Journal of Environmental Polymer Degradation, Vol. 4, No. 4, 1996

Chitin Biodegradation and Wound Healing in Tree Bark Tissues

Shigehiro Hirano,[1,3] Shuntaro Kitaura,[1] Nozomi Sasaki,[1] Hiroaki Sakaguchi,[1] Masafumi Sugiyama,[2] Kunihiko Hashimoto,[2] and Akira Tanatani[2]

Chitin biodegradation and wounded bark tissue healing were demonstrated in several evergreen and deciduous trees by dressing with a sheet of chitin-containing films or sponges. Chitinase activities in the tree bark tissues around the wounds were enhanced by this treatment up to four times those of the untreated wounds. Significant seasonal changes of chitinase activities were observed with the bark and leaf tissues of deciduous trees, but few with those of evergreen trees. A sheet of chitin films implanted or dressed in the tree bark tissues was biodegraded within 4 to 24 weeks after implantation and was assimilated into the wounded bark tissues, resulting in the stimulation of the wounded bark tissue healing.

KEY WORDS: Chitinase; chitin biodegradation; chitin film; tree; wounded bark tissue healing.

INTRODUCTION

Chitin is (1 → 4)-*N*-acetyl-β-D-glucosaminan and chitosan is the *N*-deacetyl derivative. Chitin and chitosan are biosynthesized as the main structural components of the cuticles of crustaceans, insects, and mollusks and the cell walls of some pathogens [1]. On the other hand, chitin is biodegraded by chitinase (EC 3.2.1.14) and lysozyme (EC 3.2.1.16) in the soil and hydrospheres of the earth. "The chitin circulation on the earth by biosynthesis and biodegradation" contributes significantly to the preservation of the natural environment and ecology of the earth. Chitin and chitosan are ecological and environmental active biopolymers [2]. Many investigators [3] have reported chitinase activities in plants including vegetable, potato, and flower tissues, although plant tissues do not contain any chitin as a constituent. However, little is known about chitinase activity in tree tissues or about the biodegradation of chitin and its derivatives in tree tissues. We report (a) chitinase activities in the bark, xylem, and leaf tissues of several evergreen and deciduous trees and (b) chitin biodegradation and wounded bark tissue healing in tree bark tissues by dressing with a sheet of chitin-containing films or sponges.

[1] Chitin/Chitosan R&D Room, 445-Sakuradani, Tottori 680, Japan, and Department of Agricultural Biochemistry and Biotechnology, Tottori University, Tottori 680, Japan.
[2] Nishikawa Rubber Co., Ltd., Hiroshima 733, Japan.
[3] To whom correspondence should be addressed at Chitin/Chitosan R&D Room, 445-Sakuradani, Tottori 680, Japan.

EXPERIMENTAL

Materials

Crab shell chitosan, Flonac N, a commercial product from Kyowa Technos, Chiba, Japans was used, and a regenerated chitin powder (d.s. 1.0 for NAc, 120 mesh) [4] and hydroxethyl (HE)-chitin (d.s. 0.7 for HE) [5] were prepared.

Wound Dressing Materials

Chitosan was dissolved in aqeous 2% acetic acid–methanol, acetic or propionic acid anhydride (3 mol eq per GlcN) was added, and the mixture was kept at room temperature overnight to form a gel [6]. The gel was

1064-7546/96/1000-0261$09.50/0 © 1996 Plenum Publishing Corporation

sliced into an appropriate thickness, and the sliced gel was dialyzed against running water for 2 days [7] and frozen in a deep freezer. The frozen layer was melted by standing at room temperature and dried to afford an opaque sponge sheet (3-mm in thickness). On the other hand, the above sliced gel was air-dried to give a sheet of transparent films (24–27 μm in thickness). In addition, the following composite material sheets (1-mm in thickness) containing 30% chitin or 30% chitosan were prepared by mechanical mixing with natural rubber: chitin–starch–rubber (30:35:35, w/w), chitin–cellulose–rubber (30:35:35, w/w), chitin–$CaCO_3$–rubber (30:35:35, w/w), chitin–rubber (30:70, w/w), and chitosan–rubber (30:70, w/w). A nonwoven chitin–cellulose (30:70, w/w) sheet was prepared by mixing cellulose xanthate and chitin xanthate [8]. Sheets of filter paper (cellulose) and natural rubber were used as control dressings. The films or sponges were cut into an appropriate square size (3–6 × 3–6 cm) and used in the present study.

Trees

As evergreen trees, we examined a camellia tree (*Camellia japonica* L. var. trifida Makino), a dendropanax tree (*Dendropanax trifidus* Makino), a camphor tree (*Machilus thunbergii*), a Japanese cypress tree (*Chamaecyparis pisifero* var. Plumosa aurea), and a pasania tree (*Pasania cuspidata*). As deciduous trees, we examined a cherry tree A (*Prunus sargentii* Rehd), a cherry tree B (*Prunus yedoensis* Matsumura), a liriodendron tree (*Liriodendron tulipifera* Linn), a maple tree (*Acer palmatum* Thumb subsp. matumurae Koidz), a platanus tree (*Platanus orientalis* L.), and a spear-flower tree (*Ardisia japonica* Blume). All these trees were on the Tottori University campus.

Methods

A Crude Enzyme Solution from Tree Bark Tissues

The following operation was carried out at 4°C in a cold room. A portion of the tree bark tissues (2–5 g, fresh weight) around the wounds was cut off with a knife and was homogenized with a homogenizer in 10–20 ml of a 50 mM imidazole–HCl buffer solution (pH 7.0) containing EDTA (1.0 mM). The homogenate was centrifuged at 1500*g* for 30 min. From the supernatant solution, a salting-out precipitate in the region of a 20 to 80% concentration of ammonium sulfate was collected by centrifugation at 28,000*g* for 20 min, and the precipitate was redissolved in the above buffer solution (less than 3 ml) and dialyzed in 0.02 mol imidazole–HCl buffer solution (pH 7.0, 800 ml) for 12 h and then in the above fresh buffer solution for 12 h to afford a crude enzyme solution.

Chitinase Activity Assay

A portion (0.70 ml) of the crude enzyme solution obtained above was added to a solution of HE-chitin (1.0 mg) in 1.3 ml of a 0.2 *M* Na_2HPO_4–0.1 *M* citric acid buffer solution (pH 5.0) or to a suspension mixture of a chitin powder (30 mg) in 13 ml of a 0.2 *M* Na_2HPO_4–0.1 *M* citric acid buffer solution (pH 5.0). The mixture solution (total, 2.0 ml) was incubated at 37°C for 30 min, and the increased reducing sugar value was measured by the modified [9] Schales and Schales method [10]. The reducing sugar value of the crude enzyme solution (0.70 ml) and that of HE-chitin (1.0 mg) were subtracted from the observed reducing value to give the correction value. The enzyme unit (U) is defined as the reducing sugar value of 1 μmol of *N*-acetyl-D-glucosamine per min per 1.0 g fresh tissue or mg protein. The hydrolysis rate of the water-soluble HE-chitin by chitinase was 8.9 times higher than that of the water-insoluble chitin powder under the present conditions.

Protein Assay

A portion (0.1–0.5 ml) of the above crude enzyme solution was withdrawn for the analysis of protein by the Lowry method [11] with bovine serum albumin (100 μg/ml) as a standard.

RESULTS AND DISCUSSION

Chitinase Activities in Tree Tissues

The present study was carried out from July to December 1987–1995. A square piece (3–6 × 3–6 cm) of the bark tissue was cut off carefully with a knife from trees. The wound was dressed with a sheet of the same square size of films, sponges, or nonwoven clothes of chitin and chitosan to be examined and was taped firmly with a gum tape. As a control experiment, the tree bark wounds were left without any wounded bark dressings. Chitinase activities in the bark tissues around the wounds were analyzed at weekly intervals, and the wounded bark tissue healing was examined during 9 years after the treatment.

Table I shows chitinase activities of the bark, xylem, and leaf tissues of six deciduous trees and five ev-

Table I. Chitinase Activities in the Bark, Xylem, and Leaf Tissues of Several Trees

Tree	Chitinase activity (mU/l g fresh tissue) (mU/mg protein)[a]		
	Bark	Xylem	Leaf
Deciduous trees			
Cherry A	18	4.0	n.d.
	0.7	0.6	n.d.
Cherry B	29	2.4	9.0
	0.8	Trace	Trace
Liriodendron	36	1.0	13
	0.9	Trace	3.9
Maple	8.0	Trace	Trace
	0.3	Trace	Trace
Platanus	16	3.0	8.0
	0.7	n.d.	0.9
Spear-flower	15	Trace	11
	0.6	Trace	1.1
Evergreen trees			
Camellia	20	1.0	26
	Trace	Trace	1.2
Dendropanax	12	1.4	20
	0.4	0.1	0.2
Camphor	13	1.0	21
Japanese cypress	0.6	Trace	1.3
	2.0	1.0	12
	0.5	0.2	0.8
Pasania	11	1.0	1.0
	0.5	Trace	0.8

[a] Analyzed with a chitin powder as a substrate in November 1987 and 1988. The average values of three to five samples are shown.

ergreen trees, and these analyses were carried out in November 1987 and 1988. Tree bark chitinase activities (mU/g fresh tissue and mU/mg protein) were high in the bark tissues and low in the xylem tissues. The high localization of chitinase activity in the bark tissues is probably a self-defense function of trees to prevent the pathogen attack from the outside.

In a cherry tree B and a liriodendron tree (deciduous trees), the bark and leaf chitinase activities (mU/L g fresh tissue) were 128 and 70 in July, 125 and 70 in August, 103 and 60 in September, 68 and 40 in October, 34 and 12 in November, and 30 and a trace in December (1990 and 1991) as shown in Fig. 1. Contrarily, the seasonal change of chitinase activities was small in the bark and leaf tissues of evergreen trees. In a camellia tree and a liriodendron tree (evergreen trees), the bark and leaf chitinase activities (mU/l g fresh tissue) were 27 and 26 in July, 22 and 26 in August, 12 and 15 in September, 13 and 14 in October, 16 and 19 in November, and 12 and 10 in December (1990 and 1991). No data are available on chitinase activity in the

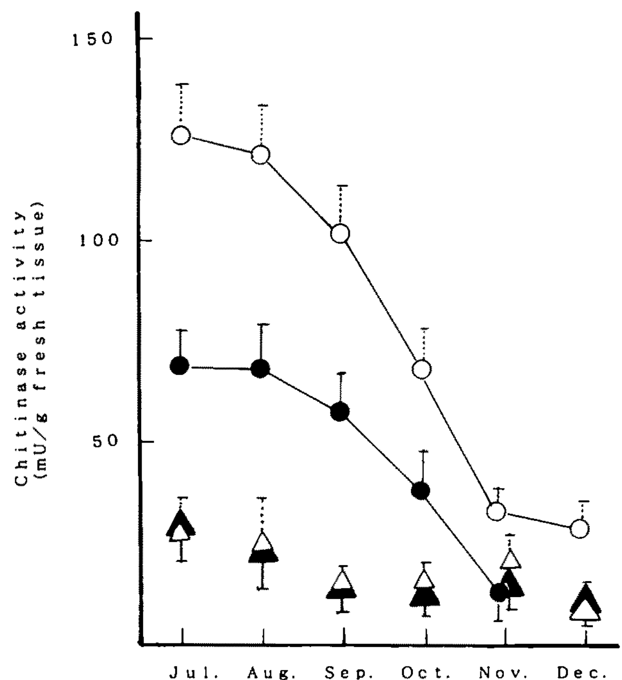

Fig. 1. Seasonal changes of chitinase activity in the bark and leaf tissues of deciduous and evergreen trees. Chitinase activities of the bark (○) and leaf (●) tissues of cherry B and liriodendron trees (deciduous trees) and the bark (△) and leaf (▲) tissues of camellia and dendropanax trees (evergreen trees).

spring to summer seasons. The chitinase activities of the bark and leaf tissues of deciduous trees were more active than those of evergreen trees in the summer season as shown in the present study. The deciduous trees sprout their new leaves in the spring season, and their leaves grow up in the summer season. During these seasons, the deciduous trees themselves prevent infection by pathogens by secreting high chitinase activity in the bark and leaf tissues, because the new young soft leaves of deciduous trees may be infected more easily with pathogens than the old hard leaves of evergreen trees. Both the bark and the leaf chitinase activities decrease in the winter season, resulting in fallen leaves.

Biodegradation and Wounded Bark Tissue Healing

The chitinase activity in the tree bark tissues around the wounds was stimulated by dressing with a sheet of chitin films, and the activity increased to about three times those of undressed wounds at 13 weeks after treatment (Table II). A similar stimulation of the bark tissue chitinase was also observed with wounded bark tissues by dressing with a sheet of 30% chitin-containing composite sponges: chitin–cellulose–rubber, chitin–$CaCO_3$–

Table II. Chitinase Activities in Wounded Bark Tissues of Several Trees by Dressing with a Sheet of Chitin Films

Tree	Weeks after dressing[a]	Chitinase activity (mU/mg protein)[b] Dressed	Not dressed (control)
Dendropanax	4	6.1	2.0
	7	5.3	3.7
Camellia	1	2.2	1.2
	13	3.9	1.0
Maple	13	3.5	Trace
Cherry A	4	4.5	1.3
	9	8.1	3.8
	13	9.0	2.5
Spear-flower	4	2.0	Trace
	9	0.6	Trace
	13	0.5	Trace

[a]Analyzed in October 1993 and 1994 with HE-chitin as substrate.

rubber, chitin-rubber, chitin-starch-rubber, and chitin-cellulose (Table III). The chitinase activity was stimulated slightly even in undressed wounded bark tissues, probably due to a wound stress in the self-defense function of trees (Tables II and III), and the activity was essentially similar to that in the wounded bark tissues dressed with a sheet of cellulose, rubber, chitosan-cellulose, and chitosan-rubber. These data indicate that only chitin is active for stimulation of chitinase in the wounded tree bark tissues. While cellulose, rubber, $CaCO_3$, and starch are inactive.

Three sides of a square portion (3 × 3 cm) were cut from tree bark tissues with a knife, the bark portion was opened from the cut sides, each sheet (3 × 3 cm, 27–75 μm in thickness) of chitosan, chitin, and *N*-propionylchitosan films was implanted under the bark tissues in July 1987, and their fate was examined for up to 31 weeks (Table IV). Under the bark tissues of a Japanese cypress tree, the chitin film was biodegraded within 17 weeks and the *N*-propionylchitosan film within 24 weeks, resulting in their assimilation into the wounded bark tissues. However, the chitosan film was not digested for up to 31 weeks under the tree bark tissues examined. The biodegradation period varied with tree ages and species, seasons, the *N*-acyl structure of chitosan [12, 13], and the sheet thickness (the result is now shown). A sheet of chitin films was biodegraded within 9 weeks under the bark tissues of a young cherry tree B and was digested within 24 weeks under those of an old cherry tree B.

Figure 2 shows a typical wound healing of the wounded bark tissues of a camellia tree. The lower wound, which had been dressed with a sheet of chitin films in August 1992, has a smooth surface of new bark tissues. However, the upper wound, which had not been dressed with the sheet, has a rough surface of new bark tissues and still had uncovered bark tissues on a central xylem part in August 1995.

A Proposed Biofunction for the Biodegradation of Chitin in Tree Bark Tissues

When the tree wounds are dressed with a sheet of chitin-containing films or sponges, chitin is biodegraded

Table III. Stimulation of Chitinase Activities in the Wounded Bark Tissues of a Camellia Tree by Dressing with a Sheet of 30% Chitin-Containing Sponges in August 1994 and 1995

Wounded bark dressing materials	Chitinase activity 9 weeks later (mU/mg protein)[a]
Chitin-cellulose	3.8
Chitin-cellulose-rubber	3.8
Chitin-$CaCo_3$-rubber	3.6
Chitin-rubber	3.4
Chitin-starch-rubber	3.0
Chitosan-cellulose	1.0
Chitosan-rubber	0.8
Chitin	3.6
Cellulose	0.8
Chitosan	0.9
Rubber	0.8
Undressed	0.9

[a]Analyzed with HE-chitin as a substrate in October 1994 and 1995, and expressed as the average value of two or three analyses.

Table IV. Biodegradation of a Sheet of Chitin, *N*-Propionylchitosan, and Chitosan Films Under the Bark Tissues of Several Trees[a]

Film	Biodegradation period after implantation (weeks)
Chitin	
Dendropanax	8
Camellia	5
Japanese cypress	17
Maple	13
Cherry A	4
Cherry B (young)	9
Cherry B (old)	24
Spear-flower	4
N-Propionylchitosan	
Japanese cypress	24
Cherry B (young)	9

[a]Implanted on June 30, 1989.

Fig. 2. The healing acceleration of wounded tree bark tissues by dressing with a sheet of chitin films in a camellia tree. The picture was taken 3 years after dressing. The lower wound was dressed with a sheet of chitin films, and the upper wound was not dressed (control).

by tree tissue extracellular chitinase into oligosaccharides. The oligosaccharides play a function in eliciting the biosyntheses of phytoalexins [14, 15] and pathogenesis-related (PR) proteins including chitinase [16] and phenylalanine ammonia-lyase [17], resulting in increases in plant growth and yields by preventing plants from pathogenic infection at cellular level. Furthermore, the biodegradation of chitin is accelerated by induced chitinase isoforms in the bark tissues [16]. Chitin and its derivatives are usable not only as a natural elicitor of PR-proteins and phytoalexins, but also as a wounded bark dressing material for trees and as a novel supporting material for the controlled release of plant hormones and agricultural chemicals.

REFERENCES

1. Jap. Soc. Chitin/Chitosan (Ed.) (1995) *A Hand Book of Chitin and Chitosan*, Gihodo, Tokyo (in Japanese).
2. S. Hirano (1995) in C. G. Gebelein and C. E. Carraher, Jr. (Eds.), *Industrial Biotechnological Polymers*, Techonomic, Lancaster, PA, pp. 189-203.
3. L. S. Graham and M. B. Sticklen (1994) *Can. J. Bot.* **72**, 1057-1083.
4. S. Hirano, Y. Ohe, and H. Ono (1976) *Carbohydr. Res.* **47**, 315-320.
5. S. Hirano (1988) *Methods Enzymol.* **161**, 408-410.
6. S. Hirano and R. Yamaguchi (1976) *Biopolymers* **15**, 1685-1691.
7. S. Hirano (1978) *Agr. Biol. Chem.* **42**, 1939-1940.
8. S. Hirano, A. Usutani, and M. Zhang (1994) *Carbohydr. Res.* **256**, 331-336.
9. T. Imoto and K. Yagishita (1971), *Agr. Biol. Chem.* **35**, 1154-1156.
10. O. Schales and S. S. Schales (1945) *Arch. Biochem.* **8**, 285-292.
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) *J. Biol. Chem.* **193**, 265-275.
12. S. Hirano and Y. Yagi (1980) *Carbohydr. Res.* **83**, 103-108.
13. S. Hirano and T. Matsumura (1987) *Carbohydr. Res.* **165**, 120-122.
14. C. R. Allan and L. D. Hadwiger (1979) *Exp. Mycol.* **3**, 285-287.
15. A. Yamada, N. Shibuya, O. Kodama, and T. Akatsuka (1993) *Biosci. Biotech. Biochem.* **57**, 403-409.
16. H. Inui, Y. Kosaki, K. Uno, K. Tabata, and S. Hirano (1991) *Agr. Biol. Chem.* **55**, 3107-3109.
17. S. Notsu, N. Saito, H. Kosaki, H. Inui, and S. Hirano (1994) *Biosci. Biotechnol. Biochem.* **58**, 552-553.