

Metal ion	Residual activity (%)
Control (EDTA)	100
Cu^{2+}	87
Ca^{2+}	100
Cd^{2+}	84
Mn^{2+}	92
Na^{+}	82
Fe^{2+}	76
K^{+}	57
Co^{2+}	54
Zn^{2+}	35

4.4 Summary

Also, the fruiting body of this medicinal mushroom, *S. crispa*, produced an esterase with high specificity towards short-chain esters, a moderate thermostability and alkaliphilic properties. To summarise, a new alkaline esterase purified from the fruiting bodies of the *S. crispa* mushroom could potentially be used as a biocatalyst in the food, chemical and pharmaceutical industries.

Chapter 5. Antifungal and antibacterial activities of lectin from *Sparassis crispa*

5.1 Introduction

Lectins are proteins or glycoproteins of non-immune origin which is widely distributed in animals, plants, microorganism, vegetables, fruits, beans and mushrooms. Some of the lectins found in higher mushrooms *Sclerotium rolfsii, Volvariella volvacea, Ganoderma capense, Tricholoma mongolium, Pleurotus ostreatus, Agrocybe cylindracea, Schizophyllum commune, Armillaria luteo-virens, Agarics blazei, Grifola frondosa, Pholiota aurivella, Hericium erinaceum, Ganoderma lucidum, Pleurotus ostreatus, and Mycoleptodonoides aitchisonii.* There is a report that mushroom lectin manifest different action including antiproliferative and antitumour properties (Zhang et al., 2009). In addition, some lectins including mushroom lectins express other potential activities such as immunoenhancing, vasorelaxing, hypotensive, and antimicrobial activities (Gozia et al., 1993; Verheyden et al., 1995; Wang et al., 1998). These clearly indicate that mushroom lectins might be employed as drugs or therapeutic reagents for pharmaceutics; mushrooms have now become a valuable source of lectins for drug discovery.

In recent years, mushroom lectins have become of more interest, mainly due to the discovery of some of these lectins exhibiting potent biological activities (Mo et al., 2000), for example, *Agaricus bisporus* lectin shows its antiproliferation activity against human colon cancer cell lines HT29 and breast cancer cell lines (MCF-7) (Yu et al., 1999). *Volvariella volvacea* lectin possesses antitumor activity to sarcoma S-180 cells (Wang, et al., 1998). *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells *in vitro* and sarcoma S-180 cells *in vivo* (Wang et al., 1995). In this study *S. crispa*

mushroom fruiting bodies lectin was isolated and purified. Also, their antibacterial and antifungal activities were determined against bacteria and fungal strains including *Fusarium oxysporum* and *Fusarium solani*. CD spectroscopy assay and Trp blue shift assay showed that lectin had interaction with microbial surfaces.

5.2 Materials and Methods

5.2.1 Protein estimation

The protein concentration was estimated with the method described by BCA, bovine serum albumin was used as a standard. Absorbance at 280 nm was also measured.

5.2.2 Hemagglutination assay (HA)

Hemagglutinating activity was assayed in 96-well microtiter plates in a final volume of 100 µl containing 50 µl of a 2% suspension of erythrocytes of human and/or animals previously washed three times in saline solution, and 50 µl of a two –fold serial dilution of lectin solution. Agglutination was scored after 1 h at 37°C as the reciprocal of the highest lectin dilution giving detectable agglutination. HA (titre), the reciprocal of the highest dilution of the sample promoting full agglutination of erythrocytes, was defined as one hemagglutination unit. Specificity HA was defined as the ratio between the titre and protein concentration (unit mg^{-1}) (Charungchitrack et al., 2011).

5.2.3 Microorganism

Bacterial and fungal cultures used in this present study were obtained from the Korean Collection for Type Cultures (KCTC). Gram negative species were *Escherichia coli* (KCTC 1682), *Salmonella typhymurium* (KCTC 1926) and *Pseudomonas aerugenosa* (KCTC 1637) while the Gram positive species were *Staphylococcus aureus* (KCTC 1621), *Bacillus subtilis* (KCTC 1918), and *Listeria monocytogenes* (KCTC

3710). The fungal strains were *Candida albicans* (KCTC 7270); *Candida catenulate*, (7642); *Candida glabrata*, (7219); *Candida rugosa*, (7324). Antibiotic resistant *E. coli* strains (CCARM 1229 and CCARM 1238), *S. aureus* strains (CCARM 3108 and CCARM 3089), and *C. albicans* (CCARM 14001 and CCARM 14007) were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University in Korea.

5.2.4 Extraction and purification of lectin

The fresh fruiting body of the mushroom was isolated and freeze-dried. Crude enzyme extracts were prepared as reported previously with some modification (Ertunga et al., 2009). Each frozen fresh fruiting body (50 g) of the mushroom was ground to a fine powder in liquid nitrogen using a pre-chilled ceramic mortar pestle. Then, the mushroom samples were separately extracted in acetate buffer (pH 5.0), phosphate buffer (pH 7.0) and Tris–HCl buffer (pH 9.0) containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. The concentration of all the buffers was 10 mM. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. The resulting supernatant was used as crude extracts.

The lectin was purified from the crude supernatant by three steps: 1) ammonium sulfate precipitation, 2) DEAE–Sepharose anion exchange chromatography, and 3) gel filtration chromatography. The crude extracts were precipitated with (65% saturation) ammonium sulfate. Then the solution was centrifuged at (8000 rpm, 15 min, and 4°C), and the precipitate was dissolved in a Tris–HCl buffer (10 mM, pH 9.0) and dialyzed in a PD-10 desalting column with the same buffer. The filtered enzyme solution was subjected to a DEAE–anion exchange column (5 ml) which was equilibrated with a Tris–HCl buffer (100 mM, pH 9.0) containing 0.15 M NaCl and then eluted with a linear salt gradient at a flow rate of 0.5 ml/min. The fractions with a

high lectin activity were collected, concentrated and loaded in the gel filtration Sephadex G–75 column for further purification.

5.2.5 Antibacterial activity

Bacterial cells were cultured at 37 ^{o}C in appropriate culture media. The antimicrobial activity of lectin was determined by the microdilution method. In brief, aliquots of bacterial suspensions (50 µl) in the mid-logarithmic phase at a concentration of 2×10^5 colony forming units (CFU)/ml in an appropriate culture medium were added to each well containing 50 µl of lectin solution that had been twofold serially diluted in buffer I (10 mM sodium phosphate buffer, pH 7.2) or buffer II (phosphate buffered saline (PBS), 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 150 mM NaCl (pH 7.2). Several wells were kept untreated as a control for monitoring bacterial growth. Inhibition of growth was determined by measuring the absorbance at 620 nm using a Versa-Max microplate Elisa Reader (Molecular Devices, Sunnyvale, CA, USA) after incubation for 18-24 h at 37 ^{o}C. The lowest concentration of lectin that completely inhibited growth was defined as the minimum inhibitory concentration (MIC) (Park et al., 2008). The MIC values were calculated as an average of several independent experiments conducted in triplicate.

5.2.6 Antifungal activity

The fungal strains of *Candida albicans* (KCTC 7270); *Candida catenulate*, (7642); *Candida glabrata*, (7219); *Candida rugosa*, (7324) were cultured at 28 ^{o}C in YPD media (dextrose 2%, peptone 1%, and yeast extract 0.5%, pH 5.0 to 5.5). Fungal cells (final concentration 2 x 10^4 CFU/ml) that were grown in 50 µl of YPD media were seeded in each well of a microtiter plate containing 50 µl of twofold serially diluted lectin in buffer I or buffer II, as described above. After incubating for 24 to 30 h at 28 ^{o}C, the lowest concentration of lectin inhibiting the growth of fungi was

microscopically determined to be the MIC (Park et al., 2007). All MIC measurements are the average 3-4 independent experiments.

5.2.7 Hyphae fungi

Fusarium oxysporum (ATCC 16909) was obtained from American Type Culture Collection. *Fusarium solani* (KCTC 6326) was obtained from the Korean Collection for Type Cultures. The fungal strains were grown at 28°C in potato dextrose broth (PDB) medium. The fungal cells (2×10^5 cells/ml) were seeded in 100 µl of potato dextrose broth per well in 96-well microtiter plates, mixed with 10 µl of the serially diluted lectin solution, and incubated for 24 h at 28°C. All assays were performed in triplicate. To visualize the fungicidal effect, morphological changes were examined by phase contrast light microscopy using an ECLIPSE TE300 microscope (Nikon, Japan).

5.2.8 Interaction of lectins with cell wall components by CD spectroscopy

S. crispa lectins were scanned in the presence or absence of LPS (0.1%) dissolved in SP buffer. The secondary structures was monitored at a concentration of 50 µg of lectin in the presence of laminarin from digitata laminarin (0.1%; sigma-aldrich, St. Luis, USA), and in the presence of mannan from *Saccharomyces cerevisiae* (0.1%; sigma-aldrich, St. Luis, USA). CD data represent average value from three separate recordings (Gopal et al., 2012).

5.2.9 Tryptophan (Trp) fluorescence assay

The fluorescence emission spectrum of Trp of the lectin was monitored in the presence of 10 mM SP buffer and cell wall components (0.1% LPS, 0.1% laminarin, and 0.1% mannan). The Trp fluorescence measurements were taken using a spectrofluorometer. Final concentration (2 µg) of lectin was added to 200 µl of above environments, and the lectin:different environment mixture was allowed to interact at

25°C for 10 min. The fluorescence was excited at 280 nm, and the emission was scanned from 300 to 400 nm (Gopal et al., 2012).

5.3 Results and discussion

5.3.1 Purification of lectin from S. crispa fruiting bodies

This present study reports the first investigation on the purification of a lectin from *S. crispa* fruiting bodies. Crude fruiting bodies extract was subjected to ammonium sulfate precipitation and dialysis. The lectin was purified by anion exchange and gel filtration chromatography. In the SDS-PAGE, *S. crispa* lectin exhibited a single band at 24 kDa indicating it is a monomer protein (Figure 5.1). Hemagglutinating activity of the *S. crispa* lectin was assayed in each purification steps. The interaction between a lectin and a sugar moiety occurs at the carbohydrate recognition domain and involves Van der walls forces and the formation of hydrogen bonds between sugar hydroxyls and amino acid residues at the active sites. The cross-linkages formed between adjacent cells through binding between lectins and glycoconjugates causes cell agglutination. When the erythrocytes are involved in this type of interaction, the phenomenon of hemeagglutination occurs. (Maria et al., 2010).

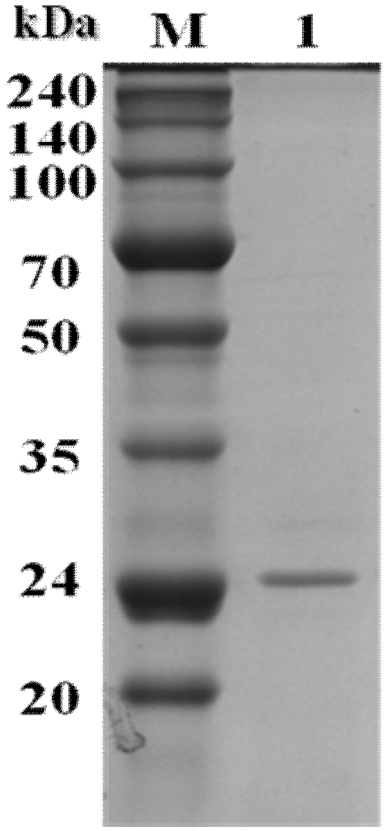


Figure 5.1 SDS-PAGE of purified lectin protein. Lectin was analyzed by SDS-PAGE on a 15% separating gel with Coomassie brilliant blue G-250 staining. Lane M molecular size marker, Lane 1 the purified protein from Sephadex G-75 Gel filtration column.

5.3.2 Hemeagglutination

Lectins agglutinate cells or mediate specific physiological or pathological process by recognition of and binding to distinct glycosylated cell ligands. Mushrooms lectins are involved in various functions such as defense against predators and parasites and they are also involved in the development of fruiting bodies and mycorrhizal formation. (Pohleven et al., 2011).

5.3.3 Antibacterial assays

Antibiotic resistance is increasing at a rate that far exceeds the development of new types of antibiotic agents. *S. crispa* showed higher activity against Gram-negative bacteria than Gram-positive bacteria. The MICs for resistant *E. coli*, resistant *S. aurues*, and *P. aeruginosa* were 100, 200, 50 µg/ml, respectively, which were higher than their corresponding normal strains. The MIC values of the lectin against bacterial strains under lower salt (without NaCl) and high salt (with 150 mM NaCl) conditions are summarized in Table 5.2. Considering the potential clinical application of this lectin, we evaluated its antibacterial in the presence of sodium chloride. This study showed that lectin has similar antibacterial activity in both buffer conditions, which suggested that salt condition did not reduced lectin antimicrobial activity. It is reported that plant lectins able to inhibit oral bacteria adherence to enamel. Also, it is reported that lectins have anti-adhesion potential which are explored as a biotechnological tool for the studies involved in therapeutics of dental diseases which are closely related to bioflim formation (Cavalcante et al., 2011).

Table 5.1 Antibacterial activities of lectin against non-resistant and drug-resistant fungal strains. Antibacterial activity was performed in 10 mM sodium phosphate buffer, pH 7.2 and phosphate buffered saline, pH 7.2 (number in the parenthesis)

	MIC (µg)			
Microorganisms	Lectin	Ampicillin	Ciprofloxacin	Oxacillin
Gram (-) bacteria				
E. coli	100 (100)	100 (100)	-	> 64 (>64)
S. typhimurium	25 (25)	50 (50)	-	8(8)
P. aeruginosa	50 (50)	-	-	16(16)
Gram (+) bacteria				
S. aureus	100 (100)	-	-	25(25)
B. subtilis	50 (100)	-	-	-
L. monocytogenes	100 (100)	-	-	-
Resistant strains				
E. coli CCARM 1229	100 (100)	>200 (>200)	-	-
E. coli CCARM 1238	200 (200)	>200 (>200)	-	-
S. aureus CCARM 3089	100 (100)	-		>200(>200)
S. aureus CCARM 3090	100 (100)	-	-	>200(>200)
P. aeruginosa 3904	50 (50)	-	>200 (>200)	-
P. aeruginosa 1034	50 (50)	-	>200 (>200)	-

5.3.4 Antifungal activity

This lectin also exerted antifungal activity against *C. albicans*, *C. catenulate*, *C. glabrata*, *C. rugusa* cells (Table 5.2). Also, this lectin showed antifungal activity against various hyphae forming fungi such as *Fusarium oxysporum* and *Fusarium solani*. It is proposed that lectin role in defense against pathogens are due to the presence of lectins at potential sites of invasion by infectious agents and the binding of lectins to various fungi and their ability to inhibit fungal growth and germination (Vaz et al., 2011). Some *Fusarium* species produce mycotoxins in cereal crops that can affect human and animal health. Isolation of a lectin with inhibitory activity toward fungi and with low environmental toxicity has important applications in vegetable biotechnology as a promising biological control agent in genetically modified plants (Charungchitrack et al., 2011). The antifungal activity of lectin was showed against all tested fungal cells. As shown in Figure 5.2 and 5.3, light microscopy confirmed that lectin strongly inhibited the growth *of F. oxysporum* and *F. solani* (Figure 5.2 and 5.3).

Table 5.2 Antifungal activities of lectin against non-resistant and drug-resistant fungal strains. Antifungal activity was performed in 10 mM sodium phosphate buffer, pH 7.2 and phosphate buffered saline, pH 7.2 (number in the parenthesis)

	MIC (µg)	
Fungal strains	**Lectin**	**Fluconazol**
C. albicans	100 (100)	25
C. catenulate	25 (25)	12.5
C. glabrata	100 (100)	12.5
C. rugusa	50 (100	25
Resistant strains		
C. albicans 14001	200 (200)	>200
C. albicans 14007	100 (100)	>200

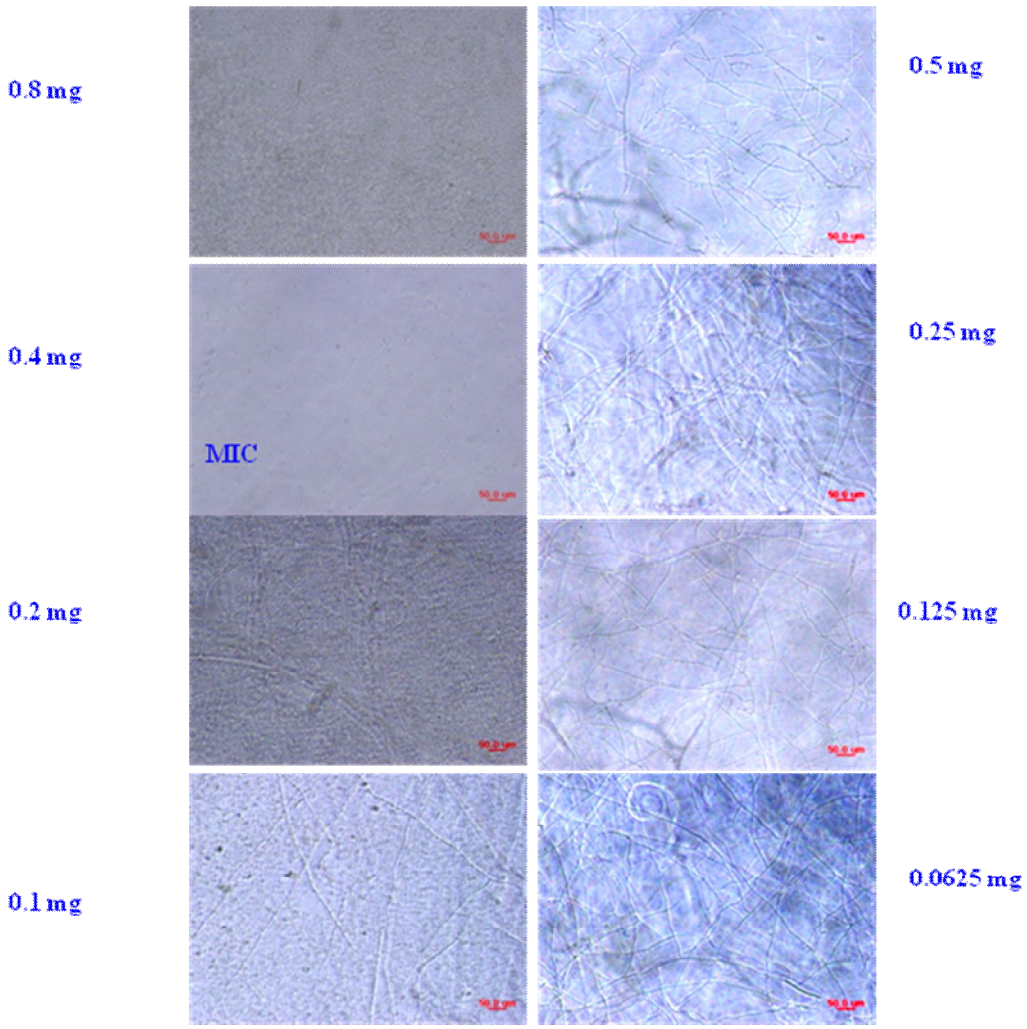


Figure 5.2 Antifungal activity of purified *S. crispa* lectin under light microscopy against *F. solani*.

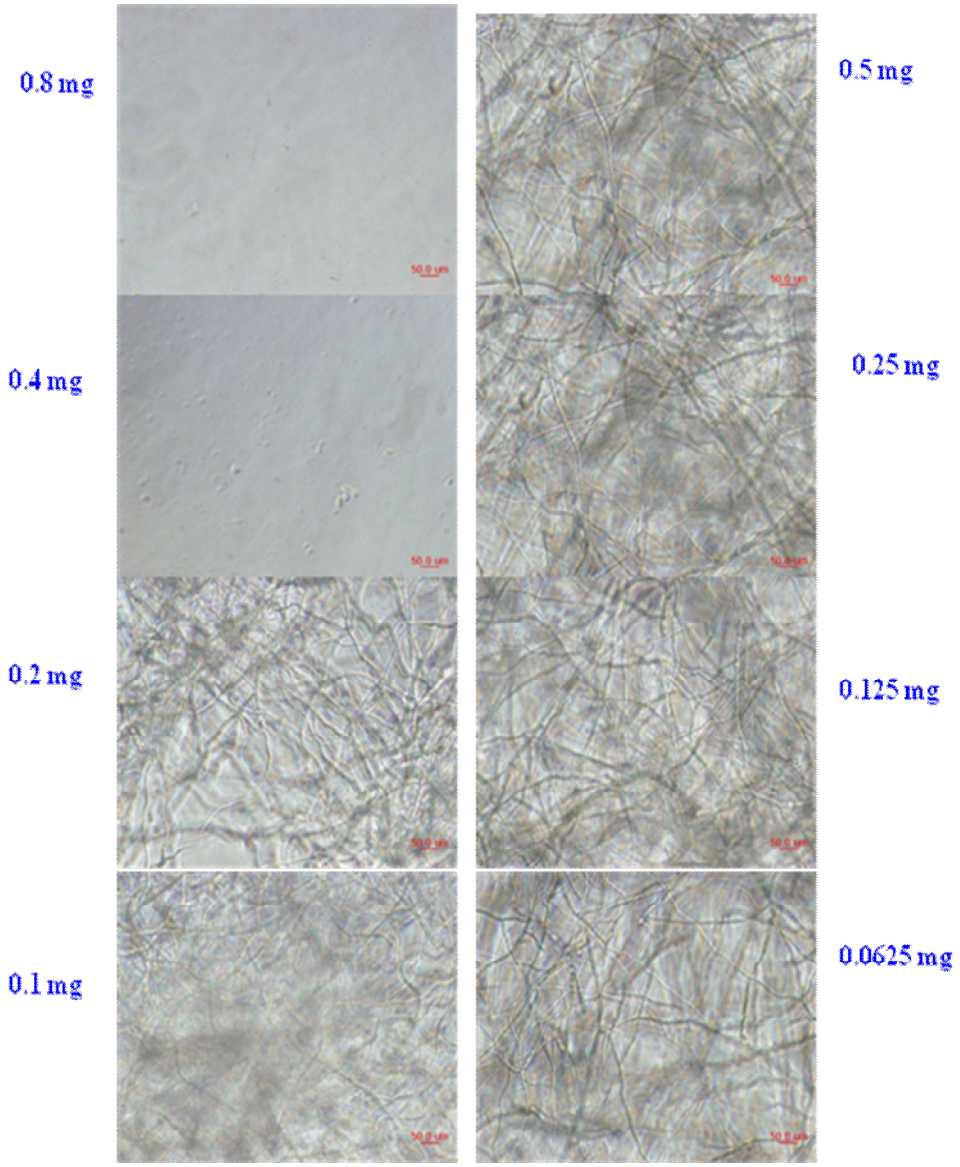


Figure 5.3 Antifungal activity of purified *S.crispa* lectin under light microscopy against *F. oxysporum*

5.3.5 Circular dichroism

Circular dichroism (CD) spectroscopy can be a valuable method for determining the secondary structures of proteins can be a valuable method for determining the secondary structures of proteins. We have used CD to investigate the conformational changes brought by lectin–cell wall components interaction. LPS is cell wall component of Gram-negative bacterial surfaces, and its antigenic portion, the lipid A. CD spectroscopy showed that lectin had a transition from random coil to α-helix upon interaction with LPS. The ability of lectin to bind LPS is a prereuisite for their antibacterial and endotoxin detoxifying activities. Glycoconjugates present on bacterial cell surfaces, such as peptidoglycans, lipopolysaccharides and trichoic acids, which constitute potential lectin targets. Lectins specifically bind teichoic and teichuronic acids, peptidoglycans and lipopolysaccharides in bacterial cell walls. (Ferreira et al., 2011).

Additionally, in order to investigate that binding of this lectin to the fungal surface occurred via interaction with mannan or laminarin, which are the major components of the fungal cell wall. CD spectroscopy showed that lectin displayed alpha-helical conformational changes associated with fungal cell wall components of mannan or laminarin (Figure 5.4). Therefore, it is clearly indicated that cell wall components is having essential role in fungicidal activity of lectin. This point is clearly supported that plant defension NaD1 killing fungal cells occurs through a cell wall dependent process (Van der Weerden et al., 2011)

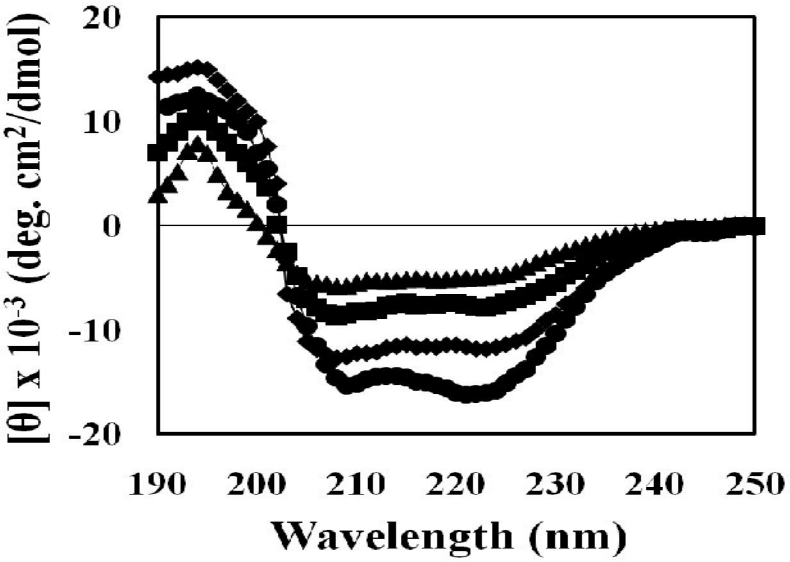


Figure 5.4 CD spectra of lectin in the presence of 10 mM Tris-HCl buffer, pH 9.0 (▲), LPS (●), mannan (■) and laminarin (♦).

5.3.5 Characterization of the tryptophan environment using fluorescence spectroscopy

Fluorescence intensity and maximum fluorescence emission of tryptophan residue data have often been used to study conformational transitions in protein structures (Sultan et al., 2005). The lectin-binding process can also be followed by the analysis of the Trp flourescence spectra (Table 5.3). In buffer, lectin had a fluorescense emission maximum at 353, indicating that the Trp residues of lectin are located in a more hydrophilic environment. When lectin bound to LPS, mannan and laminarin the fluorescence maxima for lectin was shifted to shorter wavelength, suggesting that the Trp side chain partitions preferentially into a more rigid, hydrophobic environments in LPS, mannan and laminarin. This tendency is consistent with the CD spectra of lectin in the presence of LPS indicate a much more structured conformation upon binding (Figure 5.4). The recorded blue shift suggests that tryptophan residues are involved in the interaction with cationic or hydrophobic domain of lectin. Cationic lectins were thought to react with cell wall components by means of electrostatic, cation-π interaction and hydrophobic forces. Lectin consists of positively and negatively charged as well as hydrophobic domains.

Table 5.3 Tryptophan emission maxima of 2 µg protein in the presence of LTA, LPS and Laminarin.

Protein	**λ_{max} buffer (nm)**	**Blue shift (nm)**		
		LPS	**mannan**	**laminarin**
S. crispa lectin	351	14	9	8

5.4 Summary

Therefore, the lectin isolated from this mushroom showed antimicrobial activity, which suggests that they might be useful in the development of a topical applications for many applications, including inhibition of bacterial or fungal infections at wound sites.

Overall conclusions and future studies

The partially purified enzyme from the *S. crispa* mushroom can economically perform the bioremediation and biodegradation of single walled carbon nanotubes. A new alkaline esterase purified from the fruiting bodies of the *S. crispa* mushroom could potentially be used as a biocatalyst in the food, chemical and pharmaceutical industries. The lectin isolated from this mushroom showed antimicrobial activity, which suggests that they might be useful in the development of a topical applications for many applications, including inhibition of bacterial or fungal infections at wound sites. Many different aspects of this cauliflower mushroom, *S. crispa*, paved an interest for the clinical research and industrial applications. This mushroom's significant role helped the scientists and researchers to isolate novel components. In the future, efforts should be taken to screen other novel components for potential contributions in research and food processing in the food industry.

References

Aida, F.M.N.A., Shuhaimi, M., Yazid, M., Maaruf, A.G., 2009. Mushroom as a potential source of prebiotics: a review. Trends Food Sci. Tech. 20, 567–575.

Allen, B.L., Kotchey, G.P., Chen, Y., Yanamala, N.V.K., Klein-Seetharaman, J., Kagan, V.E., Star, A., 2009. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. J. Am. Chem. Soc. 131, 17194–17205.

Allen, B.L., Kichambare, P.D., Gou, P., Vlasova, I.I., Kapralov, A.A., Konduru, N., Kagan, V.E., Star, A., 2008. Biodegradation of single-walled carbon nanotubes through enzymatic catalysis. Nano Lett. 8, 3899–3903.

Baldrian, P., Wiesche, C., Gabriel, J., Nerud, F., Zadražil, F., 2000. Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl. Environ. Microbiol. 10, 2471–2478.

Bamforth, S.M., Singleton, I., 2005. Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J. Chem. Tech. Biotechnol. 80, 723–736.

Berch, S.M., Ka, K.H., Park, H., Winder, R., 2007. Development and potential of the cultivated and wild-harvested mushroom industries in the Republic of Korea and British Columbia. BC J. Ecosys. Manage. 8, 53–75.

Bezalel, L., Hadar, Y., Cerniglia, C.E., 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 63, 2495–2501.

Bornscheuer, U. T., 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiol. Rev. 26, 73–81.

Bresler, M. M., Rosser, S. J., Basran, A., Bruce, N. C., 2000. Gene cloning and nucleotide sequencing and properties of a cocaine esterase from *Rhodococcus sp*. Strain MB1. Appl. Environ. Microbiol. 66, 904–908.

Butterfeild, W., Jong, S.C., Alexander, M.T., 1978. Polypropylene vials for preserving fungi in liquid nitrogen. Mycologia. 70, 1122–1124.

Çağlarırmak, N., 2007. The nutrients of exotic mushrooms (*Lentinula edodes* and *Pleurotus* species) and an estimated approach to the volatile compounds. Food Chem. 105, 1188–1194.

Cajthaml, T., Moder, M., Kacer, P., Sasek, V., Popp, P., 2002. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. J. Chrom. 974, 213–222.

Cavalcante, T.T.A., Anderson Matias da Rocha, B., Alves Carneiro, V., Vassiliepe Sousa Arruda, F., Fernandes do Nascimento, A.S., Cardoso S, N., Do Nascimento, K.S., Sousa Cavada, B., Holanda Teixeira, E., 2011. Effect of Lectins from *Diocleinae* Subtribe against Oral *Streptococci*. Molecules. 16, 3530–3543.

Chao, Y. -P., Fu, H., Wang, Y. -L., Huang, W. -B., Wang, J. -Y., 2003. Molecular cloning of the carboxylesterase gene and biochemical characterization of the encoded protein from *Pseudomonas citronellolis* ATCC 13674. Res. Microbiol. 154, 521–526.

Chandrasekaran, G., Kim, G.J., Shin, H.J., 2011. Purification and characterization of an alkaliphilic esterase from a culinary medicinal mushroom, *Sparassis crispa*. Food Chem. 124, 1376–1381.

Charungchitrak, S., Petsom, A., Sangvanich, P., Karnchanatat, A., Antifungal and antibacterial activities of lectin from the seeds of *Archidendron jiringa Nielsen*. Food Chem. 2011, 126, 1025–1032.

Choi, G.-S., Kim, J.-Y., Kim, J.-H., Ryu, Y.-W., Kim, G.-J. 2003. Construction and characterization of a recombinant esterase with high activity and enantioselectivity to (S)-ketoprofen ethyl ester. Protein Expres. Purif. 29, 85–93.

Choi, Y. J., Lee, B. H., 2001. Culture conditions for the production of esterase from *Lactobacillus casei* CL96. Bioproc. Biosystems Eng. 24, 59–63.

Colak, A., Camedan, Y., Faiz, O., Sesli, E., Kolcuoglu, Y., 2009. An esterolytic activity from a wild edible mushroom, *Lycoperdon perlatun.* J. Food Biochem. 33, 482–499.

Crisan, E.B., Sands, A., Nutritional value. In: Chang ST, Hayes WA, editors. The biology and ultivation of mushrooms. New York: Academic Press; 1978. p.137.

Cromex-Toribio, V., Garcia-Martin, A.B., Martinez, M.J., Martinez, A.T., Guillen. F., 2009. Enhancing the production of hydroxyl radicals by *Pleurotus eryngnii* via quinone redox cycling for pollutant removal. Appl. Environ. Microbiol. 3954–3962.

Dai, Y.C., Wang, Z., Binder, M., Hibbett, D.S., 2006. Phylogeny and a new species of *Sparassis* (*Polyporales*, Basidiomycota): evidence from mitochondrial atp6, nuclear rDNA and rpb2 genes. Mycologia. 98, 584–592.

Doyle, W.A., Blodig, W., Veitch, N.C., Piontek, K., Smith, A.T., 1998. Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. Biochemistry. 37**,** 15097–15105.

Ertunga, N.S., Cakmak, U., Colak, A., Faiz, O., Sesli, E., 2009. Characterisation of esterolytic activity from two wild mushroom species, *Amanita vaginata* var. *vaginata* and *Tricholoma terreum*. Food Chem. 115, 1486–1490.

Eibes, G., Cajthaml, T., Moreira, M.T., Feijoo, G., Lema, J.M., 2006. Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere. 64**,** 408–414.

Fahmy, A. S., Abo-Zeid, A. Z., Mohamed, T. M., Ghanem, H. M., Borai, I. H., Mohamed, S. A., 2008. Characterization of esterases from *Cucurbita pepo cv*. "eskandrani". Biores. Technol. 99, 437–443.

Faiz, O., Colak, A., Saglam, N., Canakci, S. Belduz, A.O., 2007. Determination and characterization of thermostable esterolytic activity from a novel thermophilic bacterium *Anoxybacillus gonensis* A4. J. Biochem. Mol. Biol. 40, 588–594.

Ferreira, R.S., Napoleão, T.H., Santos, A.F.S., Sá, R.A., Carneiro-da-Cunha, M.G., Morais, M.M.C., Silva-Lucca, R.A., Oliva, M.L.V., Coelho, L.C.B.B., Paiva, P.M.G., 2011. Coagulant and antibacterial activities of the water-soluble seed lectin from *Moringa oleifera*. Lett. Appl. Microbiol. 53, 186–192.

Gadd. G.M., 2001. Fungi in bioremediation (British Mycological Society Symposia), first ed. Cambridge University Press, New York.

Garibay-Orijel, R., Cordova, J., Cifuentes, J., Valenzuela, R., Estrada-Torres, A., Kong, A., 2009. Integrating wild mushrooms use into a model of sustainable management for indigenous community forests. Forest Ecol. Manag. 258, 122–131.

Giuliani, S., Piana, C., Setti, L., Hochkoeppler, A., Pifferi, P. G., Williamson, G., Faulds, C. B., 2001. Synthesis of pentylferulate by a feruloyl esterase from

Aspergillus niger using water-in-oil microemulsions. Biotechnol. Lett. 23, 325–330.

Gobbetti, M., Smacchi, E., Corsetti, A., 1997. Purification and characterization of a cell surface-associated esterase from *Lactobacillus fermentum* DT41. Int. Dairy J. 7, 13–21.

Gómez-Toribio, V., García-Martín, A.B., Martínez, M.J., Martínez, A.T., Guillén, F., 2009. Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. Appl. Environ. Microbiol. 75, 3944–3953.

Gopal, R., Park, J.S., Seo, C.H., Park, Y., 2012. Applications of circular dichroism for structural analysis of gelatin and antimicrobial peptides. Int. J. Mol. Sci. 13, 3229–3244.

Gozia, O., Ciopraga, J., Bentia, T., Lungu, M., Zamfirescu, I., Tudor, R., Roseanu, A., Nitu, F., 1993. Antifungal properties of lectin and new chitinase from potato tubers. FEBS Lett. 370, 245–249.

Guillot, J., Konska, G., 1997. Lectins in higher fungi. Biochem. Syst. Ecol. 25, 203–230.

Harada, T., Kawaminami, H., Miura, N.N., Adachi, Y., Nakajima, M., Yadomae, T., Ohno, N., 2006. Comparison of the immunomodulating activities of 1,3-β-glucan fractions from culinary-medicinal mushroom, *Sparassis crispa* Wulf.:Fr. (Aphyllophoromycetideae). Int. J. Med. Mushr. 8, 231–244.

Harada, T., Miura, N., Adachi, Y., Nakajima, M., Yadomae, T., Ohno, N., 2002. Effect of SCG, 1,3-beta-D-glucan from *Sparassis crispa* on the hematopoietic response in cyclophosphamide induced leukopenic mice. Biol. Pharm. Bull. 25, 931–939.

Harada, T., Miura, N.N., Adachi, Y., Nakajima, M., Yadomae, T., Ohno, N., 2004. Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates cytokine induction by 1,3-β-D-glucan SCG in DBA/2 mice in vitro. J Interferon Cytokine Res. 24, 478–489.

Harada, T., Miura, N.N., Adachi, Y., Nakajima, M., Yadomae, T., Ohno, N., 2002. IFN-gamma induction by SCG, 1,3-beta-D-glucan from *Sparassis crispa*, in DBA/2 mice in vitro. J. Interferon Cytokine Res. 22, 1227–1239.

Hammel, K.E., Kalyanaraman, B., Krik, T.K., 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo (p)- dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. 261, 16948–16952.

Hasegawa, A., Yamada, M., Dombo, M., Fukushima, R., Matsuura, N., Sugitachi, A., 2004. *Sparassis crispa* as biological response modifier. Gan To Kagaku Ryoho. 31, 1761–1763.

Hervey, A.H., 1947. A survey of 500 basidiomycetes for antibacterial activity. Bull Torrey Bot Club. 74, 476–503.

Hirai, H., Sugiura, M., Kawai, S., Nishida, T., 2005. Characteristics of novel lignin peroxidases produced by white-rot fungus *Phanerochaete sordida* YK-624. FEMS Microbiol. Lett. 246, 19–24.

Hoffmann, P., 1991. Cryopreservation of fungi. World J. Microbiol. Biotechnol. 7, 92–94.

Homolka, L., Lisá, L., Nerud, F., 2006. Basidiomycete cryopreservation on perlite: Evaluation of a new method. Cryobiol. 52, 446–453.

Homolka, L., Lisá, L., Nerud, F., 2003. Viability of basidiomycete strains after cryopreservation: Comparison of two different freezing protocols. Folia Microbiol (Praha). 48, 219–226.

Horie, K., Rakwal, R., Hirano, M., Shibato, J., Nam, H. W., Kim, Y. S., Kouzuma, Y., Agrawal, G. K., Masuo, Y., Yonekura, M., 2008. Proteomics of two cultivated mushrooms *Sparassis crispa* and *Hericium erinaceum* provides insight into their numerous functional protein components and diversity. J. Proteome Res. 7, 1819–1835.

Hyun, K.W., Jeong, S.C., Lee, D.H., Park, J.S., Lee, J.S., 2006. Isolation and characterization of a novel platelet aggregation inhibitory peptide from the medicinal mushroom, *Inonotus obliquus*. Peptides. 27, 1173–1178.

Johri, S., Jamwal, U., Rasool, S., Kumar, A., Verma, V., Qazi, G.N., 2005. Purification and characterization of peroxidases from *Withania somnifera* (AGB 002) and their ability to oxidize IAA. Plant Sci. 169**,** 1014–1021.

Jones, E. T., Lyunch, K. A., 2007. Nontimber forest products and biodiversity management in the Pacific Northwest. Forest Ecol. Manag. 246, 29–37.

Kaneko, T., Oguri, S., Kato, S., Nagata, Y., 1993. Developmental appearance of lectin during fruiting body formation in *Pleurotus cornucopiae*. J. Gen. Appl. Microbiol. 39, 83–90.

Kavishree, S., Hemavathy, J., Lokesh, B.R., Shashirekha, M.N., Rajarathnam. S., 2008. Fat and fatty acids of Indian edible mushrooms. Food Chem. 106, 597–602.

Kawagishi, H., Hayashi. K., Tokuyama, S., Hashimoto, N., Kimura, T., Dombo, M., 2007. Novel bioactive compounds from the *Sparassis crispa* mushroom. Biosci. Biotechnol. Biochem. 71, 1804–1806.

Kermasha, S., Bisakowski, B., Ismail, S., Morin, A., 2000. The effect of physical and chemical treatments on the esterase activity from *Pseudomonas fragi* CRDA 037. Food Res. Int. 33, 767–774.

Kim, M.Y.. Chung, L.M., Lee, S.J., Ahn, J.K., Kim, E.H., Kim, M.J., Kim, S.L., Moon, H.I., Ro, H.M., Kang, E.Y., Seo, S.H., Song, H.K., 2009. Comparison of free amino acid, carbohydrates concentrations in Korean edible and medicinal mushrooms. Food Chem. 113, 386–393.

Kim, H. E., Park, K. R., 2002. Purification and characterization of an esterase from *Acinetobacter lwoffii* I6C-1. Curr. Microbiol. 44, 401–405.

Kodani, S., Hayashi, K., Hashimoto, M., Kimura, T., Dombo, M., Kawagishi, H., 2009. New sesquiterpenoid from the mushroom *Sparassis crispa*. Biosci. Biotechnol. Biochem. 73, 228–229.

Koduri, R.S., Tien, M., 1995. Oxidation of guiacol by lignin peroxidase. Role of veratryl alcohol. J. Biol. Chem. 270, 22254–22258.

Kotchey, G.P., Allen, B.L., Vedala, H., Yanamala, N., Kapralov, A.A., Tyurina, Y.Y., Klein-Seetharaman, J., Kagan, V.E., Star, A. 2011. The enzymatic oxidation of graphene oxide. ACS Nano 5**,** 2098–2108.

Koseki, T., Fushinobu, S., Ardiansyah, Shirakawa, H., Komai, M., 2009. Occurrence, properties, and applications of feruloyl esterases. Appl. Microbiol. Biotechnol. 84, 803–810.

Kwon, A.H., Qiu, Z., Hashimoto, M., Yamamoto, K., Kimura, T., 2009. Effects of medicinal mushroom (*Sparassis crispa)* on wound healing in streptozotocin-induced diabetic rats. Am. J. Surg. 197, 503–509.

Kwon, H.S., Chung, E., Oh, J., Lee, C.H., Ahn, I.S., 2008. Optimized production of lignolytic manganese peroxidase in immobilized cultures of *Phanerochaete chrysosporium*. Biotechnol. Bioproc. Eng. 13**,** 108–114.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680–685.

Lam, Y.W., Ng, T.B., Wang, H.X., 2001. Antiproliferative and antimitogenic activities in a peptide from puff ball mushroom *Calvatia caelata*. Biochem. Biophys. Res. Comm. 289, 744–749.

Lee, D., Koh, Y. S., Kim, K. J., Kim, B. C., Choi, H. J., Kim, D. S., Suhartono, M. T., Pyun, Y. -R., 1999. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol. Lett. 179, 393–400.

Lee, S. Y., Lee, B. H., 1990. Esterolytic and lipolytic activities of *Lactobacillus casei*-subsp-*casei* LLG. J. Food Sci. 55, 119–122.

Lee, J.M., Kim, J.Y., Choi, K.D., Han, K.D., Hur, H., Kim, S.W., Shim, J.O., Lee, J.Y., Lee, T.S., Lee, M.W., 2004. Sawdust media affecting the mycelial growth and the fruiting body formation of *Sparassis crispa*. Mycobiol. 32, 190–193.

Li, X., Lin, X., Zhang, J., Wu, Y., Yin, R., Feng, Y., Wang, Y., 2010. Degradation of polycyclic aromatic hydrocarbons by crude extracts from spent mushroom substrate and its possible mechanisms. Curr. Microbiol. 60**,** 336–342.

Lindequist, U., Niedermeyer, T.H., Julich, W.D., 2005. The pharmacological potential of mushrooms. Evid.base. Compl. Alternative Med. 2, 285–299.

Mahajna, J., Dotan, N., Zaidman, B.Z., Petrova, R.D., Wasser, S.P., 2009. Pharmacological values of medicinal mushrooms for prostate cancer therapy: the case of *Ganoderma lucidum*. Nutr. Cancer. 61, 16–26.

Mansur, M., Arias, M.E., Copa-Patio, J.L., Flrdh, M., Gonzlez, A.E., 2003. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. Mycologia. 95**,** 1013–1020.

Manzi, P., Gambelli, L., Marconi, S., Vivanti, V., Pizzoferrato, L., 1999. Nutrients in edible mushrooms: an inter-species comparative study. Food Chem. 65, 477–482.

Maria, C.S., Angelita, D.C., Custodio, D.D.S., Flavia, C.A.M., Celeste, M.P.D.A., 2010. Prtical purification of leaf lectin from *Manihot esculenta* and screening its fungicidal activity. J. Agr. Biotechnol. Sustain. Dev. 8, 136–141.

Matsumoto, H., Natsume, A., Ueda, H., Saitoh, T., Ogawa, H., 2001. Screening of a unique lectin from 16 cultivable mushrooms with hybrid glycoprotein and neoproteoglycan probes and purification of a novel N-acetylglucosamine-specific lectin from *Oudemansiella platyphylla* fruiting body. Biochim. Biophys. Acta. 1526, 37–43.

Mau, J.L., Beelman, R.B., Ziegler, G.R., 1994. Aroma and flavor components of cultivated mushrooms. In Spices, Herbs and Edible Fungi Charalambous, G., Ed.; Elsevier: Amsterdam. p. 657–684.

Mau, J.L., Hwang, S.J., 1997. Effect of λ-irradiation on flavor compounds of fresh mushrooms. J. Agric. Food Chem. 45, 1849–1852.

Mehboob, N., Asad, M.J., Imran, M., Gulfraz, M., Wattoo, F.H., Hadri, S.H., Asghar, M., 2011. Production of lignin peroxidase by *Ganoderma leucidum* using solid state fermentation. Afr. J. Biotechnol. 10, 9880–9887.

Mohamed, M. A., Mohamed, T. M., Mohamed, S. A., Fahmy, A. S., 2000. Distribution of lipases in the Gramineae. Partial purification and characterization of esterase from *Avena fatua.* Biores. Technol. 73, 227–234.

Mo, H., Winter, H.C., Goldstein, I.J., 2000. Purification and characterization of Neu5Acα2-6Galβ1-4Glc/GlcNAc-specific lectin from the fruiting body of the polypore mushroom *Polyporus squamosus*. J. Biol. Chem. 275, 10623–10629.

Oguri, S., Ando, A., Nagata, Y., 1996. A novel development stage-specific lectin of the basidiomycete *Pleurotus cornucopiae*. J. Bacteriol. 178, 5692–5698.

Oh, D.S., Park, J.M., Park, H., Ka, H.K., Chun, J.W., 2009. Site characteristics and vegetation structure of the habitat of cauliflower mushroom (*Sparassis crispa*). Kor. J. Mycol. 37, 33–40.

Oh, D.S., 2009. Studies on site conditions of *Sparassis crispa* at its natural habitat, artificial cultivation characteristics and utilization [dissertation]. Graduate School of Chonnam National University.

Ohno, N., Miura, N.N., Nakajima, M., Yadomae, T., 2000. Antitumor 1,3-beta-glucan from cultured fruit body of *Sparassis crispa*. Biol. Pharm. Bull. 23, 866–872.

Ohno, N., Nameda, S., Harada, T., Miura, N. N., Adachi, Nakajima, M., Yoshida, K., Yoshida, H., Yadomae, T., 2003. Immunomodulating activity of a (β-glucan preparation, SCG, extracted from a culinary-medicinal mushroom, *Sparasis crispa* Wulf. : Fr. (Aphyllophoromycetidae), and application to cancer patients. Int. J. Med. Mushr. 5, 359–368.

Park, H., Lee, B.H., Ka, K.H., Bak, W.C., Oh, D.S., Park, J.M., Chun, W.J., 2006. Cultivation of cauliflower Mushroom (*Sparassis crispa*) by use of steam-treated coniferous sawdusts. J. Korean Wood Sci. Technol. 34, 84–89.

Park, H.G., Shim, Y.Y., Choi, S.O., Park, W.M., 2009. New method development for nanoparticle extraction of water-soluble β-(1→3)-D-glucan from edible mushrooms, *Sparassis crispa* and *Phellinus linteus*. J. Agric. Food Chem. 57, 2147–2154.

Park, S.C., Kim, M.H., Hossain, M.A., Shin, S.Y., Kim, Y., Stella, L., Wade, J.D., Park, Y., Hahm, K.S., 2008. Amphipathic alpha-helical peptide, HP (2-20), and its analogues derived from *Helicobacter pylori*: pore formation mechanism in various lipid compositions. Biochem. Biophys. Acta 1778, 229–241.

Park, S.C., Lee, J.R., Shin, S.O., Park, Y., Lee, S.Y., Hahm, K.S., 2007. Characterization of a heat-stable protein with antimicrobial activity from *Arabidopsis thaliana*. Biochem. Biophys. Res. Commun. 362, 562–567.

Park, H. -J., Jeon, J. H., Kang, S. G., Lee, J. -H., Lee, S. -A., Kim, H. -K., 2007. Functional expression and refolding of new alkaline esterase, EM2L8 from deep-sea sediment metagenome. Protein Expres. Purif. 52, 340–347.

Pasczynski, A., Huynh, V.B., Crawford, R., 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett;29, 37–41.

Petrova, R.D., Mahajna, M.A., Denchev, C.M., 2005. Potential role of medicinal mushrooms in breast cancer treatment: current knowledge and future perspectives. Int. J. Med. Mushr. 7, 141–155.

Periasamy, K., Natarajan, K., 2004. Role of lignocellulosic enzymes during basidiomata production by *Pleurotus djamor* Var. *Roseus*. Indian J. Biotechnol. 3, 577–583.

Pilz, D., Molina, R., 2002. Commercial harvests of edible mushrooms from the forests of the Pacific Northwest United States: issues, management, and monitoring for sustainability. Forest Ecol. Manag. 155, 3–16.

Pohleven, J., Brzin, J., Vrabec, L., Leonardi, A., Cokl, A., Strukelj, B., Kos, J., Sabotic, J., 2011. Basidiomycete *Clitocybe nebularis* is rich in lectins insecticidal activities. Appl. Microbiol. Biotechnol. 91, 1141–1148.

Politi, M., Silipo, A., Siciliano, T., Tebano, M., Flamini, G., Braca, A., Jiménez-Barbero, J., 2007. Current analytical methods to study plant water extracts: the example of two mushrooms species, *Inonotus hispidus* and *Sparassis crispa*. Phytochem. Anal. 18, 33–41.

Puttaraju, N.G., Venkateshaiah, S.U., Dharmesh, S.M., Urs, S.M.N., Somasundaram, R., 2006. Antioxidant activity of indigenous edible mushrooms. J. Agric. Food Chem. 54, 9764–9772.

Robbins, W.J., Hervey, A., Davidson, R.W., Roberta, M.A., Robbins, W.C., 1945. A survey of some wood-destroying and other fungi for antibacterial activity. Bull Torry Bot. Club. 72, 165–190.

Rothschild, N., Novotny, C., Sasek, V., Dosoretz, C.G., 2002. Ligninolytic enzymes of the fungus *Irpex lacteus* (*Polyporus tulipiferae*): isolation and characterization of lignin peroxidase. Enzyme Microb. Technol. 31**,** 627–633.

Sanchez, V.C., Pietruska, J.R., Miselis, N.R., Hurt, R.H., Kane, A.B., 2009. Biopersistence and potential adverse health impacts of fibrous nanomaterials: what have we learned from asbestos? Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 1, 511–529,

Siepmann, R., 1987. Wachstumshemmung von Stammfaulepilzen undvon *Gremmeniella abietina* durch *Bacillus subtilis.* E. J. Forest Pathol. 17, 59–64.

Shim, J.O., Son, S.G., Yoon, S.O., Lee, Y.S., Lee, T.S., Lee, S.S., Lee, K.D., Lee, M.W., 1998. The optimal factors for the mycelial growth of *Sparassis crispa.* Kor. J. Mycol. 26, 39–46.

Shin, H.J., Oh, D.S., Lee, H.D., Kang, H.B., Lee, C.W., Cha, W.S. 2007. Analysis of mineral, amino acid and vitamin contents of fruiting body of *Sparassis crispa*. Kor. J. Life Sci. 17, 1290–1293.

Smith, D., 1998. The use of cryopreservation in the ex-situ conservation of fungi. Cryo Letts. 19, 79–90.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Sultan, N.A.M., Swamy, M.J., 2005. Fluorescence quenching and time-resolved fluorescence studies on Trichosanthes dioica seed lectin. J. Photochem. Photobiol. B: Biol. 80, 93–100.

Tada, R., Yoshikawa, M., Kuge, T., Tanioka, A., Ishibashi, K.I., Adachi, Y., Tsubaki, K., Ohno, N., 2009. A highly branched 1,3-β-d-glucan extracted from *Aureobasidium pullulans* induces cytokine production in DBA/2 mouse-derived splenocytes. Int. Immunopharmacol. 9, 1431–1436.

Tien. M., Kirk, T.K., 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Nat. Acad. Sci. 81, 2280–84.

Vander Weerden NL, Hancock RE, Anderson MA. Permeabilization of fungal hyphae by the plant defension NaD1 occurs through a cell wall dependent process. 2010. J. Biol. Chem. 285, 37513–37520.

Vaz, A.F.M., Costa, R.M.P.B., Melo, A.M.M.A., Oliva, M.L.V., Santana, L.A., Silva-Lucca, R.A., Coelho, L.C.B.B., Correia, M.T.S., 2011. Biocontrol of *Fusarium* species by a novel lectin with low ecotoxicity isolated from *Sebastiania jacobinensis*. Food Chem. 119, 1507–1513.

Verger, R., 1997. "Interfacial activation" of lipases: Facts and artifacts. Trends Biotechnol. 15, 32–38.

Verheyden, P., Pletinckx, J., Maes, D., Pepermans, H.A.M., Wyns, L., Willem, R., Martins, J., 1995. 1H NMR study of the interaction of N,N,N' triacetyl chitotriose with Ac-AMP2, a sugar binding antimicrobial protein isolated from *Amaranthus caudatus*. FEBS Lett. 370, 245–249.

Vlasova, I.I., Vakhrusheva, T.V., Sokolov, A.V., Kostevich,V.A., Ragimov, A.A., 2011. Peroxidase-induced degradation of single-walled carbon nanotubes: hypochlorite is a major oxidant capable of in vivo degradation of carbon nanotubes. J. Phys. 291, 1–6.

Wang, J., Wang, H.X., Ng, T.B., 2007. A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*. Peptides. 28, 560–565.

Wang, Y., 2009. Prebiotics: Present and future in food science and technology. Food Res. International. 42, 8–12.

Wang, H., Ng, T.B., Ooi, V.E.C., 1998. Lectins from mushrooms. Mycol. Res. 102, 897–906.

Wang, H.X., Ng., T.B., Liua, W.K., Ooi, V.E., Chang, S.T., 1995. Isolation and characterization of two distinct lectins with antiproliferative activity from the cultures mycelium of the edible mushroom Tricholoma mongolicum. Int. J. Pept. Protein Res. 46, 508-513.

Wang, H., Ng, T.B., Liu, Q., 2001. Isolation of a new heteromeric lectin with mitogenic activity from fruiting bodies of the mushroom *Agrocybe cylindracea*. Life Sci. 70, 877–884.

Wang, Z., Binder, M., Dai, Y.C., 2004. Hibbett, D.S. Phylogenetic relationships of *Sparassis* inferred from nuclear and mitochondrial ribosomal DNA and RNA polymerase sequences. Mycologia. 96, 1015–1029.

Warheit, D. B., 2006. What is currently known about the health risks related to carbon nanotube exposures? Carbon. 44, 1064–1069.

Wasser, Wasser S., 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl. Microbiol. Biotechnol. 60, 258–274.

Wedekind, E., Fleischer, K., 1924. Zur Kenntnis des Sparassols. *Berichte der deutschen chemischen Gesellschqft.* 576 (Abt. B**)** 1121–1 123.

Wedekind, E., Fleischer, K.., 1924. Zur Kenntnis des Sparassols. *Berichte der deutschen chemischen Gesellschqft.* 576 (Abt. B**)** 1121–1 123.

Wei, Z., Kondratenko, M., Dao, L.H., Perepichka, D.F., 2006. Rectifying diodes from asymmetrically functionalized single-wall carbon nanotubes. J. Am. Chem. Soc. 128**,** 3134–3135.

Wilkins, W.H., Harris, G.C.M., 1944. Investigation into the production of bacteriostatic substances by fungi. VI**.** Examination of the larger basidiomiceytes. Ann. Appl. Biol. 31, 261–370.

Woodward, S., Sultan, H.Y., Barrett, D.K., Pearce, R.B., 1993. Two new antifungal metabolites produced by *Sparassis crispa* in culture and in decayed trees. J. Gen. Microbiol. 139, 153–159.

Xie, C. -y., Gu, Z. -x., You, X., Liu, G., Tan, Y., Zhang, H., 2010. Screening of edible mushrooms for release of ferulic acid from wheat bran by fermentation. Enzym. Microb. Technol. 46, 125–128.

Yadav, M., Yadav, P., Yadav, K.D.S., 2009. Purification, characterization, and coal depolymerizing activity of lignin peroxidase from *Gloeophyllum sepiarium* MTCC-1170. Biochemistry. 2009, 74, 1125–1131.

Yamaguchi, S.Y., 1979. The umami taste. In food Taste Chemistry. J.C., Ed.;ACS Symposium Series 115; American Chemical Society: Washington, DC, p. 33–51.

Yamamoto, K., Kimura, T., Sugitachi, A., Matsuura, N., 2009. Anti-angiogenic and anti-metastatic effects of β-1,3-D-glucan purified from *Hanabiratake*, *Sparassis crispa*. Biol. Pharmaceut. Bull. 32, 259–263.

Yao, M., Yamamoto, K., Kimura, T., Dombo, M., 2008. Effects of *Hanabiratake* (*Sparassis crispa*) on Allergic Rhinitis in OVA-Sensitized Mice. Food Sci. Tech. Res. 14, 589–594.

Yu, L.G., Fernig, D.G., White, M.R.H., Spiller, D.G., Appleton, P., Evans, R.C., Grierson, I., Smith, Davies, H., Gerasimenko, O.V., Petersen, O.H., Milton, J.D., Rhodes, J.M., 1999. Edible mushroom (*Agaricus bisporus*) lectin, which reversibly inhibits epithelial cell proliferation, blocks nuclear localization sequencedependent nuclear protein import. J. Biol. Chem. 274, 4890–4899.

Zhang, Y., Geiβen, S.U., 2010. In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. J. Hazard. Mater. 176, 1089–1092.

Zhang, G.Q., Sun, J., Wang, H.X., Ng, T.B., 2009. A novel lectin with antiproliferative

activity from the medicinal mushroom Pholiota dipose. Acta Biochim. Pol. 59, 415-421.

ABSTRACT

Biological and functional properties of proteins isolated from a brown-rot fungi, *Sparassis crispa*

Gayathri Chandrasekaran

Advisor: Prof. Hyun-Jae Shin, PhD

Department of Chemical Engineering

Graduate School of Chosun University

Sparassis crispa is an edible and medicinal mushroom. The purpose of this work is mainly focused on the proteins isolation and purification from the fruiting bodies of this mushroom. Subsequently, various applications in biodegradation of organic pollutants, food industry and biological activities of this mushroom were developed.

Chapter-1 of this thesis is the about the general introduction and background research of *Sparassis crispa* mushroom.

Chapter-2 is the review of literature about the medicinal and nutritional properties of *S. crispa* mushroom.

In the Chapter-3, lignin peroxidase (LiP) was isolated and partially purified from the fruiting bodies of the medicinal and edible mushroom *Sparassis crispa*. The degradation of both raw grade and thermally treated carboxylated single-walled carbon nanotubes (SWNTs) by the LiP from *S. crispa* was investigated. The interactions between the carbon nanotubes and LiP were examined using various techniques, and

the products of the enzymatic degradation were identified. The results demonstrated the degradation of both types of carboxylated SWNTs was enzymatically catalyzed by LiP. Also, the radicals produced by the peroxidase reaction could play a role in the degradation of both types of carbon nanotubes. Furthermore, the degradation product is accompanied by the production of CO_2 gas. Therefore, the low costs of extraction of the partially purified mushroom enzyme make it a highly promising enzyme for practical application to environmental bioremediation.

In the Chapter-4, we found that the fruiting body of the medicinal and edible mushroom *Sparassis crispa* produces an alkaliphilic esterase. The substrate specificity of this esterase was high for a *p*-nitrophenyl acetate substrate. The molecular weight of the purified enzyme was approximately 60 kDa as determined by SDS–PAGE. A zymogram analysis using a tributyrin substrate revealed that this enzyme is an esterase. The optimum pH and temperature were 8.0 and 50°C, respectively. The pH and temperature stability profiles show that this enzyme is more stable at alkaline conditions and at 30-40°C. K_m and V_{max} for this esterase enzyme acting on *p*-nitrophenyl acetate were 0.2 mM and 0.5 U/mg proteins, respectively.

In the Chapter-5 lectin has been isolated from the fruiting bodies of *S. crispa*. The purified lectin showed one protein band in SDS-PAGE and agglutinated human erythrocytes. The lectin inhibited the growth of Gram-negative and Gram-positive bacteria, including drug-resistant strains. The lectin also displayed antifungal activity against various fungal strains. Furthermore, lectin inhibited the mycelial growth of *Fusarium oxysporum* and *Fusarium solani*. The circular dichorism method demonstrated that lectin adopted alpha helical structure in the presence of buffer and cell wall components such as lipopolysaccharides (LPS), mannan, and laminarian.

Moreover, tryptophan blue shift assay indicated that lectin interacted with LPS, mannan, and laminarian. Therefore, the present study indicated that cell wall components is having essential role in antimicrobial activity of the lectin. Overall, these results indicated that lectin could be used as antibiotics against microbial strains.

In conclusion, *S. crispa* mushroom serve to be an excellent sources for various enzymes. Therefore, in the future efforts will be taken to screen other novel components for the potential contributions in clinical research, environmental hazardous cleanup, pharmaceutical and food industries.

Acknowledgements

With deep immense of gratitude, I would like to thank everyone who has been helpful in the successful completion of my Ph.D. study.

It is great pleasure to express my hearty gratitude to my esteemed supervisor, **Professor Hyun-Jae Shin** at Chosun University for giving his healthy suggestions, constant encouragement, support, kind guidance and care throughout this study. It has been a great opportunity to be part of his team and to work on an exciting topic with a lot of scientific freedom which helped me to complete this Ph.D. study successfully.

It is an honor for me to thank **Prof. Sun-Il Kim, Prof. Byung Wook Jo, Prof. Jung Gyu Kim, Prof. Jae-Wook Lee, Prof. Jung-Heon Lee** in the Department of Chemical Engineering at Chosun University for their care, encouragement and support throughout my Ph. D carrier.

I wish to express my gratitude to **Prof. Geun-Joong Kim** in the Department of Biological Science at Chonnam National University for his scientific discussions in all my works.

I wish to **Dr. Deuk-Sil Oh** in the Forest Resources Research Institute at Naju and **Prof. Young Jin Jeon** in Chosun University for their support in various ways.

I would like to thank **Dr. Soo-Kyung Choi** for her scientific discussions and offering time for all these interesting results.

Thanks also to **Dr. Tae Ho Lee, Dr. Kyung-Jun Hwang, Dr. Hee Gon Kim, Mrs. Eun-Mi Ryu, Mr. Wu Yuanzhang, Mr. Eui Jin Kim, Mr. Jin-Young Kim, Mr. Yun-Soo Kim, Ms. Lim Ga-Eun, Ms. Eun Sil Choi, Ms. Nam Bo Mee, Ms. Hyung-Hoon Chae, Ms. Me-Hwa Lee and Ms. Ga-Ryun Kim** to be my friends and to share my live story with good atmosphere in all the ups and downs during my Ph.D. carrier.

My special thanks to the president and the administrative officers in Department of Chemical Engineering at Chosun University.

I extend my thanks to all the people in the International office and Graduate school at Chosun University for their great help.

I wish to thank **Ms. Sumi Kim**, International Relations Coordinator at Chosun University.

My thanks to Korean teachers in Graduate school at Chosun University.

On a personal level, I would like to thank my parents, my husband and my family members for their continues support on my choices, encouragement and love throughout my academic career.

I convey my thanks to all my friends whom I cannot possibly name individually for their valuable support directly or indirectly and their timely help for completion of this work.

My humble thanks to the almighty God for his everlasting guidance and love.

Curriculum vitae

Name: Gayathri Chandrasekaran

Address:

Chosun University, 375 Seosuk-Dong, Dong-Ku, Gwangju, Republic of Korea

Telephone: +82-62-230-7865

E. mail: gayalaksmi@gmail.com

Personal profile

Date of Birth: May 15, 1980

Gender: Female

Educational and research experiences

1998-2000: B.SC in Biochemistry, Bharathidasan University, Trichy, India.

2000-2002: M.SC in Biochemistry, J.J. College of Arts and Science, affiliated to Bharathidasan Univeristy, Pudukkottai, Tamil Nadu, India.

2002-2004: M.Phil in Biotechnology, Bharathidasan Univeristy, Trichy, India.

9/2005- 11/2005: Short term trainee at Department of Ocular Pharmacology, All India Institute of medical sciences, New Delhi, India.

2006: Research assistant in Department of Ocular Pharmacology, All India Institute of Medical Science, New Delhi, India.

2006-2008: Women Scientist in DST under Women Scientist Scholarship Scheme in Department of Ocular Pharmacology, All India Institute of medical science, New Delhi.

3/2009-8/2012: Ph.D, Department of Chemical Engineering, Chosun University, Gwangju, Korea.

Publications

1. **Gayathri Chandrasekaran**, Hyun-Jae Shin, Antifungal and antibacterial activities of lectin from *Sparassis crispa*. (To be communicated).
2. **Gayathri Chandrasekaran**, Soo-Kyung Choi, Geun-Joong Kim, Hyun-Jae Shin, The enrichment of lignin peroxidase from the *Sparassis crispa* mushroom and its capacity to biodegrade single-walled carbon nanotubes. (To be communicated).
3. **Gayathri Chandrasekaran**, Deuk-sil Oh, Hyun-Jae Shin**,** Versatile applications of the culinary–medicinal mushroom *Mycoleptodonoides aitchisonii* (Berk.) (Climacodontaceae): A Review. (article in the press).
4. **Gayathri Chandrasekaran**, Geun-Joong Kim, Hyun-Jae Shin, Purification and characterization of an alkaliphilic esterase from a culinary medicinal mushroom, *Sparassis crispa*, Food Chemistry, 124 (2011) 1376-1381.
5. **Gayathri Chandrasekaran**, Deuk-sil Oh, Hyun-Jae Shin**,** Properties and potential applications of the culinary-medicinal cauliflower mushroom, *Sparassis crispa* Wulf.: Fr. (Aphyllophotomycetideae): A Review, International Journal of Medicinal Mushrooms, 13 (2011) 177-183.
6. **Gayathri Chandrasekaran**, Hyo-Kyung Han, Geun-Joong Kim, Hyun-Jae Shin, Antimicrobial activity of soluble aminopropyl functionalized magnesium phyllosilicates, Applied Clay Science, 53 (2011), 729-739.
7. Velpandian T, Arora B, Senthilkumari S, Ravi AK, **Gayathri C**, Azad R, Ghose S. Regional variation in the levels of macular xanthophylls and carotenoids in dietary components: comparing North and South India. Journal of Nutritional Science and Vitaminology, 56 (2010), 411-420.

Published abstracts

1. **Gayathri, C**, Velpandian, T, Ghose, S. Assessment of colors and documentation of the ocular toxicity during the festival of colors (Holi) in the northern states of India. Indian J. Pharmacol. 40 (2008), Sup.2, S188.
2. **Gayathri Chandrasekaran**, Hyun-Jae Shin. Monitoring color removal of trypan blue by AMP clay. Journal of Bioscience and Bioengineering, 108 (2009), Sup. 1, S86-S87.

Scientific Activities

Oral Presentations

1. **Gayathri Chandrasekaran**, Hyun-Jae Shin. Isolation and purification of an alkaline esterase from *Sparassis crispa*. Korean Institute of Chemical Engineers conference, 2009, Seoul, South Korea.
2. **Gayathri Chandrasekaran,** Hyun-Jae Shin, Nam Hyung Geun, Yun Soo Kim. Purification and characterization of lignin peroxidase from a versatile mushroom *Sparassis crispa* and screening their degradation property. Korean Society for Biotechnology and Bioengineering, 2011, Jeju, South Korea
3. **Gayathri Chandrasekaran,** Hyun-Jae Shin. Synthesis of organo clay and their antimicrobial properties. 9th International Marine Biotechnology Conference, 2010, Qingdao, China (Received travel fellowship award)

Poster Presentations

1. **Gayathri Chandrasekaran,** Hyun-Jae Shin. Monitoring colour removal of trypan blue by AMP clay. "Asia Pacific Biochemical Engineering conference 2009, Kobe, Japan.
2. **Gayathri Chandrasekaran,** Hyun-Jae Shin, Eun-Mi Ryu, Eun Sil Choi. Purification of a lignin peroxidase from a versatile mushroom *Sparassis crispa*

and their bioremediation aspects on carbon nanotubes. “Asian Congress on Biotechnology”, 2011, Shanghai, China.