Degradation of *cis*-1,4-Polyisoprene Rubbers by White Rot Fungi and Manganese Peroxidase-Catalyzed Lipid Peroxidation

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Abbreviations

AA:	arachidonic acid
$CO_2^{\bullet-}$:	formate anion radical
2,6-DMP:	2,6-dimethoxy phenol
DD/MAS:	dipolar decoupling/magic angle spinning
Ds-Py-GCMS:	double shot pyrolysis GC-MS
EI-GC-MS:	electron ionization-gas chromatography-mass spectrometry
Et_2O:	diethyl ether
EtOH:	ethyl alcohol
EtONa:	sodium ethoxide
FR:	fenton reaction
GC-MS:	gas chromatography-mass spectrometry
GPC:	gel permeation chromatography
1-HBT:	1-hydroxybenzothoriazole
HRP:	horseradish peroxidase
HPLC:	high performance liquid chromatography
IR:	polyisoprene
KODE:	oxo-octadecadienic acid
LA:	linoleic acid
Lac:	laccase
LOX:	lipoxygenase
LPO:	lipid peroxidation
LNA:	linolenic acid
MnP:	manganese peroxidase
NMR:	nuclear magnetic resonance
NR:	natural rubber
$O_2^{\bullet-}$:	super oxide anion radical
•OH:	hydroxyl radical
OX:	oxalate
PDA:	potato dextrose agar
ROO•:	peroxyl radical
RZ:	reinheitzahl

SEM:	scanning electron microscopy
TA:	tartrate
TGA:	thermogravimetric analysis
THF:	tetrahydrofuran
TIC:	total ion chromatography
XPS:	X-ray photoelectron spectroscopy

Preface

Natural rubber, exclusive *cis*-isoprene units linked to each other by 1,4-addition, is produced by more than 2500 different species of plants (Moyle, 1942; Archer and Audley, 1973; Mooibroek and Cornish, 2000) and some fungi such as *Lactarius* and *Peziza* (Stewart et al., 1955). Natural rubber has been commercially exploited for more than 100 years by cultivating and trapping the rubber tree, *Hevea brasiliensis*. As an alternative to biological production, synthetic *cis*-1,4-polyisoprene has also been obtained since polymerization of isoprene by the Ziegler catalyst was achieved in 1956. These raw rubber materials are converted to rubber products by the process of vulcanization that leads to cross-links between rubber chains (Chapman and Porter, 1988). Sulfur vulcanization creates strong chemical network that gives recalcitrance toward physical, physicochemical, and microbial destruction and superior physical properties of elasticity, whereas recycling of spent rubber products makes problematic due to the irreversible process (Liu et al., 2000).

Due to environmental and economical concerns, there is a continuing broad interest in recycling of scrap rubber and development of recycling technologies (Myhre and MacKillop, 2000). To date, a number of mechanical, physical, and chemical recycling processes have been developed, some of which have been scaled up for use in the rubber industry although there is concern over the negative impact of these technologies on the global environment, caused by emission of CO_2 and other hazardous by-products such as SOx (Warner, 1994). Therefore, microbial methods for the safe disposal and recycling of spent rubber products have been focus of research interests. So far, a number of reports on biological possibility for

degradation and desulfurization of rubber products have been published during the last century (Beckman et al, 1974; Warner, 1994; Löffler et al., 1994; Holst et al., 1998; Schnecko, 1998).

Studies on microbial degradation of natural rubber have been started since the beginning of 20th century. Söhgen and Fol reported the decomposition of rubber by microorganisms in 1914. (Söhgen and Fol, 1914). Spence and Van Niel first used natural rubber latex overlay plates for isolation of rubber-degrading bacteria (Spence and Van Niel, 1936). They established a method to develop rubber-degrading colonies on such plates that can be recognized by the production of clear zones through the opaque agar plates, and subsequently reported the appropriate isolation of four rubber-degrading actinomycetes. Since then, common observations reported in different studies on microbial degradation have been that isolates capable of degrading rubber mainly belong to actinomycetes such as *Nocardia* and *Streptomyces* although rubber-degrading bacteria and fungi imperfecti have also been isolated (Linos and Steinbüchel, 2001).

Enzymatic degradation of rubber has also been investigated. Tsuchii and coworker treated latex of NR and IR with the extracellular crude enzyme from an isolated Gram-negative bacterium, designated as *Xanthomonas* sp. strain 35Y (Tsuchii and Takeda, 1990). Chromatographic and ^{1}H and ^{13}C NMR analyses of enzymatic reaction revealed existence of two fractions containing high and low-molecular weight oligomer with characteristic carbonyl end-groups. Successful incorporation of ^{18}O into the degradation dimer under an $^{18}O_2$ atmosphere suggested that oxygenase catalyzed the cleavage of carbon-carbon bonds in the polyisoprene. The partially purified enzyme is associated with typical absorption

spectra of protohemeprotein of oxygenase at 404 and 533 nm, and 50 kDa of molecular mass (Tsuchii and Tokiwa, 1999a). Recently, a gene of *Xanthomonas* sp. whose gene product could be involved in the rubber degradation was cloned (Jendrossek and Reinhardt, 2003). A purified extracellular protein corded by cloned gene, *roxA* was unequivocally characterized as a novel type of oxygenase. The protein, RoxA was 65 kDa molecular mass containing two heme-binding motifs for covalent attachment of heme to the protein (Braaz et al., 2004).

Interest in rubber desulfurization also lies mainly in the area of recycling, in which biological possibilities for desulfurization of rubber products, mainly tire rubber have been demonstrated. Sulfur-oxidizing bacteria, *Acidianus brierleyi*, *Rhodococcus rhodochrous*, *Sulfolbus acidocaldarius*, *S. solfataricus*, *Thiobacillus ferrooxidans*, *T. thiooxidans*, *T. thioparus*, and sulfur-reducing bacteria, *Pyrococcus furiosus* were isolated and applied to the microbial desulfurization (Torma and Raghavan, 1990; Raghavan et al., 1990; Löffler et al., 1995; Romine and Romine, 1998; Bredberg et al., 2001b, d). However, difficulties in biodesulfurization rise from rubber size and toxicity toward microorganisms. In general, microorganism involved cannot diffuse into the rubber materials, hence desulfurization is restricted to the surface region, and toxicity of rubber additives such as curing agents to sulfur-utilizing microorganisms inhibits microbial growth (Christiansson et al., 2000). To date, the utilization of unbound sulfur on rubber vulcanizates by microorganisms has been reported. However, no reports of oxidation and utilization of bound sulfur have been recorded (Warner, 1994). There has been no report of enzymatic and microbial cleavage of sulfide linkages between isoprene chains in rubber products.

White rot fungi are a group of basidiomycete primarily responsible for

biodegradation of lignin in wood. Lignin is a bulky biopolymer composed of various carbon-to-carbon and ether linkages between monomeric phenylpropane units such as arylglycerol-β-aryl ether (β-O-4 substructure), phenylcoumaran (β-5 substructure), diarylpropane (β-1 substructure), biphenyl (5-5 substructure) and resinol (β-β' substructure). Due to its heterogeneity, lignin is resistant to biological attacks by microorganisms. However, white rot fungi are known to degrade lignin in woody plants and they have been a focus of the research interest, in respect to effective utilization of plant biomass resources and application to bioremediation of polluted environment containing various hazardous compounds (Sarkanen, 1971; Crawford, 1981; Gold and Alic, 1993; Cullen, 1997).

White rot fungi produce extracellular lignin-degrading enzymes categorized as three major enzymes, a copper containing polyphenol oxidase, laccase (Lac), and two heme-containing enzymes, manganese peroxidase (MnP) and lignin peroxidase (LiP). It has been generally recognized that lignin biodegradation is initiated by one electron oxidation by these enzymes. In addition to the direct interaction of ligninolytic enzymes to lignin, indirect lignin degradation mechanisms by white rot fungi have also been demonstrated. A white rot fungus, *Ceriporiopsis subvermispora* is known to decompose lignin without erosion of the wood cell walls even after fiber separation (Messner and Srebotnic, 1994; Srebotnic and Messner, 1994; Blanchette et al., 1997). Since the ligninolytic emzymes are not able to penetrate inside of the wood cell walls without erosion (Srebotnik, et al., 1988; Daniel, et al., 1989; Evans et al., 1991), degradation of lignin by *C. subvermispora* must be catalyzed by low molecular mass agents (Messer and Srebotnic, 1994). Extracelllar lipid peroxidation (LPO) has been studied for biodegradation of lignin

(Jensen et al., 1996; Bao et al., 1994; Srebotnik et al., 1997; Kapich et al., 1999), and evidence for the involvement of LPO process in wood decay by *C. subvermispora* has been shown (Enoki et al., 1999). The LPO is initiated by a ligninolytic enzyme, manganese peroxidase (MnP) and its oxidation product, Mn(III) (Bao et. al., 1994; Kapich et al., 1999; Moen and Hammel, 1994; Bogan et al., 1996; Watanabe et al., 2000, 2001), and LPO caused degradation of nonphenolic lignin model and polyaromatic hydrocarbons (PAHs) such as phenanthrene (Moen and Hammel, 1994) and fluorene (Bogan et al., 1996). The indirect free radical-mediated degradation of lignin by a white rot fungus, *C. subvermispora* has attracted our interest to apply the biological system to the degradation of natural and synthetic polymers (Sato et al., 2001).

In the present dissertation, the author studied degradation of vulcanized natural rubber sheets by *C. subvermispora*, and reactions of a rubber model compound, *cis*-1,4-polyisoprene and vulcanized rubbers by LPO catalyzed by oxidative enzymes and transition metals.

In Chapter I, the author presents the first evidence for the degradation of vulcanized natural rubber sheets by basidiomycetes. A lignin-degrading basidiomycete, *C. subvermispora* oxidatively cleaved sulfide bonds in the rubbers on wood cultures. Monosulfide bonds in the rubbers were preferentially cleaved by the basidiomycete.

In Chapter II, vulcanized and nonvulcanized *cis*-1,4-polyisoprene were degraded by LPO catalyzed by oxidative enzymes and transition metals. LPO degraded the vulcanized and nonvulcanized polyisoprene rubbers, depending on the initiators used. The ligninolytic free radical reaction, MnP-catalyzed LPO

depolymerized polyisoprene chains, and intensively decomposed the vulcanized polyisoprene rubber sheets to release isoprene fragments extractable with organic solvents.

In Chapter III, the author demonstrates cleavage of sulfide bonds between isoprenes by LPO catalyzed by MnP. A rubber model compound, di(2-methylpent-2-enyl) sulfide was synthesized and subjected to the reactions. A sulfide bond in the rubber model compound was degraded by LPO of LA and a oxidation intermediate of LA, 2,4-decadienal. Possible mechanisms for the free radical mediated-degradation of sulfide bonds are discussed based on the detailed analysis of the reaction products.

Table 1. Overview of rubber-degrading microorganisms.

Strain designation	Reference
Actinomycetes	
Actinomadura	
A. libanotica	Jendrossek et al. (1997)
Actinomyces	
Actinomyces sp.	ZoBell and Giant (1942)
Actinomyces sp.	Blake and Kitchin (1949)
A. albus	Shapsnikov et al. (1952)
A. aurantiacus	Kalinenko (1938)
A. candidus	Nette et al. (1959)
A. elastica	Söhgen and Fol (1914)
A. elasticus	Nette et al. (1959)
A. fuscus	Söhgen and Fol (1914)
A. longisporusruber	Kalinenko (1938)
Actinoplanes	
A. italicus	Jendrossek et al. (1997)
A. missuriensis	Jendrossek et al. (1997)
A. utahensis	Jendrossek et al. (1997)
Amycolatopsis	
Amycolatopsis sp.	Heisey and Papadatos (1995)
Dactylosporangium	
D. thailandense	Jendroseek et al. (1997)
Gordonia	
Gordonia sp.	Linos et al. (1998, 2001b)
Gordonia sp.	Berekaa et.al. (2000)
G. polyisoprenivorans	Linos et al. (1999, 2001a, b)
G. polyisoprenivorans	Berekaa et al. (2000)
G. westflica sp.	Linos et al. (2002)
Micromonospora	
Micromonospora sp.	ZoBell and Beckwith (1944)
Micromonospora sp.	Jendrossek et al. (1997)
M. aurantiaca	Berekaa et al. (2000)
M. aurantiaca	Linos et al. (2001)
Mycobacterium	
Micobacterium sp.	ZoBell and Beckwith (1944)
M. fortuitum	Linos et al. (2000b)
M. fortuitum	Berekaa et al. (2000)
M. globiforme	Shaposnikov et al. (1952)
M. lacticola	Shaposnikov et al. (1952)
Nocardia	
Nocardia sp.	Hutchinson and Ridgway (1975, 77)
Nocardia sp.	Cundell and Mulcock (1975)
Nocardia sp.	Heisey and Papadatos (1995)
Nocardia sp.	Jendrossek et al. (1997)
Nocardia sp. 835A	Tsuchii et al. (1985, 1996, 1997, 1999b, 2001)
N. asteroids	Hookey (1980)
N. asteroides	Hanstveit et al. (1988)
N. asteroides	Orchard and Goodfellow (1980)
N. brasiliensis	Jendrossek et al. (1997)
Proactinomycete	
Proactinomyces sp.	ZoBell and Beckwith (1944)
P. ruber	Nette et al. (1959)

Strain designation	Reference
Streptomyces	
Streptomyces sp.	Rook (1955)
Streptomyces sp.	Leeflang (1963)
Streptomyces sp.	Taysum (1966)
Streptomyces sp.	Cundell and Mulcock (1975)
Streptomyces sp.	Heisey and Papadatos (1995)
Streptomyces sp.	Jendrossek et al. (1997)
Streptomyces sp.	Rose et al. (2005)
S. acrimycini	Jendrossek et al. (1997)
S. albaduncus	Jendrossek et al. (1997)
S. albogriseus	Jendrossek et al. (1997)
S. antibioticus	Jendrossek et al. (1997)
S. atroolivaceus	Jendrossek et al. (1997)
S. aureocirculatus	Jendrossek et al. (1997)
S. coelicolor	Jendrossek et al. (1997)
S. coelicolor	Bode et al. (2000, 2001)
S. daghestanicus	Jendrossek et al. (1997)
S. flavoviridis	Jendrossek et al. (1997)
S. fradiae	Jendrossek et al. (1997)
S. griseus	Jendrossek et al. (1997)
S. griseus	Bode et al. (2001)
S. griseobrunneus	Jendrossek et al. (1997)
S. griseoflavus	Jendrossek et al. (1997)
S. griseoviridis	Jendrossek et al. (1997)
S. halstedii	Jendrossek et al. (1997)
S. litmanii	Hutchinson and Ridgway (1975)
S. nitrosporeus	Jendrossek et al. (1997)
S. olivaceus	Jendrossek et al. (1997)
S. olivoviridis	Jendrossek et al. (1997)
S. rochei	Jendrossek et al. (1997)
S. tauricus	Jendrossek et al. (1997)
S. violaceoruber	Jendrossek et al. (1997)
Bacteria	
Acinetobacter	
A. calcoaceticus	Bode et al. (2001)
Bacillus	
Bacillus sp.	ZoBell and Grant (1942)
B. mesentericus	Nette et al. (1959)
B. subtilis	Shaposnikov et al. (1952)
Pseudomonas	
P. aeruginosa AL98	Linos et al. (2000a)
P. aeruginosa AL98	Berekaa et al. (2000)
P. citronellolis	Bode et al. (2000)
P. fluorescens	Rook (1955)
Xanthomonas	
Xanthomonas sp. 35Y	Tsuchii et al. (1990)
Xanthomonas sp. 35Y	Braaz et al. (2004)
Fungi (fungi imperfecti)	
Aspergillus	
Aspergillus sp.	DeVries (1928)
Aspergillus sp.	Kwiatkowska and Zyska (1988)

Strain designation	Reference
Aspergillus	
A. oryzae	Kalinenko (1938)
Cladosporium	
C. cladosporioides	Borel et al. (1982)
Fusarium	
Fusarium sp.	Blake et al. (1955)
F. solani	Kwiatkowska et al. (1980)
F. solani	Borel et al. (1982)
Monascus	
M. purpreus	Schade (1937)
M. rubber	Schade (1937)
Paecilomyces	
P. lilacinus	Borel et al. (1982)
Penicillium	
Penicillium sp.	DeVries (1928)
Penicillium sp.	Kalinenko (1938)
P. variable	Williams (1982)
Phoma	
P. eupyrena	Borel et al. (1982)
Spicaria	
S. violacea	Blake at al. (1955)
Stemphyliopsis	
Stemphyliopsis sp.	Blake at al. (1955)
Stemphylium	
S. macrosporoideum	Scott (1920)
Torulopsis	
Torulopsis sp.	Shaposnikov et al. (1952)

Table 2. Overview of rubber-desulfurizing microorganisms.

Strain designation	Reference
Bacteria	
Acidianus	
A. brierleyi	Christiansson et al. (1998)
Pyroccus	
P. furiosus	Bredberg et al. (2001b)
Rhodococcus	
R. rhodochrous	Romine et al. (1995)
Sulfolobus	
S. acidocaldarius	Romine et al. (1995)
S. solfataricus	Christiansson et al. (1998)
Thiobacillus	
T. ferooxidants	Torma and Raghavan (1990)
T. ferooxidants	Löffler et al. (1995)
T. ferooxidants	Romine and Romine (1995)
T. ferooxidants	Christiansson et al. (1997),
T. ferooxidants	Kim and Park (1999)
T. thiooxidants	Torma and Raghavan (1990)
T. thiooxidants	Christiansson et al. (1998)
T. thiopanus	Löffler et al. (1995)
T. thiopanus	Christiansson et al. (1998)

Chapter I

Microbial Scission of Sulfide Linkages in Vulcanized Natural Rubber by a White Rot Basidiomycete, *Ceriporiopsis subvermispora*

I-1. Introduction

Over decades, considerable efforts have been exerted to find a satisfactory way to dispose or recycle enormous quantity of waste rubber. In particular, keen attention has been placed on processes to reuse waste rubber products as a polymer component of reclaimed rubbers. Difficulties with these processes mainly arise from the cleavage of sulfide bonds without intensive damage to the main chains. Ingredients such as curing agents, antioxidants, carbon black and reinforcing fillers also make the problem more complex.

To date, a number of physical, physicochemical and chemical devulcanization processes have been proposed, some of which have been scaled up for use in the rubber industry although there is concern over the negative impact of such technology on the global environment, caused by the emission of CO_2 and other hazardous by-products such as SOx (Warner, 1994). Therefore, microbial devulcanization processes using sulfur-oxidizing and sulfur-reducing bacteria have also attracted interest (Holst et al., 1998; Raghavan et al., 1990; Löffler et al., 1995; Christiansson et.al., 1998; Kim and Park, 1999; Torma and Raghava, 1990; Bredberg et al., 2001a; Romine and Romine, 1998; Bredberg et al., 2001b). Strains belonging to *Thiobacillus* sp (Holst et al., 1998; Raghavan et al., 1990; Löffler et al., 1995; Christiansson et.al.,1998; Kim and Park, 1999; Torma and Raghava, 1990; Bredberg et al., 2001a) and *Sulforobus acidocaldalius* (Romine and Romine, 1998) have been reported to oxidize the sulfur present in rubber to sulfate. Bredberg reported that a sulfur-reducing archaeon, *Pyrococcus furiosus*, desulfurized ethanol-leached cryo-ground tire rubber under anaerobic conditions (Bredberg et al., 2000b). In

these studies, the utilization of unbound sulfur in vulcanized rubber by the microorganisms has been demonstrated, but direct evidence for the utilization of bound sulfur between polyisoprene chains has not been obtained (Warner, 1994).

One of the difficulties with bacterial treatment is suppressing the antibacterial effects of curing and anti-ageing agents in the rubber products. The curing agents, tetramethyl thiurame monosulfide (TMTM) and tetramethyl thiurame disulfide (TMTD), and an anti-ageing agent, *N*-(1,3-dimethylbutyl)-*N*-phenyl-*p*-phenylenediamine (Dusantox 6PPD), have inhibitory effects on the growth of desulfurizng-bacteria such as *Thiobatillus ferooxidans* (Christiansson et al., 2000; Nowaczyk and Domka, 1999) and *Rhodococcus rhodochrous* (Christiansson et al., 2000). Therefore, cryo-grinding and the successive removal of toxic additives by extraction with ethanol have been applied before the bacterial treatments. Bredberg and co-workers reported that a lignin-degrading wood rotting fungus, *Resinicium bicolor*, removed the toxic agents from waste rubber materials, thereby enabling the growth of a devulcanizing-bacterium, *Thiobacillus ferooxidans* (Bredberg et al., 2001c). They reported the potential of wood rotting basidiomycetes for the detoxification of rubber prior to bacterial treatment. However, to our knowledge, there is no report of rubber-degrading basidiomycetes. In the present research, the author first reports that rubber was directly devulcanized by a lignin-degrading wood rotting basidiomycete, *Ceriporiospsis subvermsipora*. The catalysis of fungal devulcanization was analyzed by XPS, DD/MAS NMR, SEM, TGA, swelling in toluene, and elemental analysis.

I-2. Materials and Methods

I-2. 1. Fungal treatments of vulcanized rubber sheets

A natural rubber (NR) sheet (TAKL7007, Tigers Polymer Co., Ltd., Osaka, Japan) was cut into small squares (20 × 20 × 1 mm, 0.65 ± 0.03 g). The rubber was then washed with distilled water and air-dried for 24 h. Eighty grams of sea sand (30-50 mesh, Nacalai Tesque, Kyoto, Japan) containing 20 mL of distilled water was packed into the bottom of a 300 mL Erlenmeyer flask. On the sand, 10 g of beech wood chips containing 10 mL of a nutrient medium composed of 0.7% glucose (Wako Pure Chemicals, Tokyo, Japan) and 0.7% corn steep liquor (Nacalai Tesque) was added. Five of the square sheets of rubber were placed on the wood chips. The medium was sterilized by autoclaving at 121°C for 20 min.

Ceriporiopsis subvermispora FP90031 and *Dichomitus squalens* CBS432.34 were maintained on a potato dextrose agar (PDA) medium (Nissui, Tokyo, Japan) at 4°C. The two strains were inoculated on new PDA plates and cultured for 1 week at 28°C. Five pellets (6 mm in diameter) from the preculture were inoculated onto the wood chip medium containing the squares of NR. Cultivation was carried out statically with 70% humidity for 250 days. After 100, 150, 200 and 250 days, the NR sheets were recovered and the fungal mycelia covering the surface were carefully removed. The rubber sheets were washed with distilled water, dried *in vacuo*, and weighed.

I-2. 2. Scanning electron microscopy (SEM)

The NR sheets were cut into even small pieces (2 × 2 × 1 mm). Each sample was fixed on brass stubs, sputter-coated with a JFC-1200 Fine Coater (JEOL, Tokyo,

Japan), and analyzed with a JSM 5310 Scanning Electron Microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV.

I-2. 3. Thermogravimetric analysis (TGA)

TGA was performed with a TA 2000 (TA Instruments, New Castle, De, USA). The rubber (15 mg) was heated at a rate of 50 °C/min to 650 °C, and maintained in air for 1 min and then in N_2 for the next 7 min at the same temperature.

I-2. 4. Determination of total sulfur content

The total sulfur content of the NR sheets was determined using a total sulfur meter, the EMIA-820W (Horiba, Kyoto, Japan). The NR (50 mg) was weighed in a crucible and mixed with 1 g of iron, 1.5 g of tungsten and 0.5 g of tin. The sample was heated to 2000 °C in a microwave at 18 MHz with 2.3 kW under an oxygen atmosphere. The amount of SO_2 formed by the combustion was determined by IR included in the total sulfur meter.

I-2. 5. Determination of swelling ratio

To analyze the changes in cross-linking density with fungal treatment, the NR sheet was soaked in toluene at room temperature for 24 h. The swelling ratio was calculated as the weight ratio between the dried and swollen state. The measurements were performed in triplicate.

I-2. 6. X-ray photoelectron spectroscopy (XPS)

Covalent bonds in the inner part of the NR sheets were analyzed by XPS. The

NR sheets were cut down the middle into two pieces along with the top and bottom surfaces. Sulfur bonds on the cut surface were determined using an AXIS-ULTRA spectrometer (Shimadzu/Kratos, Japan) equipped with monochromated Al Kα exciting radiation. The cut surface of the rubber was scanned at 1.0×10^{-8} Pa, 40 eV pass energy, 0.1 eV step width and 150 W pass energy (20 mA and 15 kV) with a 1 mm^2 mapping area. Binding energies (BEs) were calibrated using a sulfur 2p signal. The peak area of S-C, S-S, and S-O bonds corresponding to the binding energy at 161.9-162.9 eV, 163.3-164.8 eV, and 165.3-165.9 eV was determined.

I-2. 7. Dipolar decoupling (DD)/magic angle spinning (MAS) solid state ^{13}C nuclear magnetic resonance (NMR) spectroscopy

Quantitative solid state ^{13}C NMR spectra were recorded on a Bruker CMX 300 NMR spectrometer with a DD/MAS probe at a carbon frequency of 75.5 MHz, scanning time of 32000, spinning rate of 3.5 kHz, and a recycled delay of 7 sec.

I-3. Results

I-3. 1. Effect of fungal treatment on the surface morphology and chemical composition of NR sheets

White rot fungi, *C. subvermispora* and *D. squalenes*, were cultivated on a natural rubber (NR) sheet/beech wood meal medium for 250 days. Figure I-1 shows the decrease in the weight of the NR sheets cultured with the two white rot fungi. In the cultivation with *C. subvermispora*, the weight of the NR rubber sheets had decreased by 35.4% after 200 days. SEM demonstrated that the fungal

treatment with *C. subvermispora* caused deep cracks on the surface of the sheets (Figure I-2). In contrast, the weight decrease for the rubber sheets cultured with *D. squalens* for 200 days was just 2.7%, and no morphological changes were observed.

TGA has been used to determine the chemical composition of rubber products. TGA provides information regarding the polymer, oil, carbon black, mineral filler, and ash content of rubber vulcanizates (Maurer, 1969). The author analyzed changes in the chemical composition of the original NR sheets following fungal cultivation (Figure I-3). The weight percentage of organics, inorganics, and carbon black in the NR sheet was 37.3%, 54.4%, and 8.3%, respectively (Figure I-3, A). The ratio of organic and inorganic components for carbon black in the original rubber was calculated to be 4.5 and 6.5, respectively (Figure I-3, B and C). Incubation without fungi for 250 days resulted in a spontaneous decrease in the ratio of organic and inorganic components by 4.4 and 7.6%, respectively (data not shown). Treatment with *D. squales* for 200 days decreased these values by 4.4 and 21.2%, respectively. *C. subvermispora* decreased the organic components by 20.0 %. The fungus caused a significant decrease in the ratio of inorganic components by 74.2% during the 200 day-incubation. X-ray fluorescent analysis demonstrated that the inorganic ingredients were composed of $CaCO_3$ (92.6%), ZnO (4.0%), TiO_2 (1.6%), SiO_2 (1.6%), and Al_2O_3 (0.2%). These results indicate that calcium carbonate used as a filler was preferentially removed by *C. subvermispora*.

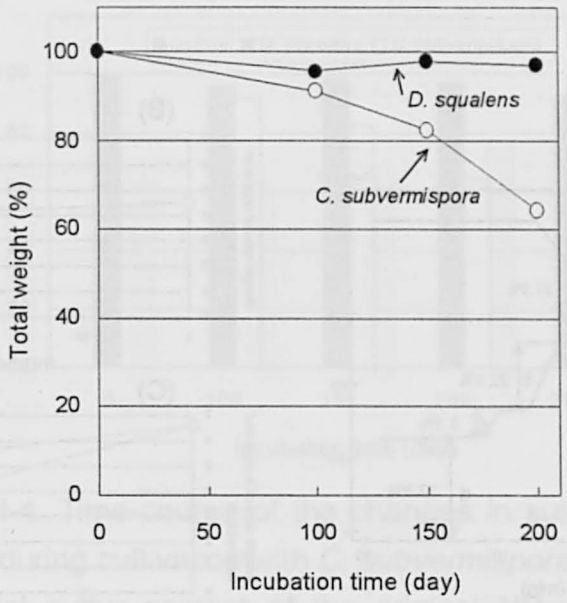


Figure I-1. Weight loss of the NR sheets during cultivation with *C. subvermispora* and *D. squalens*. *Control samples of the NR rubber sheets were maintained for 250 days under the same culture conditions without inoculation by fungi. Weight loss of the control samples was 0.6%.

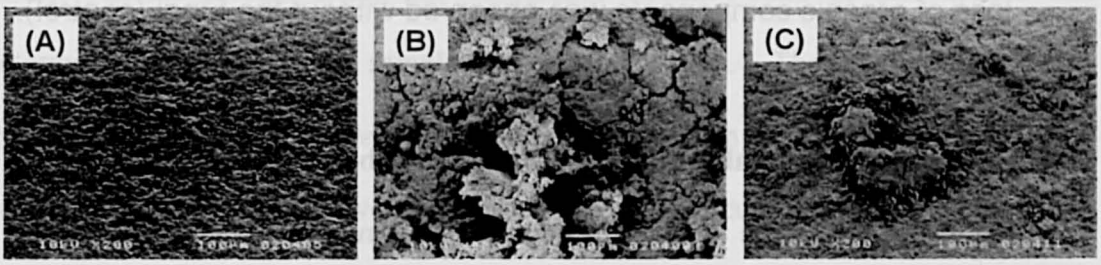


Figure I-2. Scanning electron micrographs of the NR sheets: (A) incubated without fungi for 250 days (control); (B) incubated with *C. subvermispora* for 200 days; (C) incubated with *D. squalens* for 200 days.

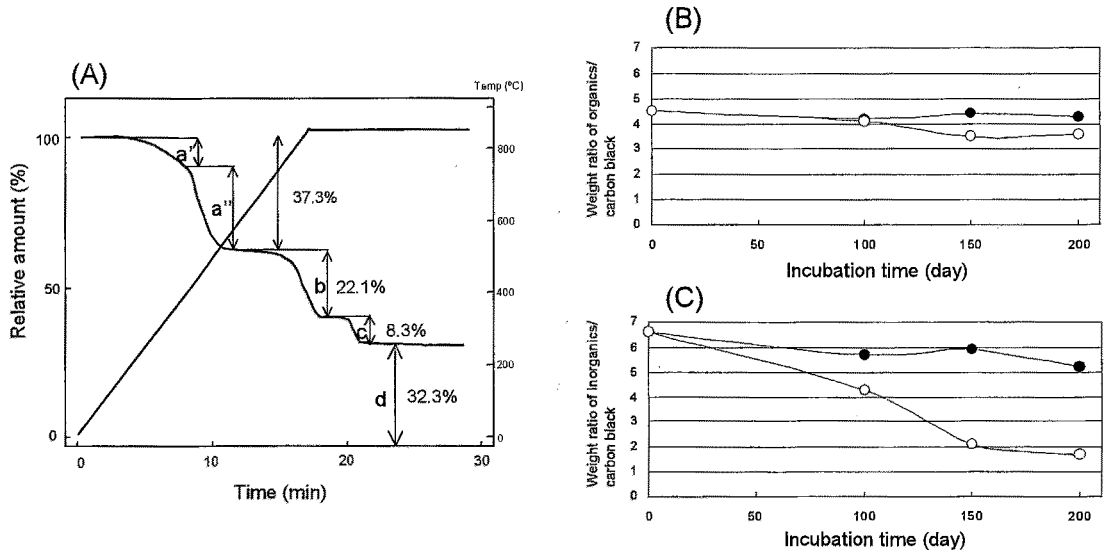


Figure I-3. Analysis of rubber components in the original NR sheet (A) by TG* and changes in the weight ratio of (B) organics/carbon black and (C) inorganics/carbon black by TG**: *(a') volatile organic components; (a") polymer components; (b) CO_2 from $CaCO_3$; (c) carbon black; (d) CaO from ash (d). **_C. subvermispora_ (Open symbol) and _D. squalens_ (Closed symbol). Incubation for 250 days without fungal inoculation caused a decrease in the ratio of inorganics/carbon black from 6.6 to 6.1. No weight loss of organic components was observed without the fungi (control).

I-3. 2. Decreases in total sulfur content of the rubber

Figure I-4 shows the total sulfur content of the rubber. The sulfur content of the original rubber sheets was 1.4%. After 200-days-culture with *C. subvermispora* and *D. squalens*, the sulfur content of the rubber had decreased by 28.9 and 13.4%, respectively. Without the fungal strains, the decrease in sulfur content during the 200 days was 7.0%.

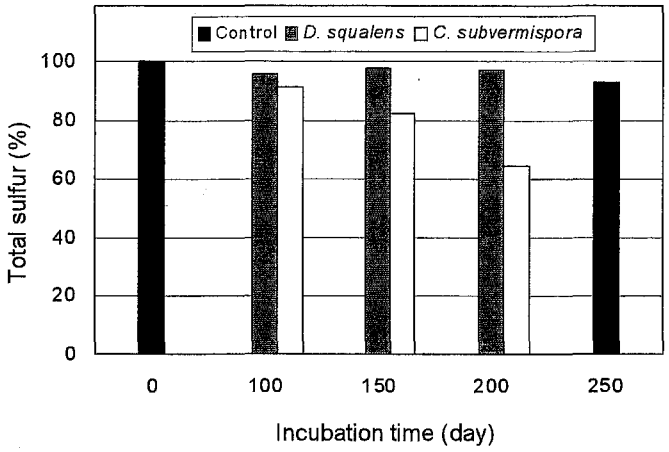


Figure I-4. Time-course of the changes in sulfur content of NR sheets during cultivation with *C. subvermispora* and *D. squalens*. The total sulfur content of the original NR sheets and those incubated for 250 days without inoculation by fungi was measured (control).

I-3. 3. Cleavage of sulfide linkages by white rot fungi

The swelling ratio in organic solvents is correlated with the cross-linkage density of the polymer as explained by the Flory-Rehner equation (Flory and Rehner, 1943). In the present study, the volumetric swelling ratio of the rubbers was determined after soaking the samples in toluene for 24 h. The swelling ratio of the sheets cultured with *C. subvermispora* increased with an increase in the cultivation period (Figure I-5). The swelling ratio of the rubbers cultured with the fungus for 200 days reached 97.9%. In contrast, the swelling ratio of those cultured with *D. squalens* decreased by 11.3% after 150 days. Incubation for 250 days without the fungi decreased the swelling ratio by 6.4%. These results demonstrate that *C. subvermispora* decreased the cross-linking density of the vulcanized rubber.

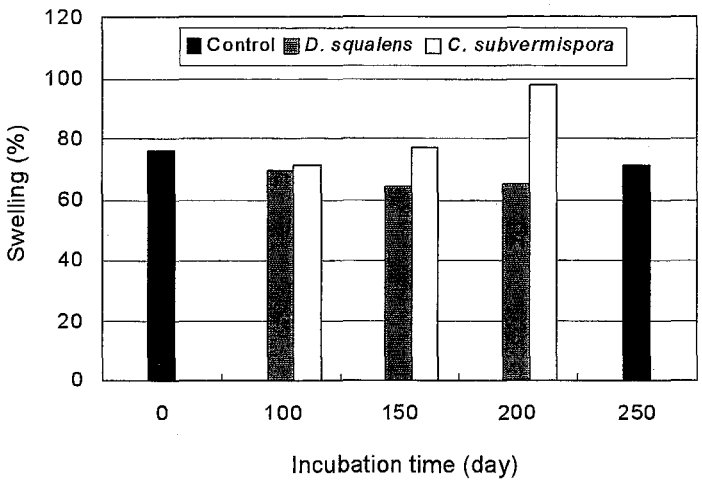


Figure I-5. Swelling ratio of the NR sheets in toluene: The rubber was soaked in toluene for 24 h. The swelling ratio was determined as a weight ratio between the dried and swollen state. The swelling ratio was measured for the original rubber and the rubber samples incubated for 250 days without fungi (control).

I-3. 4. X-ray photoelectron spectroscopy (XPS)

Structural changes in sulfide bonds in the inner part of the NR rubbers were analyzed by XPS. Three-second splits of S 2p peaks at 162.3, 163.7 and 165.7 eV were assigned as the bonding energy for S-C, S-S and S-O bonds, respectively (Moulder et al., 1992). For the analysis by XPS, the rubber sheets were cut horizontally in two along the middle (Figure I-6). Chemical bonds of the newly formed surface were analyzed. Without fungal inoculation, the ratio of S-C to S-S bonds was un-changed after 250-days-incubation. The formation of S-O bonds by autoxidation was not observed in the control experiments. When *C. subvermispora* were inoculated on the same medium, it was found that the fungus decreased the frequency of S-C bonds by 69.0% with the concomitant formation of S-O bonds during cultivation for 200 days. The spectral changes due to the sulfide bond

cleavage became marked after 150 days. In contrast, the ratio of S-C to S-S bonds did not change in the rubber cultured with *D. squalens* although the formation of S-O bonds was observed in the culture.

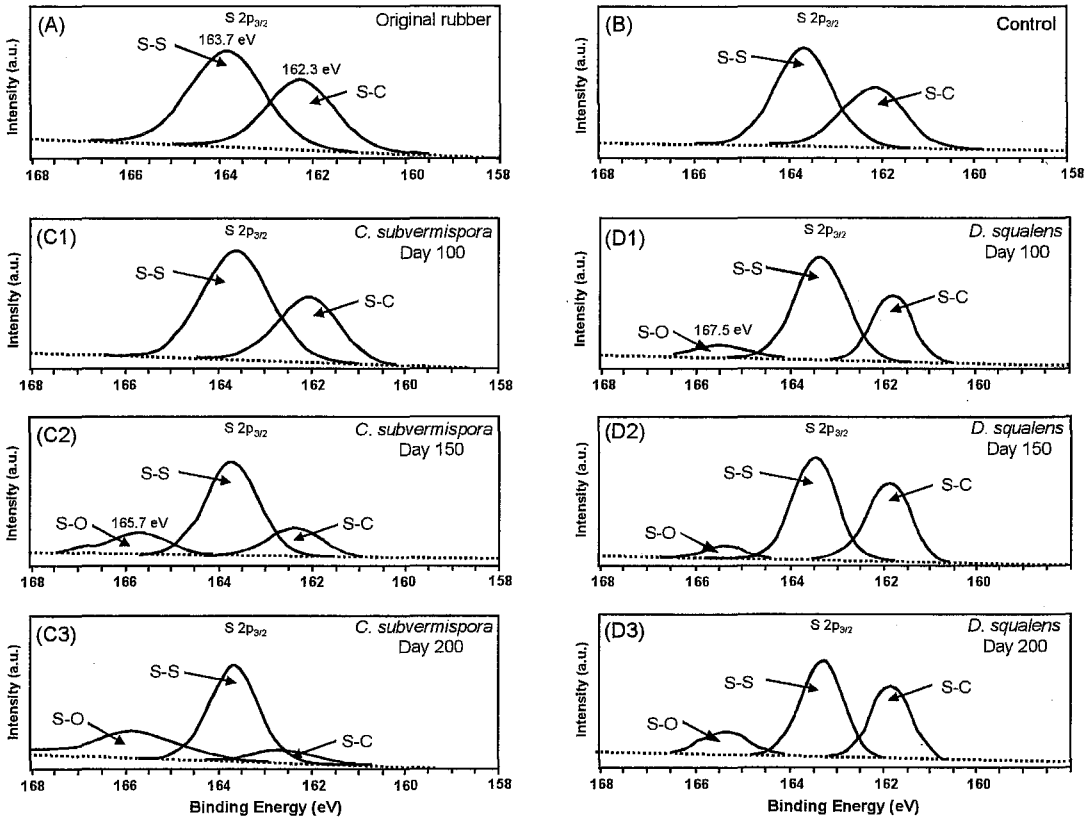


Figure I-6. X-ray photoelectron spectroscopy (XPS) of sulfide bonds in the NR sheets: (A) original NR sheets; (B) incubated for 250 days without inoculation by fungi (control); (C1-3) cultivated with *C. subvermispora* for 100, 150, and 200 days; (D1-3) cultivated with *D. squalens* for 100, 150, and 200 days.

I-3. 5. DD/MAS solid state ^{13}C NMR

^{13}C NMR signals at 44.8, 58.1, 51.0, and 64.5 ppm were assigned as *cis*-mono, *trans*-mono, *cis*-poly and *trans*-polysulfide bonds of polyisoprene according to a

reference (Krejsa and Koenig, 1993) (Figure I-7). In the original rubber, the monosulfide structure constitutes 79.4% of all sulfur-carbon bonds. *C. subvermispora* reduced the frequency of *cis*-mono and *trans*-monosulfide bonds by 34.9% and 39.4%, respectively. The change in the frequency of polysulfide bonds was smaller than that of monosulfide bonds. The decrease in the signal intensity of sulfide bonds caused by *D. squalens* was negligible except for that of *trans*-monosulfide bonds.

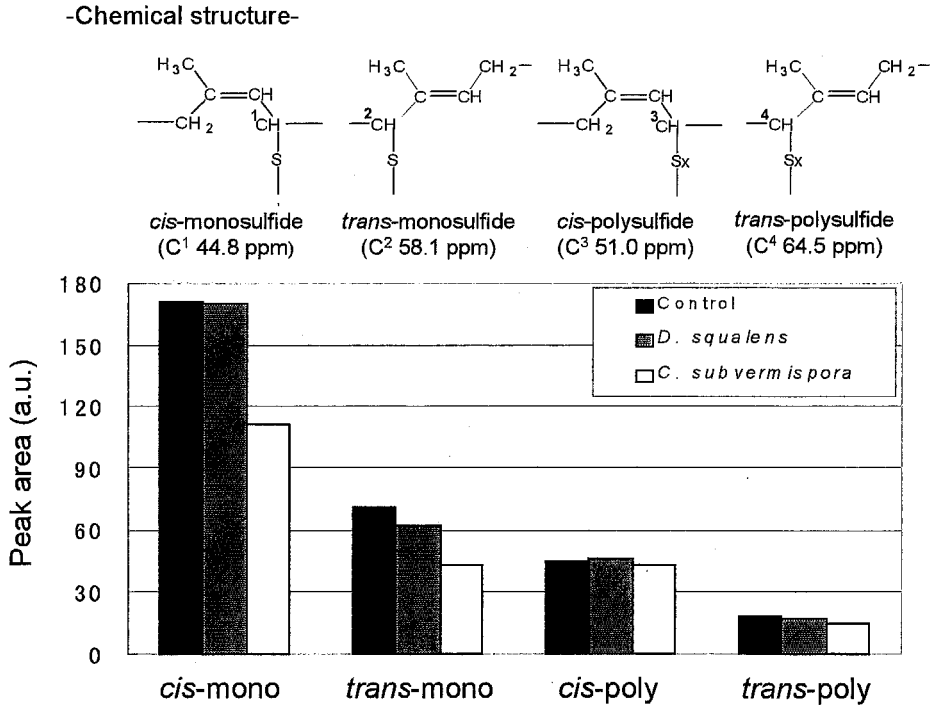


Figure I-7. Analysis of sulfide linkages in the NR sheets by DD/MAS solid state ^{13}C NMR: The rubbers were analyzed after cultivation of *C. subvermispora* and *D. squalens* for 250 days. *Control samples of the rubber were maintained for 250 days under the same culture conditions without inoculation by fungi.

I-4. Discussion

Extensive studies have been made on the microbial degradation of rubbers. The information accumulated to date demonstrates that microorganisms capable of degrading vulcanized or nonvulcanized rubbers are mostly *Actinomycets*, and other Gram-positive and negative bacteria such as *Actinomadura*, *Actinoplanes*, *Gordonia*, *Micromonospora*, *Nocardia*, *Streptomyces*, *Bacillus*, *Xanthomons*, and hundreds of other species as reviewed (Linos and Steinbüchel, 2001). In addition to the bacterial species, several fungi imperfecti such as *Fusarium solani* (Kwiatkowska et al., 1980) and *Penicillium variable* (Williams, 1982) are known to decompose rubber. To our knowledge, there has been no report of basidiomycetes that decompose rubber.

Despite the extensive study of the microbial degradation of rubber, it is still unclear if these microorganisms are able to cleave the sulfide bonds of rubber (Warner, 1994). The desulfurization of old ground rubber crumbs by sulfur-oxidizing or sulfur-reducing bacteria has been widely studied. The sulfur-oxidizing or sulfur-reducing bacteria utilized unbound sulfur in vulcanized rubbers. However, there is no direct evidence for the utilization of bound sulfur between polyisoprene chains (Warner, 1994).

In the present study, the author has clearly demonstrated that a wood rotting basidiomycete, *C. subvermispora*, oxidatively decomposed sulfide bonds in vulcanized NR sheets to decrease their cross-linkage density. The scission of S-C bonds was observed even in the inner part of the rubber sheets as shown in the XPS and DD/MAS ^{13}C NMR spectra (Figures I-6 and I-7). The fungus decomposed S-C

bonds more favorably than S-S bonds in polysulfide bonds.

In contrast to *C. subvermispora*, a white rot fungus, *D. squalens*, did not decrease the cross-linkage density of the vulcanized rubbers as shown in the swelling experiments. XPS and DD/MAS ^{13}C NMR spectra also showed that the cleavage of S-C and S-S bonds by this fungus is negligible (Figures I-6 and I-7). However, S-O bonds were formed during the cultivation as shown in the XPS spectra. These results demonstrate that *D. squalens* is not able to decompose sulfide bridges in polyisoprene chains but can oxidize unbound sulfides in the rubber. *D. squalens* is a strong lignin-degrader like *C. subvermispora*. Both of these fungi secrete manganese peroxidase (MnP) and laccase (Lac) as a lignin-degrading enzyme. In the experiments on rubber degradation, the oxidative cleavage of sulfide bonds by *C. subvermispora* became remarkable after cultivation for 150 days although lignin degradation by this fungus proceeds from an incipient stage of the cultivation to exhibit biopulping effects within 2-4 weeks (Messner and Srebotnik, 1994). It is of importance to elucidate the mechanisms of sulfide bond scission by *C. subvermispora* to develop biological and biomimetic devulcanization processes for the safe disposal and recycling of rubber products.

In conclusion, the author provided the first evidence for microbial cleavage of sulfide bonds in vulcanized rubber. The devulcanization was achieved by a new class of rubber-degrading microorganisms, belonging to basidiomycetes. Most of the rubber-degrading microorganisms described to date have been characterized by an ability to decompose or grow on NR latex or NR films, although several strains such as *Nocardia* (Tsuchii et al., 1985), *Streptomyces* (Rook, 1955), *Fusarium solani* (Kwatkwska et al., 1980), and *Penicillium variable* (Williams, 1982) are known to

degrade solid rubber vulcanizates. In addition to these microorganisms, wood rotting basidiomycetes are potential microorganisms that degrade solid rubber vulcanizates. Biomimetic reactions for the rubber degradation by wood rotting fungi should also be investigated to develop environmentally benign rubber recycling systems (Chapter II and III, Sato et al., 2003).

I-5. Summary

A white rot basidiomycete, *Ceriporiopsis subvermispora*, degraded vulcanized natural rubber (NR) sheets on a wood medium. The fungus decreased the total sulfur content of the rubber by 29% in 200 days, accompanied by the cleavage of sulfide bonds between polyisoprene chains. X-ray photoelectron spectroscopy (XPS) demonstrated that *C. subvermispora* reduced the frequency of S-C bonds by 69% with a concomitant formation of S-O bonds during the culture. Dipolar decoupling/magic angle spinning (DD/MAS) solid state ^{13}C NMR revealed that the fungus preferentially decomposed monosulfide bonds linked to a *cis*- and *trans*-1,4-isoprene backbone but the cleavage of polysulfide bonds was also observed. In contrast, no decrease in weight or devulcanization of rubber was observed in cultures of a white rot fungus, *Dichomitus squalens*. The oxidative cleavage of sulfide bonds by *C. subvermispora* demonstrates that ligninolytic basidiomycetes are potential microbes for the biological devulcanization of rubber products.

Chapter II

Degradation of Vulcanized and Nonvulcanized Polyisoprene Rubbers by Lipid Peroxidation Catalyzed by Oxidative Enzymes and Transition Metals

II-1. Introduction

cis-1,4-Polyisoprene, a main constituent of natural rubber (NR) is biosynthesized by more than 2000 species of plants mostly belonging to the *Euphorobiaceae* (Mooibroek and Cornish, 2000) and by some fungi such as *Lactarius* and *Peziza* (Stewart et al., 1955). As an alternative to biological production, *cis*-1,4-polyisoprene is synthesized chemically to obtain the so-called isoprene rubber (IR). These raw rubber materials are converted to elastic rubber products by the process of vulcanization that leads to cross-links between the elastomer chains (Chapman et al., 1988). The vulcanization gives the rubber elasticity and recalcitrance to chemical and biological degradation.

Global concerns about recycling and safe disposal of waste rubber products have initiated research to decompose recalcitrant rubber products by biological methods. Many microorganisms have been reported to degrade polyisoprene rubbers. Common observations reported in different studies on microbial degradation are that isolates capable of degrading rubber generally belong to actinomycetes such as *Actinomyces*, *Nocardia*, and *Streptomyces* although rubber-degrading fungi and bacteria have also been isolated. Despite a large number of microorganisms responsible for rubber biodegradation, gram-negative bacterium, *Xanthomonas* sp. 35Y is the only microorganism that secretes an extacellular polyisoprenoid-degrading enzyme. (Tsuchii et al., 1990, 1999a; Braaz et al., 2004). The characterized enzyme degraded NR latex and chemosynthetic poly(*cis*-1,4-isoprene) by initiation of oxidative cleavage of one double bond in the polymer chains. The resulting low-molecular-mass oligo(*cis*-1,4-isoprene)

derivatives then are further degraded, presumably by β-scission. In contrast to degradation of nonvulcanized polyisoprere by an extracellular protein with polyisoprene oxygenase activity, there has been no report for degradation of vulcanized soild rubber products by enzymatic systems.

A selective white rot fungus, *Ceriporiopsis subvermispora* degrades a recalcitrant natural polymer, lignin, by producing free radicals from lipids (Srebotnik et al., 1997; Jensen et al., 1996; Bao et al., 1994; Kapich et al., 1999) The lipid peroxidation is initiated by manganese peroxidase (MnP) or its oxidation products, Mn(III) (Bao et al., 1994; Kapich et al., 1999; Watanabe et al., 2000; Moen et al., 1994; Bogan et al., 1996; Watanabe et al., 2001). Degradation of lignin by enzymatic lipid peroxidation attracted our interest into the application of this enzymatic system to the degradation of polyisoprene rubbers.

In addition to the lipid peroxidation by MnP and Mn(III), the author examined rubber degradation by free radicals produced from unsaturated fatty acids by a laccase (Lac)/mediator system, lipoxygenase (LOX), horseradish peroxidase (HRP), and the Fenton reaction. The results demonstrate that the free radicals from unsaturated fatty acids are potent agents which degrade polyisoprene rubbers and that control of the free radical chain reactions by oxidative enzymes and transition metals provides a new strategy for degrading the recalcitrant vulcanized rubbers. The first evidence for degradation of vulcanized rubber products by the catalytic action of a fully characterized enzyme is reported.

II-2. Materials and Methods

II-2. 1. General methods

Linoleic acid (LA) was purchased from Nacalai Tescque (Kyoto, Japan). Arachidonic acid (AA), linolenic acid (LNA), Mn(III) acetate dihydrate and 1-hydroxybenzotriazole (1-HBT) were purchased from Sigma-Aldrich (St. Louis, USA). Synthetic *cis*-1,4-polyisoprene (IR) and vulcanized rubber sheets were supplied by Sumitomo Rubber Industries Co. Ltd. (Kobe, Japan). The vulcanized rubber sheets were prepared by the JIS K 6299 method using a two-roll mill (D = 6 inch) at a composition of *cis*-1,4-polyisoprene rubber 100 phr, stearic acid 2 phr, zinc oxide 3 phr, sulfur 1.5 phr and *N-tert*-butyl-2-benzothiazolylsulfenamide 1 phr. Vulcanization of the rubber was carried out at 150 °C for 30 min.

II-2. 2. Gel permeation chromatography (GPC)

THF solutions of rubbers (1%, w/v) were analyzed by GPC on tandemly-linked Shodex GPC K-803L and GPC KF-806L (8 mm × 300 cm each, Showa Denko, Tokyo, Japan) columns using a Hitachi L-7100 HPLC system (Tokyo, Japan) connected to a Differential Refractomater (Model R401, Waters, Milford, USA). Elution was carried out with THF at a flow rate of 1 mL/min at 40 °C. Weight average (*Mw*) and number average (*Mn*) molecular weights were estimated using a polystyrene standard of *Mw* 761, 4000, 90000, 170000 and 575000 (Pressure Chemical Co., Pittsburgh, USA) and those of *Mw* 2430 and 13700 (Sigma-Aldrich).

II-2. 3. Double shot pyrolysis GC-MS

Double shot pyrolysis GC-MS (Ds-Py-GC-MS) was performed on a Shimadzu GCMS-QP5050A Mass Spectrometer (Kyoto, Japan) equipped with a Frontier Lab Double-shot Pyrolyser PY-2020D (Fukushima, Japan). Separation of compounds was done on a fused silica capillary column, CP-Sil 24CB (30 m × 0.25 mm i.d., Chrompack, Netherlands) using helium as a carrier gas. The temperatures of the injection port and separator were 200 and 300 °C, respectively. Double-shot pyrograms were obtained using a two-step temperature program. For the analysis of volatile compounds, the temperature of the pyrolyzer was first maintained at 50 °C for 1 minute, and then raised to 250 °C at a rate of 8 °C min^{-1}. The programming of the column oven temperature for GC-MS was synchronized with the temperature program of the pyrolyzer. The column oven temperature for GC-MS was first maintained at 50 °C for 1 min, and then raised to 250 °C at a rate of 8 °C min^{-1}. After the analysis of volatile compounds, the column oven was cooled to 50 °C. Pyrolysis was carried out by dropping the sample holder into a pyrolysis port that had been heated to 500 °C. The pyrolysis was done for 1 min. Pyrograms were obtained by GC-MS with the same temperature program described for the analysis of volatile compounds.

II-2. 4. Enzyme preparation

Crude MnP from *Ceriporiopsis subvermispora* FP90031 was collected from 14 day-cultures grown on a wood medium composed of beech wood (4.5 g), wheat bran (0.5 g) and water (15 mL) at 28 °C (Watanabe et al., 2000). The culture filtrate was dialyzed against 20 mM sodium succinate buffer (pH 4.5). The dialyzate was

concentrated by ultrafiltration, and then purified by gel filtration on Superdex 75 PG (1.6 × 60 cm, Amersham Pharmacia Biotech, Sweden) using 20 mM sodium succinate buffer containing 0.1 M NaCl as an eluent. Fractions showing MnP activity were collected and purified on a MONO-Q (HR 10/10) (Amersham Pharmacia Biotech) column. Elution of the MONO-Q column was carried out using a linear gradient program from (A) 20 mM succinate buffer to (B) the same buffer solution containing 1 M NaCl. Fractions showing MnP activity were collected, concentrated and desalted by ultrafiltration with Centriprep YM-30 (cut off, 30,000, Millipore, USA). The RZ value of the purified enzyme was 2.5. Laccase from *Coriolus* sp, lipoxygenase from soybean (112,000 U/mg) and horseradish peroxidase (266 U/mg) were obtained from Daiwa Chemical Industry (Osaka, Japan), Toyobo Co. Ltd., (Osaka, Japan) and Nacalai Tesque Co. Ltd. (Kyoto, Japan), respectively. The commercial enzymes were used without further purification.

II-2. 5. Enzyme assay

Activities of MnP and Lac were assayed with 0.2 mM 2,6-dimethoxyphenol (2,6-DMP) in 25 mM sodium tartrate buffer with and without 0.5 mM $MnSO_4$ and 0.1 mM H_2O_2 (Watanabe et al., 2000). Horseradish peroxidase was assayed in a 25 mM sodium phosphate buffer (pH 6.0) containing 0.2 mM 2,6-DMP and 0.1 mM hydrogen peroxide. Reactions were started by adding enzymes and were quantified by monitoring the initial rate of increase in A_{470}. One unit (U) of these enzymic activities is defined as the amount of enzyme that oxidizes 1 μM 2,6-DMP in one minute. The activity of lipoxygenase (LOX) was measured by monitoring the formation of lipid hydroperoxide from 100 μM linoleic acid in 25 mM sodium borate

buffer (pH 9.0) (Axelrod et al., 1981). One unit of LOX activity is defined as the amount of enzyme that produces 1 μmol of linoleic acid hydroperoxide per minute.

II-2. 6. Degradation of nonvulcanized polyisoprene (IR)

In a test tube (30 × 200 mm), 250 μL of a 10% solution of IR dissolved in $CHCl_3$ was added. The solution was evaporated under reduced pressure below 30 °C to form a cast film of IR around the inner surface of the test tube. In all the reactions, the pre-coated test tube was incubated at 35 °C and 100 rpm for 48-96 h in a buffer (5 mL) solution containing unsaturated fatty acids (9 mM) and the surfactant Tween 20 (0.05%) under an oxygen atmosphere. Linoleic acid (LA), linolenic acid (LNA) and arachidonic acid (AA) were used as an unsaturated fatty acid. After the reaction, 5 mL of $CHCl_3$ was added to the reaction mixture. The solution was shaken vigorously and partitioned into organic and aqueous phases. The organic layer was separated, dehydrated with sodium sulfate and evaporated.

Reactions of MnP were carried out in a 10 mM sodium tartrate buffer (pH 4.5) containing Tween 20, unsaturated fatty acids, manganese sulfate (1 mM), H_2O_2 (400 μM) and the purified MnP (2 U) from *C. subvermispora*. The reaction was started by addition of the enzyme. For the reactions with Mn(III), 50 mM Mn(III)-tartrate was prepared by dissolving Mn(III)-acetate in 50 mM tartaric acid. Reactions were started by mixing the Mn(III)-tartrate (pH 4.5) solution with 500 mL of the 10 mM sodium-tartrate buffer (pH 4.5) containing Tween 20 and unsaturated fatty acids in the pre-coated test tube. The final concentration of Mn(III) in the reaction mixture was 20 mM. Reactions in a laccase-mediator system were started by adding laccase (2 U) to the 10 mM sodium tartrate buffer (pH 4.5) containing Tween 20,

1-hydroxybenzotriazole (0.4 mM) and unsaturated fatty acids. In reactions with HRP, the enzyme (2 U) was added to a 10 mM sodium phosphate buffer (pH 6.0) containing Tween 20, unsaturated fatty acids and H_2O_2 (400 μM) in the pre-coated test tube. Degradation of IR by •OH-induced lipid peroxidation was carried out in 10 mM sodium tartrate buffer (pH 4.5) containing Tween 20, unsaturated fatty acids, ferrous sulfate (1 mM) and H_2O_2 (20 mM) in the pre-coated test tube. Reactions with lipoxygenase (LOX) were performed with soybean lipoxygenase (2 U) in a 10 mM sodium borate buffer (pH 9.0) containing Tween 20 and unsaturated fatty acids.

II-2. 7. Degradation of vulcanized rubber sheets by lipid peroxidation

Treatments of vulcanized rubber sheets by lipid peroxidation were carried out using the reaction systems described for the degradation of IR, except that the pre-coated IR was replaced with vulcanized rubber sheets. The reactions with MnP and Mn(III) were done in 10 mM oxalate buffer (pH4.5), in addition to the tartrate buffer (pH4.5) system described for IR degradation. In the experiments on the vulcanized rubber sheet, two different molar concentrations of linoleic acid (9 and 90 mM) were examined. Control experiments without linoleic acid were also carried out. All the reactions were performed at 35 °C and 100 rpm for 96 h under an oxygen atmosphere. Two pieces of vulcanized rubber sheet (10 mm × 20 mm × 0.5 mm, approx. 100 mg) were placed in a test tube and allowed to react with the reaction systems described for the degradation of IR. After the reaction, the vulcanized rubber sheets were rinsed with acetone and extracted with 20 mL of $CHCl_3$ for 24 h. Insoluble materials were washed with fresh $CHCl_3$, dried, and weighed. The $CHCl_3$ extract was analyzed by double shot pyrolysis GC-MS. The

insoluble materials were analyzed by SEM.

II-2. 8. Scanning electron microscopy

The rubbers were fixed on brass stubs and sputter-coated (JFC-1200 FINE COATER, JEOL, Japan) with a gold layer having a thickness of approximately 15 nm by using argon gas as an ionizing plasma. Imaging was performed with a Scanning Electron Microscope JSM 5310 (JEOL, Tokyo, Japan) with secondary electrons at a 10 kV acceleration voltage and at room temperature.

II-3. Results

II-3. 1. Degradation of IR by lipid peroxidation with MnP and Mn(III)

Nonvulcanized synthetic polyisoprene (IR) was treated with enzymatic and nonenzymatic lipid peroxidation catalyzed by MnP and Mn(III) under an oxygen atmosphere.

Figure II-1 shows the GPC profiles of IR before and after lipid peroxidation with MnP in tartrate buffer. In the MnP-catalyzed lipid peroxidation, intensive degradation of IR was observed in the reactions with linoleic (LA), linoleinic (LNA) and arachidonic (AA) acids. The degradation depended on the presence of peroxidizable fatty acids and an active MnP (Figure II-2). The degradation of IR was also observed in the lipid peroxidation by Mn(III) in tartrate buffer (Figure II-3). As observed in the enzymatic lipid peroxidation by MnP, the degradation of IR by Mn(III) depended on the presence of unsaturated fatty acids.

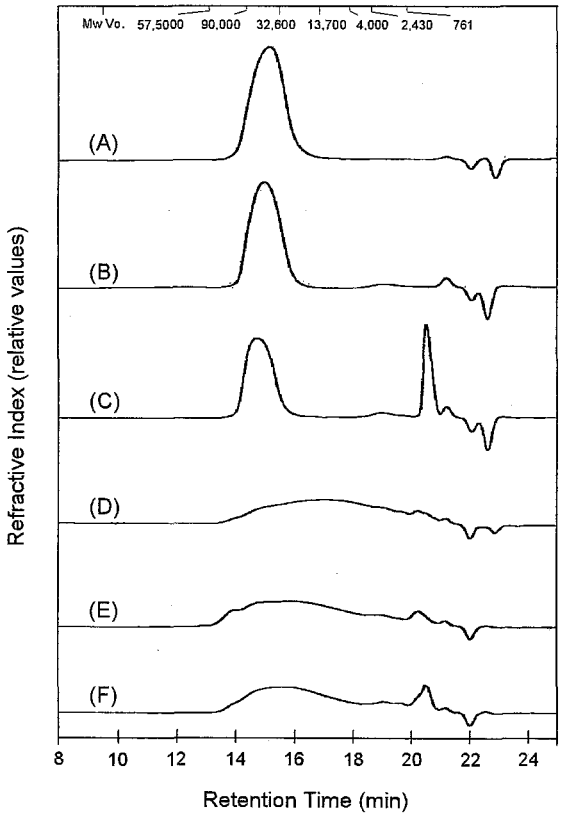


Figure II-1. GPC profiles of IR in the MnP-catalyzed lipid peroxidation of unsaturated fatty acids for 48 h. (A) Control, (B) MnP, (C) inactivated MnP/LA, (D) MnP/LA, (E) MnP/AA, (F) MnP/LNA.

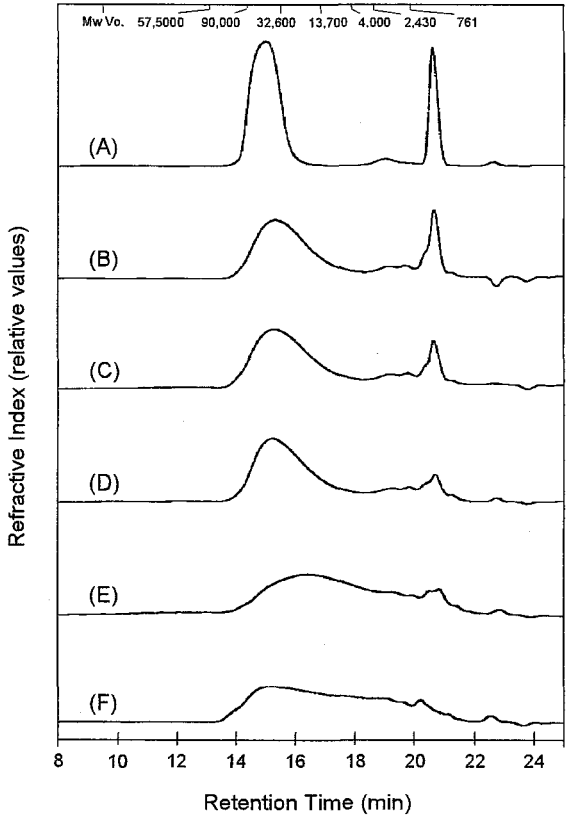


Figure II-2. GPC profiles of IR in the MnP-catalyzed lipid peroxidation of LA. (A) Control (- MnP, 48 h), (B-F) Reactions with MnP/LA. (B) 6 h, (C) 12 h, (D) 24 h, (E) 48 h, (F) 96 h.

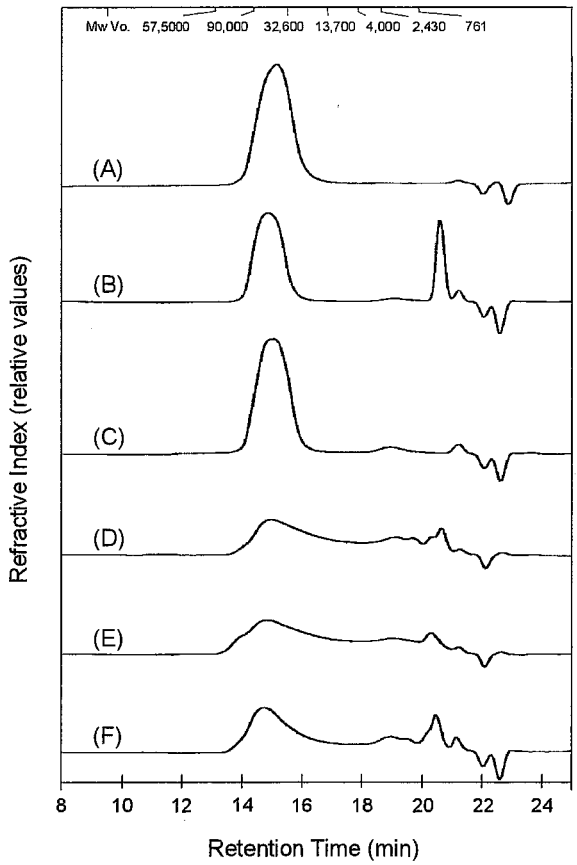


Figure II-3. GPC profiles of IR in the Mn(III)-catalyzed lipid peroxidation of unsaturated fatty acids for 48 h. (A) Control (original IR), (B) autoxidation of LA (- Mn(III)), (C) Mn(III) (-LA), (D) Mn(III)/LA, (E) Mn(III)/AA, (F) Mn(III)/LNA.

II-3-2. Degradation of IR by lipid peroxidation with a laccase/mediator system

Laccase (Lac) is a copper-containing phenol oxidase. In the presence of redox mediators like 1-HBT, Lac is able to oxidize nonphenolic lignin model compounds and recalcitrant polycyclic aromatic hydrocarbons (PAHs) that are not the substrate of Lac due to a high ionization potential. Böhmer and coworkers reported that the Lac/mediator system oxidized unsaturated fatty acids to oxidize phenanthrene into phenanthrene-9,10-quinone and 2,2'-diphenic acid within 182 hours (Böhmer et al.,

1998). In the present study, the lipid peroxidation by the Lac/mediator system was applied to the degradation of IR.

Figure II-4 shows the GPC profiles of IR after the treatment with Lac, 1-HBT and unsaturated fatty acids. IR was degraded by the lipid peroxidation catalyzed by Lac/1-HBT. The degradation of IR depended on the coexistence of Lac, mediators and peroxidizable lipids.

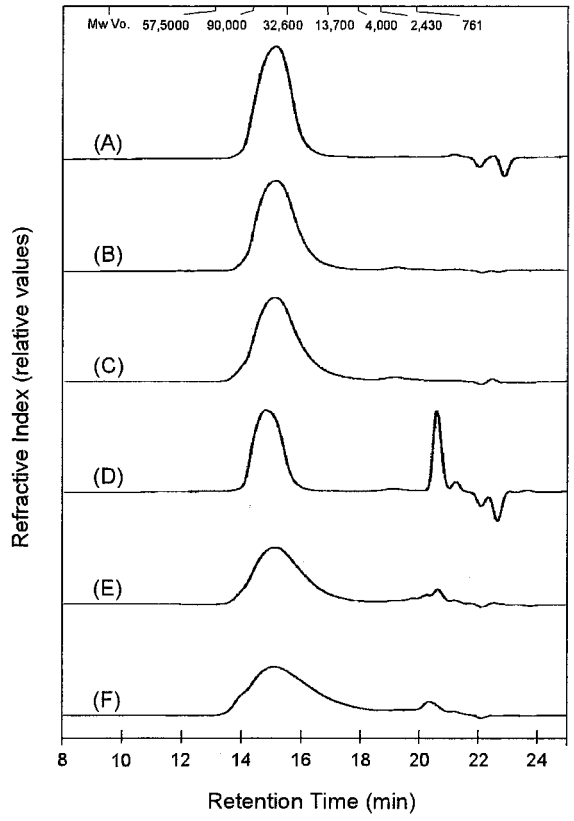


Figure II-4. GPC profiles of IR in the Lac or Lac/1-HBT-catalyzed lipid peroxidation of unsaturated fatty acids for 48 h. (A) Control (original IR), (B) Lac, (C) Lac/1-HBT, (D) Lac/LA, (E) Lac/1-HBT/LA, (F) Lac/1-HBT/AA.

II-3-3. Degradation of IR by lipid peroxidation with the Fenton reaction, HRP and LOX

Figure II-5 depicts GPC profiles of IR after lipid peroxidation of linoleic acid initiated by the Fenton reaction, lipoxygenase (LOX) and horseradish peroxidase (HRP). The Fenton reaction generates hydroxyl radicals according to scheme (I).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \bullet OH \qquad (I)$$

Due to the high redox potentials, hydroxyl radicals directly attack various biomolecules such as DNA, proteins, polysaccharides and lipids to cause cellular injury. In the reactions with unsaturated fatty acids, •OH abstracts hydrogen from the allylic or bisallylic position of unsaturated fatty acids to initiate lipid peroxidation. Figure II-5-(B, C) shows the GPC profiles of IR after the Fenton reaction in the presence and absence of linoleic acid. As shown in Figure II-5-(B), •OH was not able to degrade IR. However, addition of linoleic acid to this system caused intensive degradation of the polymer due to the •OH-induced lipid peroxidation. In contrast to the Fenton system, lipid peroxidation by LOX was not effective for the degradation of IR although LOX initiates peroxidation of linoleic acid by abstracting hydrogen from the *bis*-allylic position. Partial degradation of IR was observed when lipid peroxidation was initiated by HRP. The differences in IR degradation among the peroxidation systems demonstrate that free radical chain reactions after the initial hydrogen abstractions control the breakage of the *cis*-1,4-polyisoprene backbone.

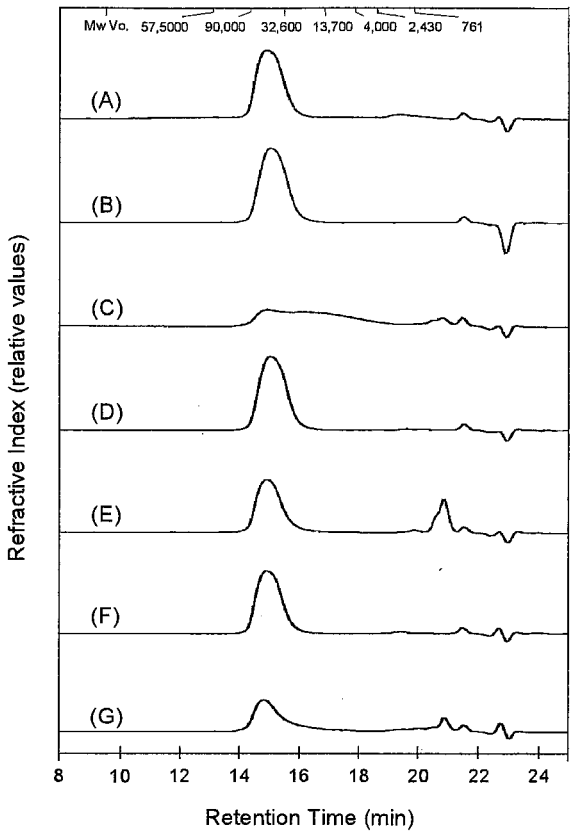


Figure II-5. GPC profiles of IR in lipid peroxidation of LA catalyzed by the Fenton reaction, LOX and HRP for 48 h. (A) Control (original IR), (B) the Fenton reaction (- LA), (C) the Fenton reaction/LA, (D) LOX (- LA), (E) LOX/LA, (F) HRP (- LA), (G) HRP/LA .

II-3. 4. Degradation of vulcanized rubber sheets

Cross-linking of polyisoprene chains by sulfur bonds makes the rubber recalcitrance to chemical and biological degradation. In the present study, enzymatic and nonenzymatic lipid peroxidation was applied to the degradation of vulcanized rubber sheets made from chemically defined components including the IR the same as that used for the degradation of nonvulcanized polyisoprene.

When the vulcanized rubber sheets were treated with unsaturated fatty acids and initiators for the lipid peroxidation, it was found that some of the unsaturated fatty

acids and their degradation products were adhered to the surface of the rubber sheets. This affected the analysis of weight decrease and observations by SEM. Therefore, degradation of the rubber sheets was analyzed after $CHCl_3$ extraction.

Figure II-6 shows the decrease in weight of the vulcanized rubber sheets after lipid peroxidation and subsequent $CHCl_3$ extraction. Oxidation of 90 mM LA by the Fenton reaction in the presence of the vulcanized rubbers resulted in a weight decrease of 80%. Without the unsaturated fatty acids, the vulcanized rubber sheets were not degraded by the Fenton reaction. Hydroxyl radicals are highly oxidative oxygen species. The radicals decompose poly(α-hydroxy acids) through abstraction of hydrogen atoms adjacent to the carbonyl groups. Thus, the Fenton reaction degraded polycaprolactone and poly(DL-lactic acid) (Ali et al., 1994) which have a relatively labile hydrogen located vicinal to the carbonyl group. In contrast to the polyesters having electron-withdrawing carbonyl groups, IR and vulcanized rubbers were not degraded by the Fenton reaction (Figure II-5 and II-6). However, in the presence of unsaturated fatty acids, the •OH-producing system induced lipid peroxidation to decompose the vulcanized rubbers.

Oxidation of 90 mM linoleic acid by HRP resulted in a decrease in the weight of the vulcanized rubbers by 14%. This enzymatic degradation depended on the presence of unsaturated fatty acids. Lipid peroxidation by lipoxygenase (LOX) caused no significant decrease in weight of the vulcanized rubbers. Lipid peroxidation by MnP in tartrate (TA) buffer caused no weight decrease but the reactions by MnP in oxalate (OX) buffer at the same pH resulted in a weight decrease of 48%. Differences of reactivity between the chelators for manganese ions were also observed in the reactions with Mn(III). Mn(III)-oxalate decreased

the weight of the vulcanized rubbers by 81% while no weight decrease was observed in the reactions with Mn(III)-tartrate. Degradation of rubbers by MnP and Mn(III) depended on the presence of unsaturated fatty acids.

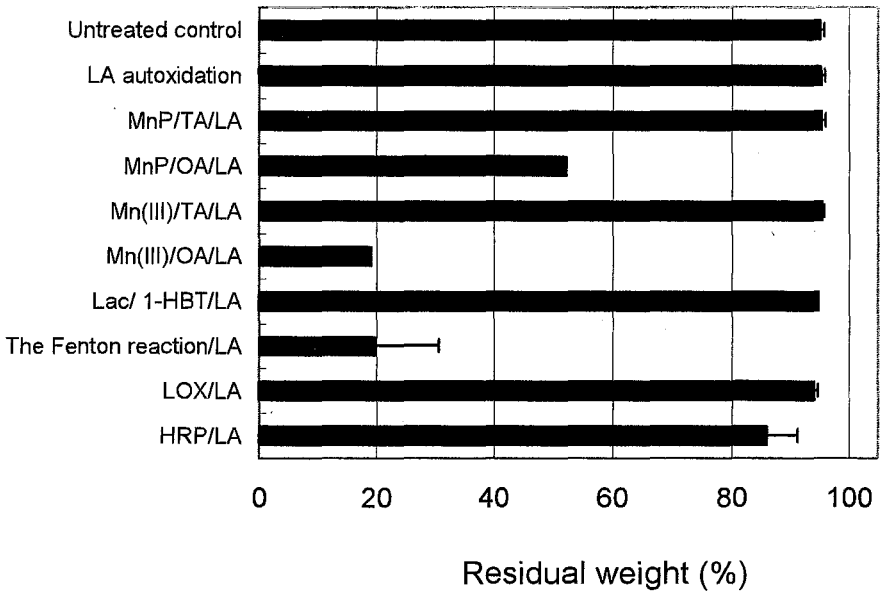


Figure II-6. Weight of residual polymer after lipid peroxidation and subsequent $CHCl_3$-extraction of vulcanized rubber sheets. TA: tartrate, OX: oxalate.

II-3. 5. Analysis of vulcanized rubber sheets by DS-Py-GCMS

Double shot pyrolysis GC-MS (DS-Py-GCMS) is a method to distinguish pyrolyzed fragments from volatile compounds that had been originally present in the sample. Degradation of the vulcanized rubber sheets by lipid peroxidation was analyzed by DS-Py-GCMS. After the removal of volatile compounds, pyrolysis GC-MS of the $CHCl_3$ extract was carried out. In the lipid peroxidation with the Fenton reaction, (a) isoprene, (b) 1,4-dimethyl-4-vinylcyclohexene, (c) 1-methyl-5-(1-methylethenyl)-cyclohexene and (d) limonene were detected at 3.95,

10.68, 11.80 and 12.28 min, respectively (Figure II-7). The pyrograms were similar to those reported for polyisoprene rubbers (Choi, 1999) The dimers (b)-(d) are formed by intermolecular cyclization and subsequent chain scission of the CH_2-CH_2 bond in polyisoprene structures. The Diels-Alder reaction of two isoprene monomers also gives the dimer (d) (Figure II-8) (Choi, 1999; Groves et al., 1991; Hackathorn et al., 1972). In the degradation of vulcanized rubbers by lipid peroxidation with the Fenton system, HRP and MnP in oxalate buffer, the marker compounds for isoprene structures (a)-(d) were produced at more than 10 times the level observed in the control. This demonstrates that isoprene fragments were liberated from the vulcanized rubbers by enzymatic and nonenzymatic lipid peroxidation. Pyrolysis of the residual polymer after the $CHCl_3$ extraction gave isoprenoid fragments with DP 1-6 as reported for the pyrolysis of polyisoprene rubbers (Groves et al., 1991). However, no marked differences in the pyrograms were found between the control and any of the reaction systems (data not shown).

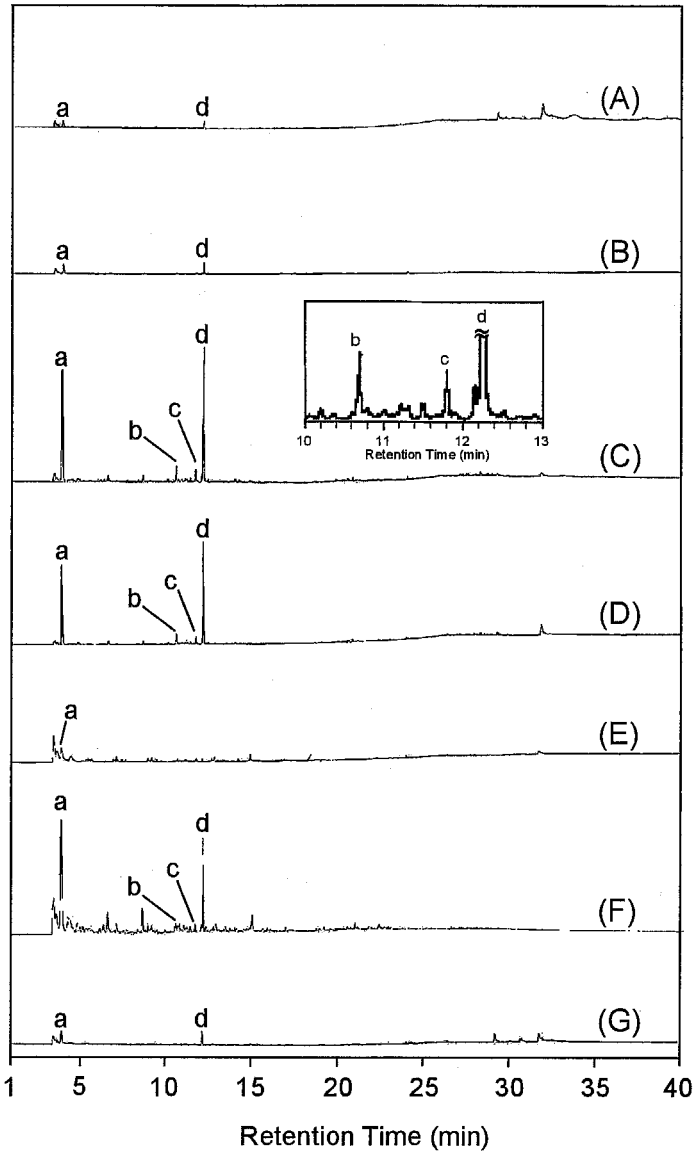


Figure II-7. Pyrograms of $CHCl_3$ extracts from vulcanized IR sheets before (A) and after the lipid peroxidation catalyzed by (B) autoxidation, (C) the Fenton reaction, (D) HRP, (E) MnP/TA, (F) MnP/OX and (G) Mn(III)/TA. Peaks, a, b, c and d were identified as pyrolyzed polyisoprene products. a; isoprene, b; 1,4-dimethyl-4-vinylcyclohexene, c; 1-methyl-5-(1-methylethenyl)-cyclohexene, d; limonene.

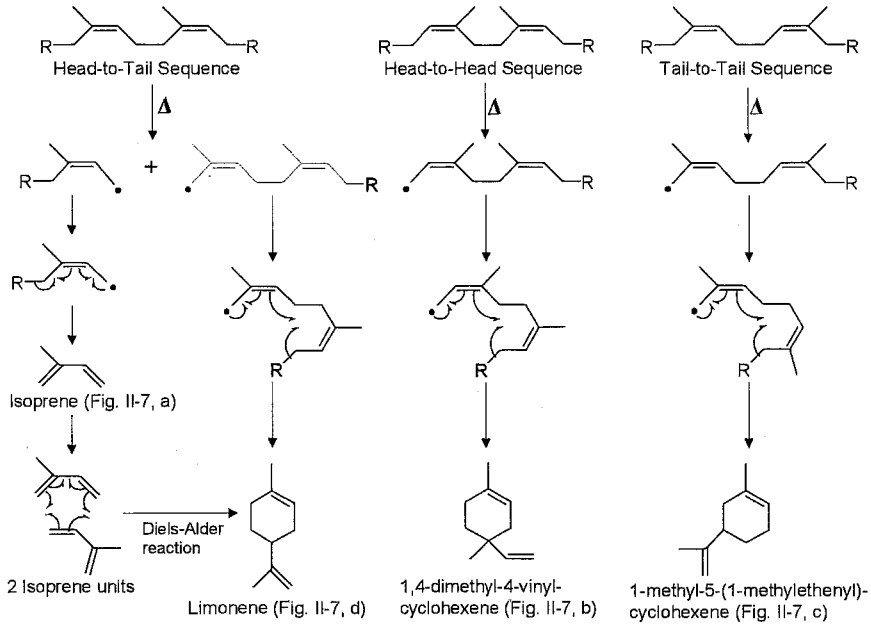


Figure II-8. Pathways for the formation of monomer and dimer components from polyisoprene rubbers by pyrolysis.

II-3-6. Analysis of vulcanized rubber sheets by SEM

Surface morphology changes were analyzed by SEM. In the presence of unsaturated fatty acids, the Fenton reaction caused lipid peroxidation to erode the vulcanized rubber sheets (Figure II-9). Deep cracks and erosion were observed. In contrast, flaky structures were observed on the surface of the rubbers treated using the HRP/lipid system. If unsaturated fatty acids were omitted, the rubber sheets were not degraded by the Fenton reaction and HRP. The analysis by SEM unequivocally proved that rubber sheets are decomposed by lipid peroxidation with MnP in oxalate (OX) buffer. Surface morphology changes were also observed in the lipid peroxidation by Mn(III) and MnP in tartrate (TA) buffer. In the lipid peroxidation by Mn(III) in oxalate buffer, observation by SEM was impossible due to gelation of the residual rubber. The surface morphological changes to the rubber

sheets caused by MnP and Mn(III) depended on the presence of unsaturated fatty acids (data not shown).

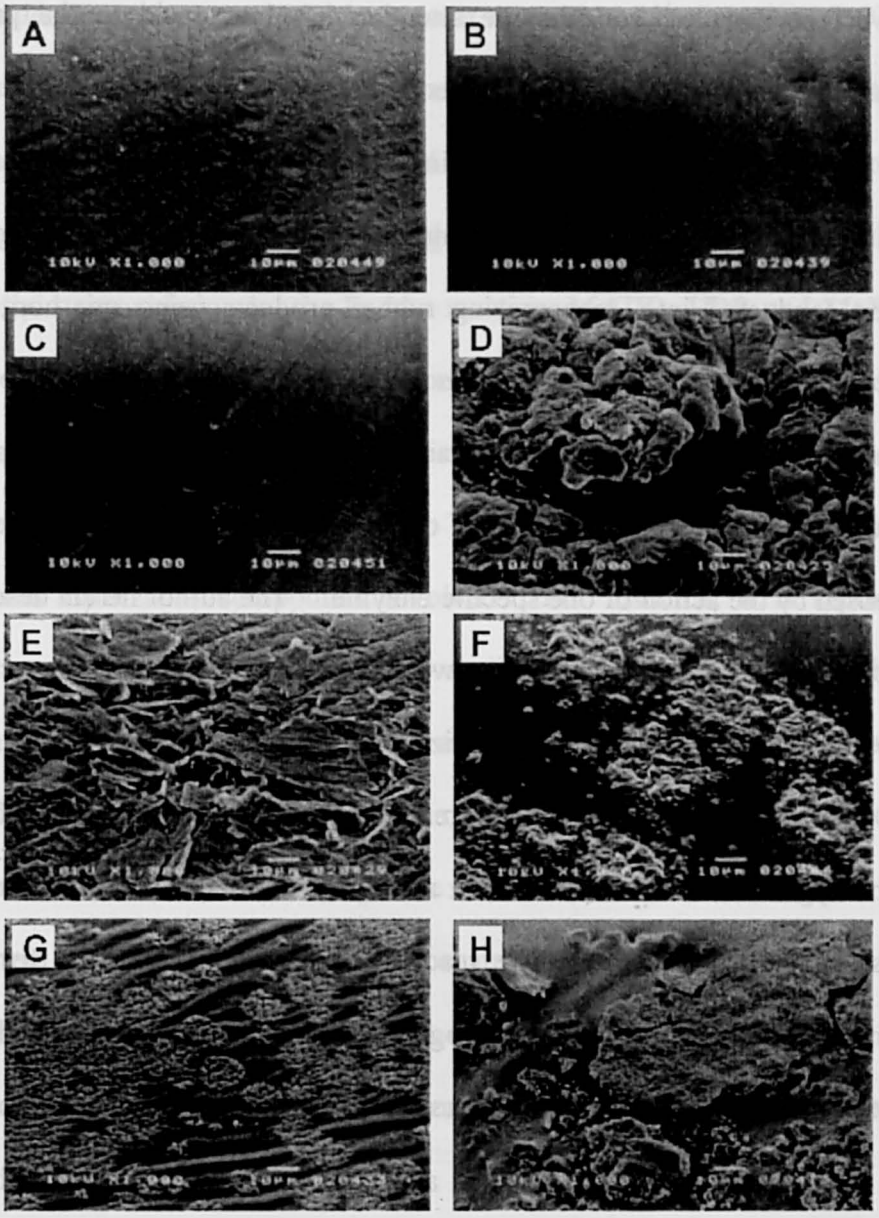


Figure II-9. Scanning electron micrographs of the surface of vulcanized rubber sheets. (A) nontreated control, (B) LOX/LA, (C) the Fenton reaction (- LA), (D) the Fenton reaction/LA, (E) HRP/LA, (F) Mn(III)/TA/LA, (G) MnP/TA/LA and (H) MnP/OX/LA.

II-4. Discussion

Over the decades, considerable attention has been paid to the problem of disposing or recycling rubber products. Biotechnological methods in general present promising prospects for finding solutions to the future demands of environmentally benign rubber recycling systems. Therefore, extensive research has been conducted into decomposing rubber products using microorganisms or enzymes. In spite of numerous studies on the microbial degradation of rubbers, there has been no report of the degradation of solid rubber products by fully characterized enzymes. It was an open question whether rubbers can be decomposed by the action of one specific enzyme. The author herein described that nonvulcanized and vulcanized rubbers were degraded by the catalytic action of an oxidative enzyme that has been characterized at the molecular level.

In rubber degradation, many different reactions have been suggested to occur between oxygen and rubbers (Barnard et al., 1972; Morand, 1977; Mead et al., 1978; DeVries, 1979; Boon, 1988). The reactions proposed involve the formation of rubber hydroperoxides initiated by hydrogen abstraction from the α-methylene group adjacent to the double bond by ROO•, responsible for the destruction of the network due to the scission of main chains and crosslinks. A nitroxylradical from 1-hydroxybenzotriazole is also reported to initiate scission of isoprene units in *cis* and *trans*-1,4-polyisoprene (Enoki et al., 2003). The process of deterioration strongly suggests that rubbers are susceptible to attack by free radicals from peroxidizable organic compounds under an oxygen atmosphere. Therefore, we applied lipid peroxidation to the degradation, and found that rubbers were degraded

by free radical reactions controlled by oxidative enzymes or by transition metals.

Striking differences in the degradation of rubber have been found between the lipid peroxidation systems used in the present research. Lipoxygenase (LOX) is an oxygenase that abstracts hydrogen directly from the *bis*-allylic positions of unsaturated fatty acids. Although LOX catalytically oxidized unsaturated fatty acids, degradation of the IR and rubber sheets by this enzyme was negligible. In contrast, lipid peroxidation by the Fenton reaction, Mn(III), HRP, and MnP caused intensive degradation of the IR. Lipid peroxidation is a chain reaction producing free radicals. A wide variety of free radical species with different redox potentials are produced during the chain reactions. Type of the free radicals depends on the catalyst and oxidizable compounds involved. For instance, it is reported that peroxidation of linoleic acid by LOX was not effective for pulp bleaching but oxidation of the same fatty acid with MnP effectively bleached the pulp (Ehara et al., 2000). Both of these two enzymes can oxidize linoleic acid but the free radical species produced in these two enzymatic chain reactions were different. In the LOX system, peroxyl radicals serve as a chain-carrying radical while acyl radicals play a central role in the MnP-catalyzed chain reactions (Watanabe et al., 2000). Roles of the free radicals in the degradation of recalcitrant compounds are not fully understood.

A number of significant differences were observed in the thermo-oxidative degradation of sulfur vulcanizates, as compared with nonvulcanizates and peroxide vulcanizates. In the present study, the author found that the sulfur-cross linkages affected the degradation of rubbers differently depending on the catalysts for lipid peroxidation. The suppressive effects of sulfur cross-linkages on the rubber

degradation were remarkable in the MnP-catalyzed lipid peroxidation, while the Fe(II)/H_2O_2/lipid system degraded the vulcanized rubbers to the same extent as observed in the degradation of IR. In the degradation of vulcanized rubber sheets by MnP and Mn(III), the rubber sheets were decomposed intensively in oxalate (OX) buffer while the same reactions in tartrate (TA) buffer resulted in attack of surface of the rubber sheets with no substantial weight loss. Mn(III) oxidizes oxalate to produce $CO_2^{\bullet-}$ and $O_2^{\bullet-}$ under an oxygen atmosphere (Khindaria, 1994) while tartrate is decomposed by Mn(III) to form glyoxal, accompanied by emission of chemiluminesence (Watanabe et al., 2001). Oxalate promotes redox cyling of manganese by reducing MnO_2 (Perez-Benito et al., 1996). Different degradation pathways of these oxidizable chelators should change the chain reactions of manganese-dependent lipid peroxidation to affect on the decomposition of vulcanized rubbers. This raises a new issue, to elucidate the interaction between the free radicals from lipids and chelators.

II-5. Summary

In spite of numerous reports concerning the biodegradation of rubber materials, there has been no report of degradation of solid rubber products by fully characterized enzymes. In the present paper, the author presented a new method to decompose nonvulcanized and vulcanized polyisoprene rubbers by controlling the free radical chain reactions of lipids using oxidative enzymes, manganese peroxidase (MnP), laccase (Lac), and horseradish peroxidase (HRP). Nonvulcanized synthetic polyisoprene (IR) was degraded by the free radicals from unsaturated fatty acids

produced by MnP, HRP, and a combination of Lac/1-hydroxybenzotriazole (1-HBT). In contrast, lipoxygenase caused no apparent degradation. Degradation of IR was also observed in lipid peroxidation initiated by the Fenton reaction (FR) and Mn(III), an oxidation product produced by MnP. Vulcanized polyisoprene rubber sheets were degraded by the lipid peroxidation initiated by HRP, MnP, Mn(III), and FR. Pyrolysis GC-MS analysis demonstrated that the lipid peroxidation liberated isoprenoid fragments from the vulcanized rubbers.

Chapter III

Degradation of Aliphatic Sulfide Linkages by Lipid Peroxidation Catalyzed by Manganese Peroxidase

III-1. Introduction

Transition metal-induced lipid peroxidation (LPO) has been extensively studied, in relation to various chronic diseases, aging, and cell signaling. The lipid peroxidation by a lignin-degrading enzyme, manganese peroxidase (MnP) is involved in lignolysis by a selective white rot fungus, *Ceriporiopsis subvermispora*. The lipid peroxidation of an unsaturated fatty acid, linoleic acid (LA) is initiated by MnP or its oxidation products, Mn(III). In the oxidation of LA with MnP, formation of alkoxyl radicals from pentadienyl radical is an initial key step in the lipid peroxidation process. In the oxidation of LA with MnP, products originating from C_9-C_{10} bond cleavage of the 9-alkoxyl radical, octanoic acid and decadienal were produced, together with a C_8-C_9 bond cleavage product of the 9-alkoxyl radical, octanoic acid. The key intermediate, 2,4-decadienal was directly oxidized by MnP to yield glyoxal, glycolaldehyde, and 2-octenal *via* C_4-C_5 cleavage of 4,6-epidioxy-2-decenal intermediate. The reaction of 2,4-decadienal with MnP also produced 2-butendial, hexanal, and pentanal as a C_5-C_6 cleavage product of 3-hydroperoxy-4,6-epidioxydecanal. 2-Butendial was directly oxidized by MnP to produce glyoxal. Thus, lipid peroxidation by MnP proceeds a unique aldehydic lipid peroxidation pathway from LA to glyoxal involving β-scission of the alkoxyl radicals and direct oxidation of the alkadienal by MnP.

In Chapter I, the author described that a white rot fungus, *Ceriporiopsis subvermispora* degraded vulcanized natural rubber sheets on a wood medium. The fungus changed surface morphology of the rubber sheet and decreased the total sulfur content of the rubber, accompanied by cleavage of sulfide bonds between

polyisoprene chains (Sato et al., 2004). In Chapter II, the author demonstrated that vulcanized and nonvulcanized *cis*-1,4-polyisoprene rubber were extensively degraded by lipid peroxidation of unsaturated fatty acids catalyzed by manganese peroxidase from *C. subvermispora*, and its oxidation product, Mn(III) (Sato et al., 2003). The free radical chain reactions involving carbon-centered, alkoxyl, and peroxy radicals from unsaturated fatty acids degraded polyisoprene chains. In this Chapter, the author synthesized di(2-methylpent-2-enyl) sulfide as a rubber model compound, and analyzed scission of a sulfide bond in the rubber model by the MnP-catalzyed lipid peroxidation of linoleic acid and its breakdown products, 2*E*,4*E*-decadienal, 2*E*-butenal, 2*E*-butene-1,4-dial, to obtain the direct evidence for the cleavage of a aliphatic sulfide linkages by lipid peroxidation by MnP.

III-2. Materials and Methods

III-2. 1. General methods

2-Methyl-2-pentenal was purchased from Sigma-Aldrich (St Luis, USA). Linoleic acid was obtained from Nacalai Tesque (Kyoto, Japan). 2*E*,4*E*-Decadienal and 1,1,4,4-tetramethoxy-2*E*-butene were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 2*E*-Butenal was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Milli-Q™ water and peroxide-free organic solvent were used throughout this study. All of the chemicals used were of analytical reagent grade. 2*E*-Butene-1,4-dial was synthesized by hydrolysis of 1,1,4,4-tetramethoxy-2*E*-butene according to the method by Chen et al. (Chen et al., 1995).

III-2. 2. Product analysis

EI-GC-MS analysis was performed on Shimadzu GCMS QP-5050A mass spectrometer (Kyoto, Japan) with ionization energy of 70 eV. Separation was carried out on CP-SIL 8CB (50 m × 0.25 mm i.d. Chrompack) column using herium as a carrier gas. Temperatures of the injection and interface of GC were 280 and 300 °C, respectively. The column oven temperature was maintained at 50 °C for 4 min, and then raised to 250 °C at a rate of 5 °C $min^{-1}$and maintained for 9 min. ^1H NMR spectra of synthesized compounds were recorded with a JEOL λ-400 NMR spectrometer (^1H: 400 MHz) in $CDCl_3$ at 22 °C using TMS as an international standard.

III-2. 3. Synthesis of a rubber model compound

2-Methyl-2-pentene-1-ol, *S*-methylpent-2-enylthiuronium chloride, 2-methyl-2-pentene-1-thiol, and di(2-methylpent-2-enyl) sulfide were synthesized as reported (Evans et al., 1962). 2-Methyl-2-pentene-1-chloride was synthesized according to the method reported by Calzada (Calzada and Hooz, 1988). The synthetic route for the rubber model compound is shown in scheme III-1.

III-2. 3. 1. 2-Methyl-2-pentene-1-ol (2)

A diethyl ether solution of 2-methyl-2-pentenal (1) (0.204 mol) (20 mL) was added to 60 mL of diethyl ether containing $LiAlH_4$ (0.0612 mol), and the solution was stirred at 22 °C. After 40 min, ice water was added to dissolve $LiAlH_4$ salt. The solution was acidified with a few drops of 15% sulfuric acid and extracted with diethyl ether. The solution was dried over sodium sulfide, filtrated, concentrated,

and distilled under reduced pressure to obtain 2-methyl-2-pentene-1-ol in a yield of 91.8%. Mass spectral data of the compound (2): *m/z* (relative intensity) 100 (M^+, 18), 85 (8), 82 (8), 71 (78), 57 (22), 43 (76), 41 (100). ^{1}H NMR spectra: δ (CDCl3) 0.97 (3H, t, J = 6.7 Hz, $CH_2C\underline{H}_3$), 1.66 (3H, s, $C(CH_3)$), 1.94 (1H, s, OH), 2.04 (2H, m, $CHC\underline{H}_2CH_3$), 3.98 (2H, s, $C\underline{H}_2OH$), 5.40 (2H, t, J = 6.7 Hz, CH).

III-2. 3. 2. 2-Methyl-2-pentene-1-chloride (3)

2-Methyl-2-pentene-1-chloride was synthesized by adding triphenylphosphine (0.13 mol) to 2-methyl-2-pentene-1-ol (0.1 mol) (2) in carbon tetrachloride (90 mL). The mixture was refluxed for 1 h, and cooled to room temperature. Dry pentane (100 mL) was added and stirred for 5 min. Precipitates of triphenylphosphine oxide formed were filtrated and washed with 50 mL of pentane. The product was concentrated by a rotary evaporator to remove pentane and the residual part was distilled under reduced pressure to give 2-methyl-2-pentene-1-chloride in a yield of 37%. Mass spectral data of the compound (3): *m/z* (relative intensity) 118 (M^+, 13), 83 (12), 69 (100), 55 (42), 41 (95). ^{1}H NMR spectra: δ (CDCl3) 0.97 (3H, t, J = 7.6 Hz, $CH_2C\underline{H}_3$), 1.73 (3H, s, $C(CH_3)$), 2.05 (2H, m, $CHC\underline{H}_2CH_3$), 4.02 (2H, s, $C\underline{H}_2Cl$), 5.52 (1H, t, J = 7.1 Hz, CH).

III-2. 3. 3. *S*-Methylpent-2-enylthiuronium chloride (4)

A mixture of thiourea (1.1 mol), 2-methyl-2-pentene-1-chloride (1.0 mol), and ethanol (200 mL) was heated under reflux for 6 h. Around a half of the ethanol was removed under reduced pressure, and the residual part was cooled to 0 °C to obtain crude *S*-methylpent-2-enylthiuronium chloride crystallines (4). Recrystallization of

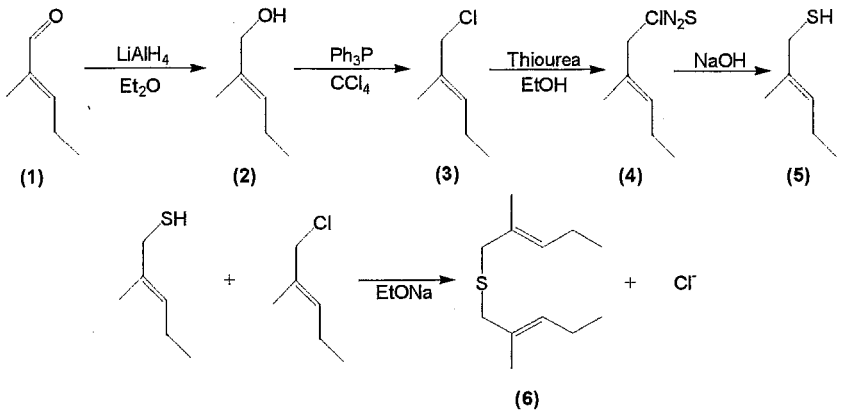
the solution was carried out twice from a boiling ethylacetate (300 mL) containing a few drops of acetic acid to obtain purified crystallines of *S*-methylpent-2-enylthiuronium chloride in a yield of 60%.

III-2. 3. 4. 2-Methyl-2-pentene-1-thiol (5)

The crystalline of *S*-methylpent-2-enylthiuronium chloride (0.6 mol) was added slowly to an aqueous 2.5 N sodium hydroxide (300 mL) containing 0.1 mg of sodium cyanide. After 2 h, the reaction mixture was steam-distilled. The distillate was washed well with water and dried over Na_2SO_4 to give 2-methyl-2-pentene-1-thiol in a yield of 50%. Mass spectral data of the compound (5): *m/z* (relative intensity) 116 (M^+, 1), 87 (5), 82 (65), 67 (28), 55 (100), 41 (66). ^{1}H NMR spectra: δ ($CDCl_3$) 0.96 (3H, t, J = 7.6 Hz, $CH_2C\underline{H}_3$), 1.21 (1H, t, J = 7.0, SH), 1.72 (1H, s, $C(CH_3)$), 2.01 (2H, m, $CHC\underline{H}_2CH_3$), 3.11 (2H, d, J = 7.8 Hz, $C\underline{H}_2SH$), 5.33 (1H, t, J = 7.1 Hz, CH).

III-2. 3. 5. Di(2-methylpent-2-enyl) sulfide (6)

2-Methylpent-2-ene-1-thiol (0.11 mol) was added to a dry ethanol solution of 2.5 M sodium ethoxide (40 ml). 2-Methyl-2-pentene-1-chloride (0.1 mol) was added dropwisely to the solution at 0 °C for 15 min, and the mixture was refluxed for 15 min. The reaction mixture was poured into water, extracted with diethyl ether, and distilled to give di(2-methylpent-2-enyl) sulfide in a yield of 85%. Mass spectral data of the main isomer: *m/z* (relative intensity) 198 (M+, 11), 115 (37), 114 (15), 99 (28), 83 (23), 82 (72), 81 (43), 72 (18), 67 (31), 59 (31), 55 (100), 52 (15). ^{1}H-NMR spectra: δ ($CDCl_3$) 0.94 (3H, t, J = 6.9 Hz, $CH_2C\underline{H}_3$), 1.67 (s, 3H, $C(CH_3)$), 2.01 (m, 2H, $C\underline{H}_2$), 2.96 (s, 1H, $C\underline{H}_2S$), 5.21 (t, 3H, J = 7.6 Hz, CH).



Scheme III-1. Synthetic route for di(2-methyl-2-pent-2-enyl) sulfide (6)

III-2. 4. Enzyme preparation and assay

Crude MnP from *Ceriporiopsis subvermispora* FP90031 was collected from 14 day-cultures grown on a wood medium composed of beech wood (4.5 g), wheat bran (0.5 g), and water (15 mL) at 28 °C (Watanabe et al., 2000; Sato et al., 2002). The culture filtrate was dialyzed against 20 mM sodium succinate buffer (pH 4.5). The dialyzate was concentrated by ultrafiltration, and then purified by gel filtration on Superdex 75 PG (1.6 × 60 cm, Amersham Pharmacia Biotech, Sweden) using 20 mM sodium succinate buffer containing 0.1 M NaCl as an eluent. Fractions showing MnP activity were collected and purified on a MONO-Q (HR 10/10) (Amersham Pharmacia Biotech) column. Elution was carried out using a linear gradient from 0 to 1 M NaCl in 20 mM succinate buffer. Fractions showing MnP activity were collected, concentrated, and desalted by ultrafiltration with Centriprep YM-30 (cut off, 30,000, Millipore, USA). The RZ value of the purified enzyme was 2.5. Activity of MnP was assayed with 0.2 mM 2,6-dimethoxyphenol (2,6-DMP, ε_{470} = $1.0 \times 10^4 M^{-1}cm^{-1}$) in 25 mM sodium tartrate buffer with and without 0.5 mM $MnSO_4$

and 0.1 mM H_2O_2 (Watanabe et al.; 2000; Sato et al., 2002). One unit (U) of MnP is defined as the amount of enzyme that oxidizes 1 μM 2,6-DMP in one minute.

III-2. 5. Degradation of the rubber model compound

The rubber model compound (1 mM), manganese sulfate (0.2 mM), hydrogen peroxide (0.2 mM), and aldehydes (1 mM) were mixed in 1 mL of 10 mM sodium acetate buffer in a test tube. 2*E*,4*E*-Decadienal, 2*E*-butenal, and 2*E*-butene-1,4-dial were used as an aldehyde for the reaction. The purified MnP from *C. subvermispora* (0.4 U) was added to this solution to start the reaction. The reactions were performed for 0.5-48 h at 35 °C using an incubator at 150 rpm. After reaction, 5 mL of chloroform was added and the mixture was vigorously shaken. The organic layer was separated, concentrated and analyzed by GC-MS.

III-3. Results

III-3. 1. Lipid peroxidation of linoleic acid by MnP

Lipid peroxidation (LPO) of linoleic acid (LA) by manganese peroxidase (MnP) has been applied to degradation of lignin model compounds (Bao et al., 1994; Kapich et al., 1999), polyaromatic hydrocarbons (PAHs) (Moen et al., 1994; Bogan et al., 1996), and polyisoprene rubber (Sato et al., 2003). LPO of LA produces a series of aldehydes during breakdown of LA with MnPs. The aldehydic LPO proceeds by acyl radical chain reaction (Watanabe et al., 2000). Watanabe reported that initiation of LA oxidation by MnP starts from hydrogen abstraction from an enolizable methylene vicinal to a terminal carboxyl group, leading to the abstraction

of *bis*-allylic hydrogen by secondary radicals (Watanabe et al., 2000). The resultant KODE is oxidized by Mn(III) to start acyl radical chain reactions. This reaction produced predominantly versatile aldehydic compounds, 2*E*,4*E*-decadienal, 2*E*,4*Z*-decadienal, glycolaldehyde, glyoxal, *n*-pentanal, *n*-hexanal, *n*-heptanal, 2*E*-heptenal, 2*E*-octenal, *n*-nonanal, 2*E*-nonenal, *n*-dodecanal, 2*E*-butendial, and 4-oxo-2-nonenal. 2,4-Decadienal is a key intermediate for the aldehydic lipid oxidation via 9-KODE.

In the present study, the aldehydic LPO by MnP was applied to degrade a rubber model compound, di(2-methylpent-2-enyl) sulfide to elucidate scission of the sulfide bond between isoprene chains by free radicals produced by the oxidation of LA and its degradation intermediates, alkanals, alkenals, and alkadienals. In the LPO of LA with MnP, 99% of the rubber model compound was degraded for 6 h to produce 2,4-dimethylthiophene and isoprene dimers (Figure III-1, D). The formation of sulfur-containing compounds, 2,4-dimethylthiophene and the isoprene dimers indicated cleavage of the sulfide bond by lipid-derived radicals. The rubber model compound was also degraded by Mn(III), dependently on the presence of LA (Data not shown). The rubber model compound was not decomposed by autoxidation of LA without MnP for 48 h. The extensive degradation of the rubber model in combination of LA and MnP is in agreement with previous observation that lipid peroxidation of by MnP degraded vulcanized rubber sheets (Sato et al., 2003).

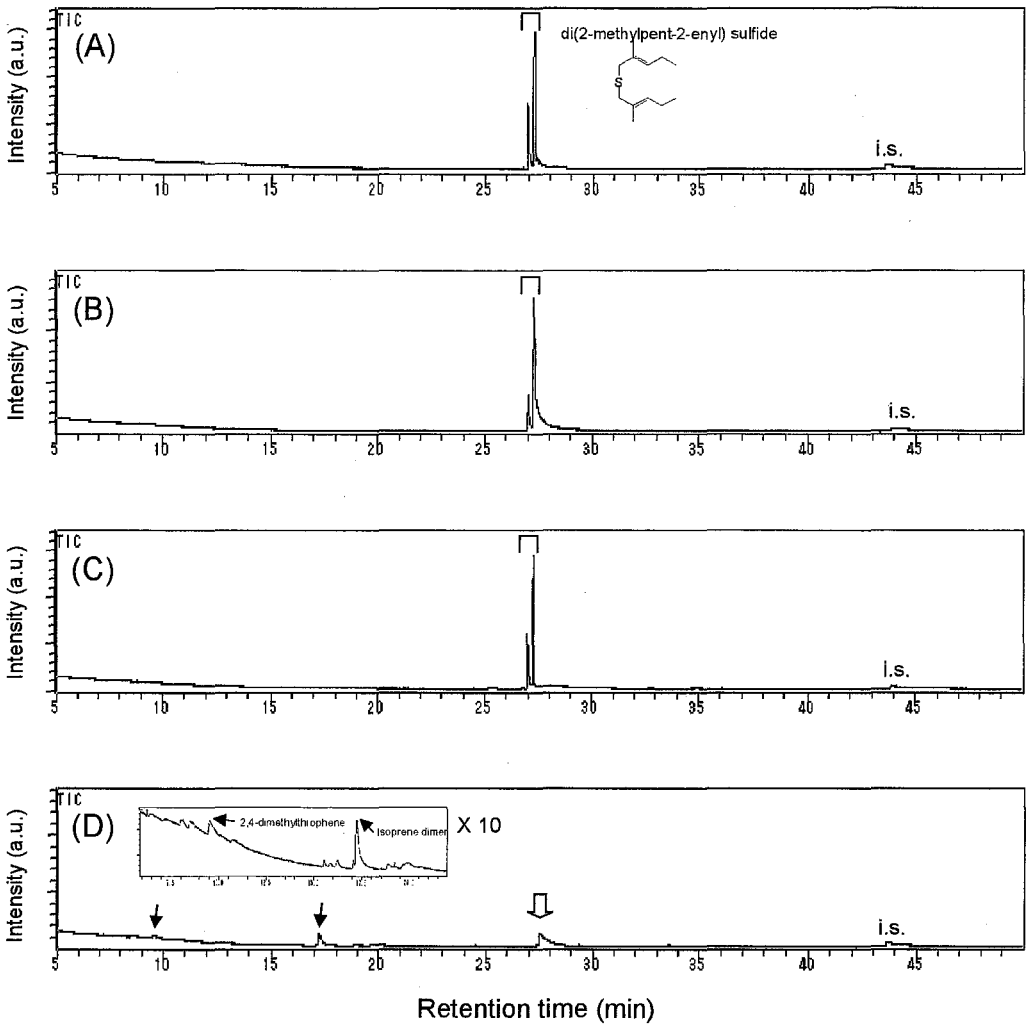


Figure III-1. Total ion chromatograms of the rubber model compound in LPO catalyzed by MnP for 6 h: (A) control without LPO of LA with MnP; (B) MnP; (C) LA autoxidation; (D) LPO of LA with MnP. i.s.; 0.5 mM fluoranthene.

III-3. 2. Lipid peroxidation of aldehydes by MnP

2*E*,4*E*-Decadienal, *n*-butenal, 2*E*-butene-1,4-dial, hexanal, pentanal, heptanal, 2-heptenal, malondialdehyde, 4-hydroxylnonenal, and glyoxal were identified as intermediate aldehydes formed during breakdown of LA with MnP. In the present study, LA, 2*E*,4*E*-decadienal, 2*E*-butene-1,4-dial, and 2*E*-butenal were directly oxidized with MnP in the presence of the rubber model compound, and products formed were analyzed by GC-MS. Reaction of 2*E*,4*E*-decadienal with MnP degraded 80% of the rubber model compound for 6 h and formation of 2,4-dimethylthiophene and di(2-methylpent-2-enyl) disulfide were observed by GC-MS (Figure III-2 and 3). An isomeric mixture of isoprene dimer was also found in the reaction products (Figure III-3). Formation of the two sulfur-containing products and the isoprene dimer suggests that peroxidation of 2*E*,4*E*-decadienal by MnP produced free radicals capable of decomposing the sulfide bond in the rubber model compound. In contrast to 2*E*,4*E*-decadienal, 2*E*-butene-1,4-dial and 2*E*-butenal caused no degradation of the rubber model compound (Figure III-2).

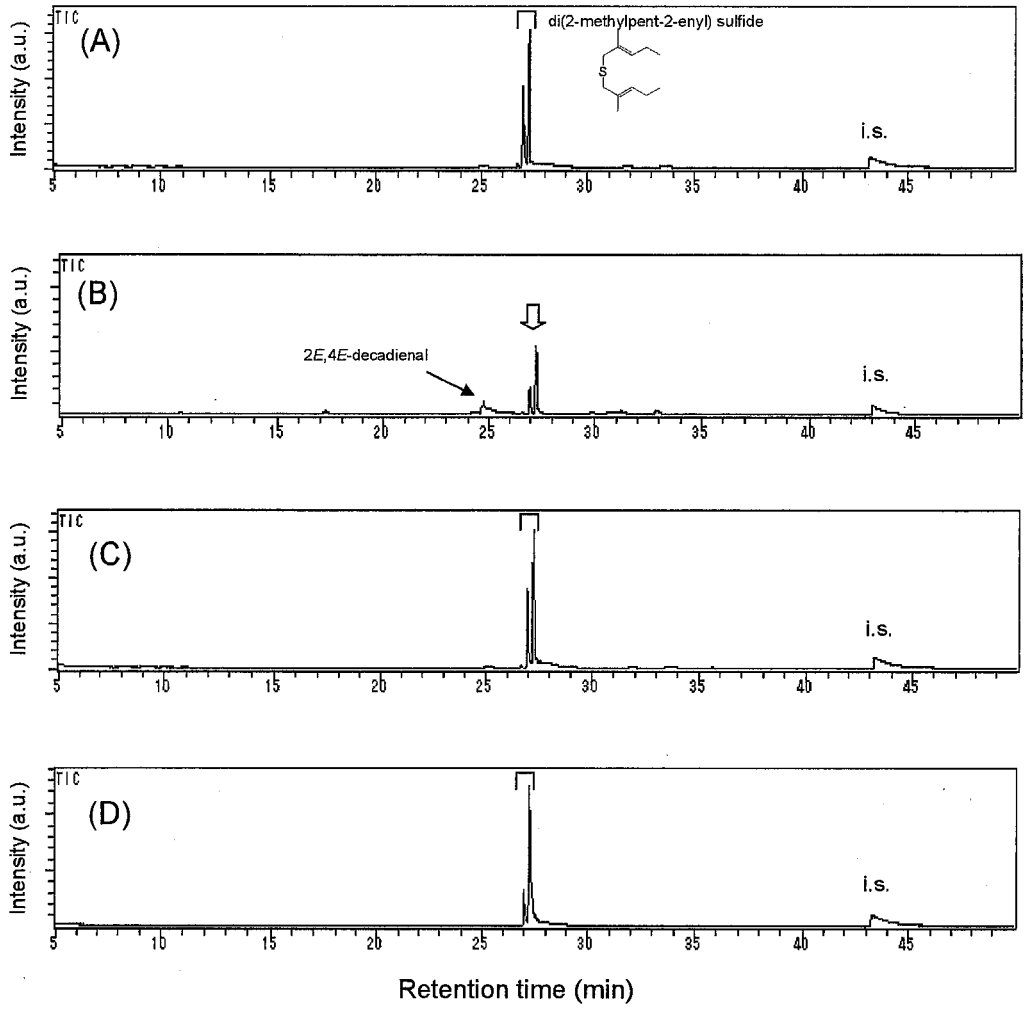
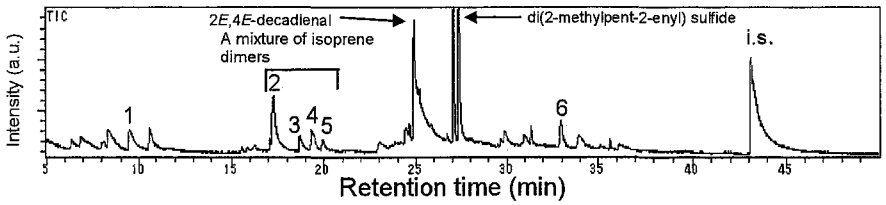


Figure III-2. Total ion chromatograms of the rubber model compound in LPO catalyzed by MnP for 6 h: (A) MnP without LPO; (B) LPO of 2*E*,4*E*-decadienal with MnP;(C) LPO of 2*E*-butene-1,4-dial with MnP; (D) LPO of 2*E*-butenal with MnP. i.s.; 0.5 mM fluoranthene.



TIC of the rubber model compound/MnP/2*E*,4*E*-decadienal for 6 h

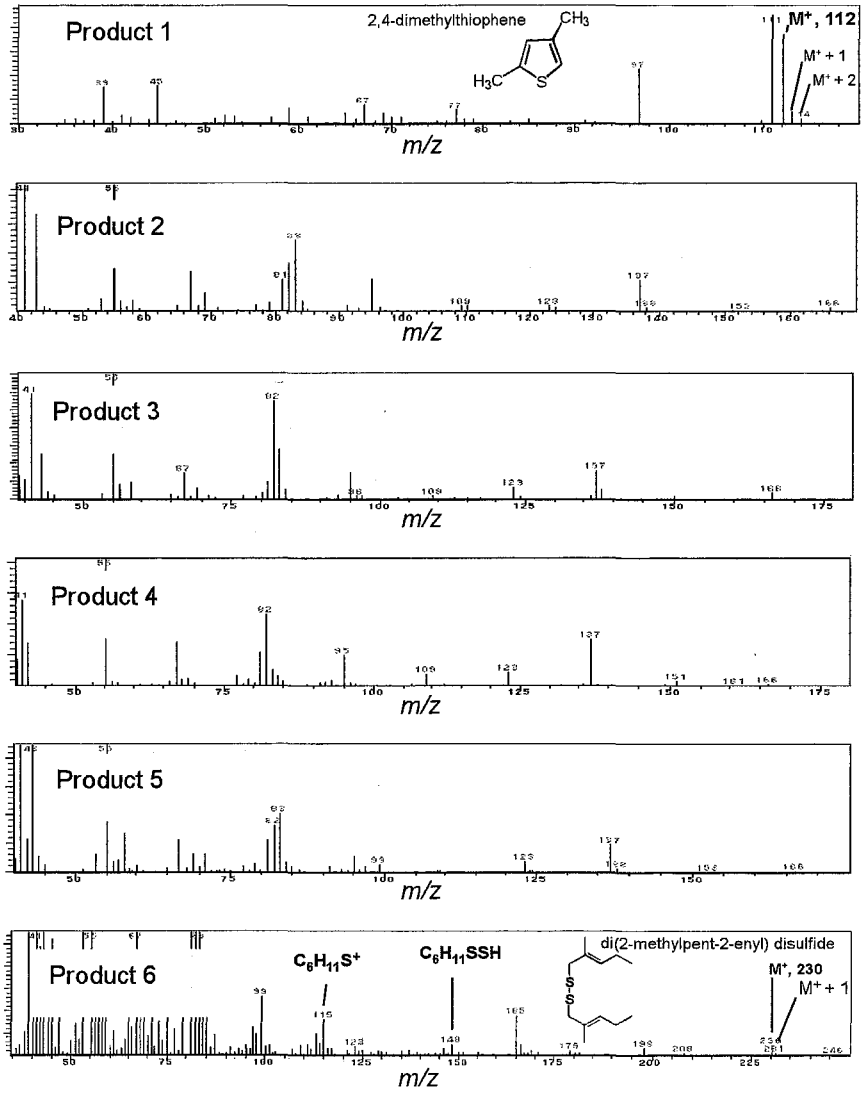


Figure III-3. Mass spectra of the products formed from the rubber model compound in LPO of 2*E*,4*E*-decadienal with MnP for 6 h.

Table III-1. Mass spectra of the products formed from the model compound in LPO of 2*E*,4*E*-decdienal with MnP for 6 h.

Products	Retention time (min)	Mass spectral data *m/z* (%)
Products 1	9.3	112 (M^+, 59), 114 (2), 113 (6), 111(100), 97 (41), 77(15), 59 (15), 58 (21), 45 (29),
Products 2	17.2	166 (M^+, 2), 151 (1), 137 (14), 123 (2), 110 (3), 95 (11), 83 (17), 82 (14), 81 (11), 67 (19), 55 (100), 43 (24), 41 (54),
Products 3	18.7	166 (M^+, 1), 151 (1), 137 (10), 124 (5), 123 (6), 109 (3), 95 (15), 83 (11), 82 (33), 81 (11), 67 (20), 56 (13), 55 (100), 41 (44)
Products 4	19.1	137 (M^+, 17), 123 (4), 109 (1), 107 (1), 95 (8), 83 (13), 82 (25), 81 (16), 67 (22), 55 (100), 41 (59)
Products 5	19.6	137 (M^+, 20), 123 (1), 109 (2), 107 (1), 83 (20), 82 (13), 81 (11), 67 (11), 55 (100), 41 (38)
Products 6	32.7	230 (M^+, 1), 231 (1), 198 (1), 166 (1), 165 (3), 148 (1), 129 (1), 115 (3), 114 (1), 99 (3), 83 (47),82 (11), 67 (12), 55 (100), 41 (53)

III-4. Discussion

Enzymatic degradation of *cis*-1,4-polyisoprene rubber was studied. It was reported that natural and synthetic rubber latices were degraded by extracellular oxygenase secreted from the culture of *Xanthomonas* sp. strain 35Y. (Tsuchii and Takeda, 1990; Braaz et al., 2004). Thus, polyisoprene chains were cleaved by the purified oxygense *in vitro*. However, to our knowledge, there has been no report of enzymatic degradation of polyisoprene products cross-linked with sulfur. In Chapter II, the author first demonstrated that vulcanized *cis*-1,4-polyisoprene rubber was degraded by lipid peroxidation of linoleic acid by MnP (Sato et al., 2003). Free radicals from linoleic acid cleaved carbon-carbon bonds in polyisoprene chains, but there has been no direct evidence for the scission of sulfide bonds by the free radicals. In this Chapter, the author synthesized di(2-methylpent-2-enyl) sulfide as a rubber model compound, and analyzed scission of S-C bonds in the rubber model by MnP-catalyzed lipid peroxidation.

GC-MS analysis demonstrated that lipid peroxidation (LPO) of linoleic acid by MnP extensively degraded di(2-methylpent-2-enyl) sulfide. LPO of 2*E*,4*E*-decadienal by MnP degraded di(2-methylpent-2-enyl) sulfide to di(2-methylpent-2-enyl) disulfide and 2,4-dimethylthiphene (Figure III-3). In addition to the formation of these sulfur-containing compounds, isoprene dimers were also detected in the chromatograms. In contrast, the rubber model compound was not degraded by LPO of 2*E*-butene-1,4-dial and 2*E*-butenal with MnP. This suggests that radicals generated in the initiation phase of LPO of 2*E*,4*E*-decadienal by MnP are involved in the degradation of the rubber model compound.

Formation of disulfide from monosulfide is explained by oxidative cleavage of S-C bond (Oae, 1982). It was reported that the oxidation is initiated by one electron abstraction of sulfur atom by hydroxyl radical and P-450 (Gilbert et al., 1973; Watanabe et al., 1981). In the LPO of LA and 2,4-decadienal by MnP, LPO produces a series of oxidative radicals. The mechanism for S-C bond scission in the model compound by MnP-catalyzed LPO is proposed as shown in Figure III-4. A sulfur atom of the model compound is oxidized by electronphillic radical like alkokyl radical to form a sulfur cation inermediate (Figure III-4, A). Abstraction of a proton from α-methylene of the intermedate produces α-carboanion (Figure III-4, B), which in turn reacts with molecular oxygen to yield a hydroperoxide. (Figure III-4, C). A O-O bond in the hydroperoxide is homolytically cleaved and an alkyl thyil radical is formed by S-C bond cleavage of the alkoxyl radical. (Figure. III-4, D). Di(2-methylpent-2-enyl) disulfide and 2,4-dimethylthiophene are formed by coupling of the alkyl thyil radical. When a sulfur cation radical reacts with oxygen before abstraction of α-proton, sulfoxide is formed (Oae, 1981). In the LPO of LA and 2*E*,4*E*-decadienal by MnP, sulfoxide was not detected on GC-MS chromatograms.

Unequivocal evidence for enzymatic scission of a sulfide bond in the rubber model compound has been shown. Control of the radical chain reactions in combination of peroxidizable compounds and catalysts should change selectivity of the sulfide bond cleavage in vulcanized rubbers. Further study is needed to understand the radical species responsible for the sulfide bond cleavage. The enzymatic devulcanization will lead to the development of new devulcanizing systems for safe disposal and recycling of waste vulcanized rubber products.

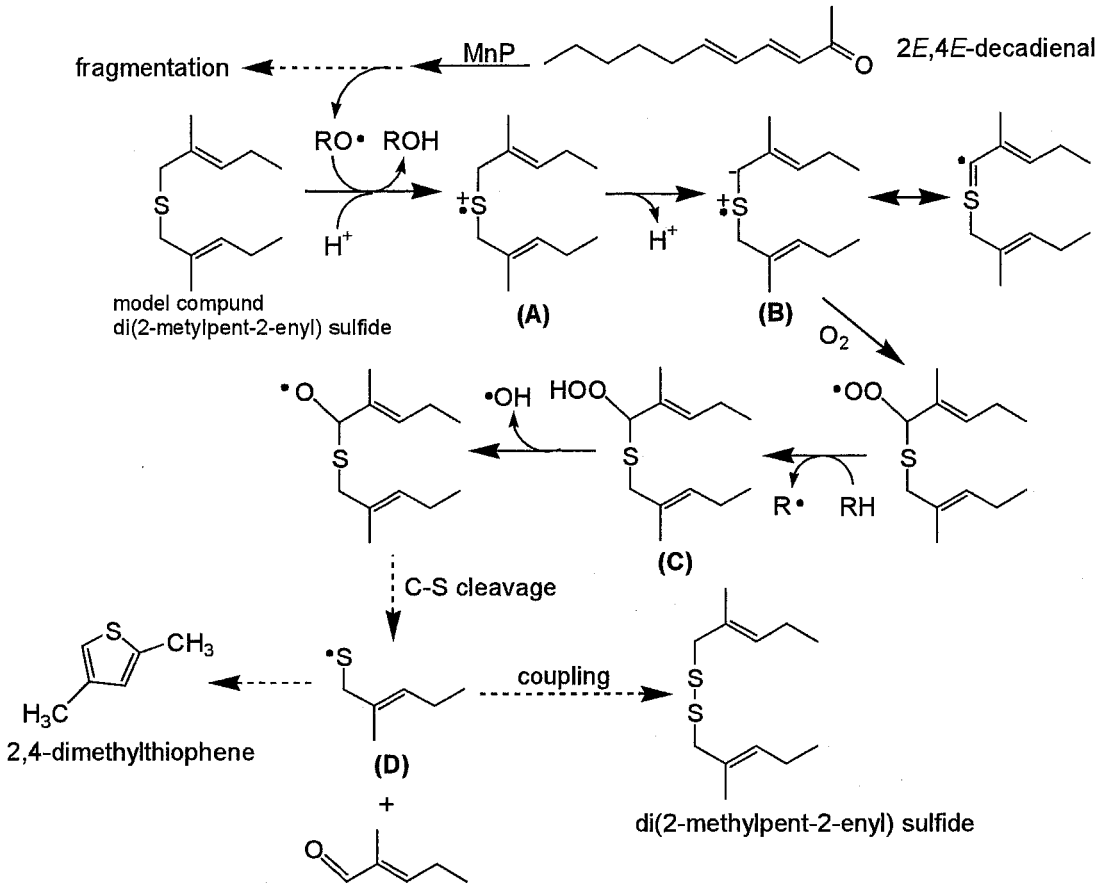


Figure III-4. Proposed pathway for the formation of di(2-methylpent-2-enyl) disulfide and 2,4-dimethylthiophene from the rubber model compound by oxidation of 2*E*,4*E*-decadienal with MnP.

III-5. Summary

Lipid peroxidation (LPO) of linoleic acid and 2*E*,4*E*-decadienal by manganese peroxidase (MnP) decomposed a rubber model compound, di(2-methylpent-2-enyl) sulfide. LPO of 2,4-decadienal by MnP degraded the model compound to di(2-methylpent-2-enyl) disulfide, 2,4-dimethylthiphene, and a mixture of isoprene dimers, while the rubber model compound was stable in LPO of 2*E*-butene-1,4-dial and 2*E*-butenal by MnP. This suggests that radicals generated in the initiation phase of 2,4-decadienal oxidation by MnP are involved in the degradation of the rubber model compound. In this mechanism, one electron oxidation of a sulfur atom of the model compound with electrophillic radicals like alkoxyl radical produces stable radical cation intermediates. The intermediate radicals react with molecular oxygen to cleave the sulfide bonds, followed by formation of a disulfide compound. In the present study, unequivocal evidence for the enzymatic scission of sulfur-carbon bond in the aliphatic sulfide by LPO with MnP was demonstrated. Control of the free radical process by MnP will give us a new strategy for safe disposal and recycling of waste vulcanized rubber products.

Conclusions

White rot basidiomycetes have been the focus of research with respect to the biodegradation of lignin in wood and the bioremediation of environmental hazards. Although white rot bacidiomycetes and ligninolytic systems have also been reported to degrade natural and synthetic polymers, to our knowledge, the degradation of rubber products by basidiomycetes has not been elucidated. In the present doctoral dissertation, the degradation of vulcanized natural rubber sheets by basidiomycetes was investigated. Lipid peroxidation (LPO), a ligninolytic system proposed for selective white rot was applied to the degradation of vulcanized and nonvulcanized synthetic polyisoprene. In addition, the cleavage of aliphatic sulfide linkages by LPO catalyzed by a ligninolytic enzyme, manganese peroxidase, was also studied.

In Chapter I, the author found that a white rot basidiomycete, *Ceriporiopsis subvermispora*, degraded vulcanized natural rubber sheets in wood cultures. The fungus decreased total sulfur content of the rubber by 54% in 200 days, accompanied by the cleavage of sulfide bonds between the polyisoprene chains. X-ray photoelectron spectroscopy (XPS) demonstrated that *C. subvermispora* reduced the frequency of S-C bonds by 69% with the concomitant formation of S-O bonds during the culture. Dipolar decoupling/magic angle spinning (DD/MAS) solid state ^{13}C NMR revealed that the fungus preferentially decomposed monosulfide bonds linked to a *cis*- and *trans*-1,4-isoprene backbone. When the rubber sheets were exposed to a culture of a white rot fungus, *Dichomitus squalens*, for 200 days, a 15% decrease in the total sulfur content and the formation of S-O bonds in the rubber was observed. However, a decrease in S-C bonds and an increase in the volume swelling ratio in

toluene were not observed. These results indicate that *D. squalens* did not cleave S-C bonds but removed unbound sulfur or oxidized sulfide to sulfoxide. The oxidative cleavage of sulfide bonds by *C. subvermispora* demonstrates that ligninolytic basidiomycetes are microbes with the potential to biologicaly devulcanize rubber products.

In Chapter II, the author showed that nonvulcanized and vulcanized polyisoprene rubber materials were degraded by controlling the free radical chain reactions of lipids using oxidative enzymes, manganese peroxidase (MnP), laccase (Lac), and horseradish peroxidase (HRP). Nonvulcanized synthetic polyisoprene was degraded by the free radicals from an unsaturated fatty acid, linoleic acid (LA) produced by MnP, HRP, and a combination of Lac/1-hydroxybenzotriazole (1-HBT). The degradation of nonvulcanized polyisoprene was also observed in the LPO of LA initiated by the Fenton reaction (FR) and Mn(III), an oxidation product produced by MnP. In contrast, lipoxygenase caused no apparent degradation due to the differences in radical chain reactions in the LPO. Vulcanized polyisoprene rubber sheets were degraded by the LPO of linoleic acid (LA) initiated by HRP, MnP, Mn(III) and FR. Pyrolysis GC-MS analysis demonstrated that the LPO liberated isoprenoid fragments extractable with $CHCl_3$. The mechanism for the decomposition of polyisoprene was explained by hydrogen abstraction from a β-position of double bonds in isoprene chains by free radicals from lipids and the β-oxidation of the alkoxyl radical.

In Chapter III, the author synthesized a vulcanized rubber model compound, di(2-methylpent-2-enyl) sulfide from 2-methyl-2-pentenal, and subjected it to degradation by LPO. It has been shown that the LPO of LA with MnP degraded

99% of the model compound within 6 h, and the LPO of an alkadienal, 2*E*,4*E*-decadienal by MnP also degraded the model compound. The model compound was decomposed by the LPO of LA or 2*E*,4*E*-decadienal with MnP to yield di(2-methylpent-2-enyl) disulfide, 2,4-dimethylthiophene, and an isomeric mixture of isoprene dimer. No degradation of the model compound was found in the LPO of 2*E*-butenal and 2*E*-butene-1,4-dial, catalyzed by MnP. It is suggested that radicals generated after the LPO of 2*E*,4*E*-decadienal play a key role in the sulfide bond cleavage. The scission of sulfide bonds was explained by the one electron oxidation of a sulfide bond by electronphilic radicals, such as the alkoxyl radical originating from LA and 2*E*,4*E*-decadienal. A thyil radical formed after the cleavage of the sulfide bond is responsible for the production of di(2-methylpent-2-enyl) disulfide and 2,4-dimethylthiophene.

In addition to the actinomycetes, bacteria, and fungi imperfecti previously reported as rubber-degrading microorganisms, the author herein is the first to report basidiomycetes capable of degrading vulcanized rubber. A white rot basidiomycete, *Ceriporiopsis subvermispora* oxidatively cleaved sulfide bonds in vulcanized polyisoprene rubber. The author also demonstrated that *cis*-1,4-polyisoprene, a sulfide bridge cross-linking two isoprenes, and vulcanized rubber products were degraded by the oxidation of unsaturated fatty acids and alkadienals with oxidative enzymes and transition metals.

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List of Publications

-Original articles-

1, Shin Sato, Yoichi Honda, Masaaki Kuwahara, and Takashi Watanabe (2003) Degradation of Vulcanized and Nonvulcanized Polyisoprene Rubbers by Lipid Peroxidation Catalyzed by Oxidative Enzymes and Transition Metals. *Biomacromol.* **4**, 321-329.

2, Shin Sato, Yoichi Honda, Masaaki Kuwahara, Hiroyuki Kishimoto, Noriko Yagi, Kiyoshige Muraoka, and Takashi Watanabe (2004) Microbial Scission of Sulfide Linkages in Vulcanized Natural Rubber by a White Rot Basidiomycete, *Ceriporiopsis subvermispora*. *Biomacromol.* **5**, 511-515.

3, Shin Sato, Yasunori Ohashi, Takahito Watanabe, Yoichi Honda, and Takashi Watanabe (2005) Degradation of Aliphatic Sulfide Linkages by Lipid Peroxidation Catalyzed by Manganese Peroxidase. (in preparation)

-Preliminary article-

1, Shin Sato, Yoichi Honda, Masaaki Kuwahara, and Takashi Watanabe (2001) Degradability of Natural and Synthetic Polymers by the White Rot Fungus, *Ceriporiopsis subvermispora*. *Wood Res.* **88**, 48-49.

-Review-

1, Shin Sato and Takashi Watanabe (2002) Degradation of Vulcanized and Nonvulcanized Rubbers by White Rot Fungi and its Biomimetic Radical Reactions. *Eco Industry* 7, 25-31. (in Japanese).

-Book-

1, Shin Sato and Takashi Watanabe (2003) Degradation of Vulcanized and Nonvulcanized Rubbers by White Rot Fungi and its Biomimetic Radical Reactions, in: Environmental Restoration and Useful Substance Production, 126-132, CMC-shuppan, Tokyo (in Japanese).

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