This is to certify that the

dissertation entitled

MOLECULAR STUDIES OF THE SITES AND MODE OF ACTION OF THE GENE-SPECIFIC TOXIN VICTORIN

presented by

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has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Botany & Plant pathology
Natural Science

L. Patl Vant
Major professor

Date 5-7-92

O-12771

MOLECULAR STUDIES OF THE SITES AND MODE OF ACTION OF THE GENE-SPECIFIC TOXIN VICTORIN

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1992

ABSTRACT

MOLECULAR STUDIES OF THE SITES AND MODE OF ACTION OF THE GENE-SPECIFIC TOXIN VICTORIN

By

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Cochliobolus victoriae Nelson, which produces the host-specific toxin victorin, causes victoria blight of oats (*Avena sativa*). Immunological methods with anti-victorin polyclonal antibody and anti-victorin polyclonal anti-idiotypic antibody were used for the characterization of the victorin receptor in oat cells.

Polyclonal antibody against victorin produced in rabbits immunized with victorin-BSA conjugates was highly sensitive, and could detect as little as 10 pg/ml of victorin in an indirect ELISA. *In vivo* and *in vitro* covalent binding of victorin to proteins in susceptible and resistant oat tissues was examined by western blotting assays using anti-victorin antibody and second antibody conjugated with ^{125}I or alkaline phosphatase. Victorin binding to proteins of 100 and 45 Kd *in vivo*, and 100, 65 and 45 kd *in vitro* were detected in both susceptible and resistant cultivars of oats.

For the localization of victorin binding proteins in oat tissues, homogenates of dark-grown oat tissues were separated in continuous/ discontinuous sucrose gradients, and an aqueous two phase method, treated with victorin, and covalent binding sites detected by western blotting.

The 100 kd victorin binding protein was located in the high density (37 to 44%) sucrose fraction in a continuous sucrose gradient, but was not located in isolated plasma membrane nor mitochondria membrane. The 65 kd and 45 KD victorin binding proteins were detected in all separated cell fractions.

Anti-victorin polyclonal anti-idiotypic antibodies were generated in rabbits immunized with anti-victorin antibody-ovalbumin conjugates. The polyclonal anti-idiotypic antibodies added with or without victorin to susceptible and resistant oat protoplasts acted as an agonist and also an antagonist against the effects of victorin in only susceptible cells. Since antibodies are too large to enter the cells, these results indicate that the receptor site of victorin exists on the surface of oat cells.

We hypothesize that a victorin receptor on the surface of susceptible oat cells is involved in the susceptibility of oat cells to victorin. However, since victorin binds to the same proteins in both susceptible and resistant cultivars of oats, host-specificity may be explained by some other component in the victorin-transduction pathway.

To my wife Miyuki, son Junichiro, and our parents.

ACKNOWLEDGMENTS

I would like to greatly thank Dr. L. Patrick Hart, who trusted me and gave me a chance to start my studies in Michigan State University in 1988. Since then, he always treated me and my family like a part of his family, and his encouragement, support, guidance, and patience have continued throughout my Ph. D. program. Dr. Jonathan D. Walton, who also helped me to have a chance to study in MSU, gave me continuous encouragement and valuable comments, technically and philosophically, which taught me a way of thinking on host-specific toxin research. Dr. Rawle I. Hollingsworth, and Dr. Raymond Hammerschmidt, who were the members of my guidance committee, were always available for discussion and gave many worthy suggestions. I also thank all the members of Pat's lab., especially Mr. Joseph clayton, and Jonathan's lab., especially Dr. John Scott-Crag and Dr. Dan Panaccion, for many suggestions and stimulating discussions. Your passions and critical attitude for research always encouraged me and braced me up. Finally, as a foreign graduate student in the United States of America, I would like to thank the Department of Botany and Plant Pathology, and MSU for various kinds of support.

I would like to give special thanks for the cheer and help of my wife Miyuki, and our parents.

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CHAPTER 1:

PRODUCTION OF ANTI-VICTORIN POLYCLONAL ANTIBODY AND DETECTION OF COVALENT BINDING SITES OF VICTORIN BY WESTERN BLOTTING.

INTRODUCTION

Cochliobolus victoriae Nelson causes victoria blight of oats (26). Victoria blight caused a severe loss of oats throughout North America in 1946 as a consequence of the introduction of cultivars carrying the Pc-2 gene for resistance to crown rust caused by *Puccinia coronata* (26, 37). Because of the excellent resistance to a major pathogen of oats, *P. coronata*, the cultivars possessing the Pc-2 gene constituted approximately 80 % of all oats grown in the United State by 1945 (37). However, the gene determining the resistance to *P. coronata* was either closely linked or controlled by the same locus for susceptibility to *C. victoriae (24, 31)*, and growers had to abandon the cultivars within a few years after the new disease "victoria blight" appeared in 1946 (37). Although no longer economically important, the study of this disease has continued because of the nature of victorin, the host-specific toxin produced by the fungus (34, 36, 41), and because of the genetic linkage between victorin sensitivity and resistance to *Puccinia coronata* in oats (24, 31). The gene which is responsible for victorin sensitivity (and hence *C. victoriae* susceptibility) and resistance to *P. coronata* in the host is the so called *Vb* or *Pc* gene (34, 36). Early attempts to isolate victorin resistant mutants showed that the gene for *C. victoriae* susceptibility is closely linked if not identical to the one for crown rust resistance (24). More recently, victorin resistant oat plants were generated by the selection of oat tissue culture in the presence of victorin (31). All of the mutated

oat plants also lost their resistance to crown rust, and major genomic rearrangements were at least cytologically not found (31). A similar observation to the one-gene hypothesis was seen in the selection experiments of HMT toxin resistant maize (4, 14, 39). Texas cytoplasm male sterility, and sensitivity to HMT toxin are strictly correlated (4, 14, 39). Both traits are maternally inherited and attempts to separate the two traits by Gengenbach *et al.* were unsuccessful (14, 39). All of the selected toxin insensitive clones from maize callus in tissue culture with or without HMT toxin were male fertile. Male sterile and toxin insensitive or male fertile and toxin sensitive mutants have not been obtained (4, 39). Thus, the one gene hypothesis that the two traits are controlled by the same gene is favorable in other examples of a host-specific toxins and sensitive plants, even though the mechanism of sensitivity to toxins in plants has not been determined.

A current hypothesis to explain the sensitivity to victorin is that the product of the *Vb* gene is a victorin receptor and that the lack of the receptor leads to resistance (29, 34). The receptor for victorin could be also a receptor for *P. coronata*. Litzenberger first proposed that *P. coronata* might make victorin or a victorin-like compound (22). During the interaction of the receptor with *P. coronata*, the victorin or victorin-like compound would cause cell death, which would result in death of an obligate parasite, such as *P. coronata*, and the interaction would cause localized cell death (hypersensitive death) rather than whole tissue/plant death. Mayama *et al.* also reported that victorin works as an elicitor of an oat phytoalexin, avenalumins, which is toxic to *P.*

coronata but not *to C. victoriae* (25). If *P. coronata* produced victorin or a victorin-like compound, the interaction with receptor might induce not only the hypersensitive reaction but also phytoalexin production. Some evidence indirectly suggests the existence of victorin receptor sites. Pre-treatment with various protein synthesis inhibitors (cycloheximide, puromycin, and MDMP), or RNA synthesis inhibitors, (actinomycin D, 6-methylpurine), or SH-residue blocking reagents (N-ethylmaleimide, dinitroflourobenzene, iodoacetate, and sodium arsenite), protected susceptible oats from victorin (12, 30, 40). Heat-treatment, which causes denaturing of proteins in tissues, also reduced the sensitivity to victorin (5, 6). Pre-treatment with toxoid of victorin; ie., victorin analog containing a reduced aldehyde (45), also reduced the toxicity of the native form of victorin (45). Early effects of victorin on susceptible tissue include membrane potential change (28), permeability changes of the plasma membrane (18), rapid leakage of electrolytes (5), lysis of protoplasts (30), and extracellular synthesis of callose from protoplasts (40). Although none of these data indicates directly the existence of host-specific receptor sites, the modification of proteins which may include a receptor site in oat tissue clearly affects the sensitivity to victorin. Thus, the understanding of the nature of the victorin receptor relates directly to the search for the mechanism of toxin sensitivity and of disease susceptibility.

The structures of the major and four minor forms of victorin have been characterized as chlorinated, partially cyclic pentapeptides (46,47). The structure consists of glyoxylic acid, 5,5-dichloroleucine, *erythro-β-*

hydroxyleucine, victalanine, *threo*-β-hydroxylysine, and α-amino-β-chloro acrylic acid (46)(Fig. 1). The hydrated aldehyde function of the glyoxylic acid residue of victorin is required for toxic activity (45). Since many aldehydes have been known to bind covalently to proteins with biological functions (33), it has been speculated that victorin binds irreversibly to its receptor by the functional aldehyde residue. Wolpert and Macko (43) conjugated victorin to ^{125}I Bolton-Hunter reagent and examined the binding of this victorin analog to oat tissue proteins. The radiolabeled analog covalently bound to a 100 kd protein only from the susceptible oat genotype *in vivo*, but bound to a 100 kd protein in both susceptible and resistant oats *in vitro* (43). The 100 kd protein in susceptible and resistant oat cultivars was immunologically the same protein (44), but a mechanism to explain the differential binding of victorin *in vivo* and *in vitro* was not determined.

The objective of this study was to identify victorin binding proteins using native victorin and immunological methods rather than a modified form of victorin. We have generated polyclonal antibodies against the isolated major form of victorin, and the binding of native victorin to oat and other non-host plant tissue proteins were detected with the anti-victorin antibody by western blotting assays. Since the pre-treatment of cycloheximide dramatically reduced the toxicity of victorin (12, 18, 40), the effect of the protein synthesis inhibitor on the binding of victorin was also determined.

A part of this study has been published (1).

MATERIALS AND METHODS

Plant Materials

Park and X469 are *C. victoriae* susceptible and victorin sensitive oat cultivars, and Garry, Korwood, and X424 are *C. victoriae* resistant and victorin insensitive oat cultivars. X469 and X424 are near-isogenic lines differing at the *Vb* allele (10, 11), and kindly provided by Dr. V. Macko, Boyce Thompson Institute, Cornell University. Wheat (*Triticum*) cv. ionia and *Arabidopsis* cv. colombia, were used for *in vivo* binding assays. For *In vivo* and *in vitro* victorin binding experiments, oats and wheat were grown for 9 days in the green house. 3 week old *Arabidopsis* plants were kindly provided by Mr. Jun Tsuji, MSU. For protoplast assays, oats were grown 20 to 25 days in a growth chamber under fluorescent and incandescent lamps (florence rate: 140 μmol/m/sec) for a 12 hr photo-period at 18°C.

Victorin C preparation

Victorin C was isolated from 40 L of culture filtrates of *C. victoriae* (HV1146A) by the slightly modified methods described by Mayama et al. (25). Briefly, the isolate was grown in 125 ml of Wheeler's oat flake medium in a 1 liter flask for 14 days at room temp. The culture filtrate was filtered through cheesecloth and 2 layers of Whatman No.1 filter paper. The clear culture fluid was passed through 5.5 X 52 cm column of amberlite XAD-7 at a flow rate of 15 ml per min, washed with deionized

water, and the toxin was extracted with 1 liter of 40 % EtOH with 0.05 % TFA. The eluant was concentrated to a volume of 30 ml, and directly loaded on a 2.6 X 23 cm column of vydac adsorbent 218TPB at a flow rate of 10 ml per min (about 15 to 25 psi) with the following solvent system, and 100 ml fractions were collected. The solvent system was: 150 ml water containing 0.05 % TFA, 300 ml 10 % acetonitrile (ACN) containing 0.05 % TFA, 300 ml of 14 % ACN/0.05 % TFA, 850 ml of 17.5 % ACN/0.05 %TFA, 400 ml of 25 % ACN. The biologically active 100 ml fractions were concentrated to 6 to 14 ml and further purified by HPLC. One to 1.5 ml of the sample was injected in a reverse-phase HPLC column (Chemcosorb 5-ODS-H (20mm X 250 mm)), and eluted with 20 to 80 % ACN gradient containing 0.05 % TFA for 30 min at a flow rate of 4 ml per min. The peaks were detected at UV 270 nm.

Bioassays to detect victorin fractions throughout this purification process were the root growth inhibition assay (35) and protoplast death assay (13, 30, 40). For the root growth inhibition assay, fractions from each purification step were diluted to 10^{-3} and 10^{-4} with water in 35 X 10 mm polystyrene disposable dishes, and 5 pre-germinated seeds (root length 1 to 2 mm) of susceptible or resistant oats were placed into the solution. After 48 hr incubation in the dark at room temp., root growth was observed. Usually, no root growth in the susceptible oats was observed in the fractions containing victorin, but root growth of 1 to 3 cm was observed in other fractions or when resistant oat seeds were used.

For the protoplast assay, 1 ml of prepared protoplast (see Protoplast preparations in Materials and Methods) solution (5 X 10^5 protoplast/ml) was

mixed with 1 μl of each fraction and incubated for 12 hr at room temp. in the dark. 40 μl of each sample and protoplast mixture was then mixed with 2 μl of FDA solution (0.5 %) and the percentage of live cells per total cells was examined under the fluorescent microscope (average of 100 cells were counted). Most of the time, 90 to 100 % of the susceptible cells died in the victorin containing fractions. An M_r 796 of isolated victorin by mass spectrum analysis was confirmed by Dr. D. Gage, Mass Spectrometry Facility, MSU.

Anti-victorin antibody production

Conjugation and immunization for anti-victorin polyclonal antibody preparation

Five milligrams of victorin was conjugated through the epsilon amino group to 10 mg of BSA with 3 mg of N-(m-maleimidobenzoyloxy)-succinimide (MBS) as the coupling reagent by the method described by Kitagawa et al. (19). Briefly, 5 mg of victorin was added in 1 ml of 0.05 M sodium phosphate buffer solution (pH 7.0), and mixed with 3 mg of MBS dissolved in 0.5 ml of tetrahydrofuran at 30°C for 30 min with occasional stirring. Tetrahydrofuran was removed by nitrogen gas, and the solution was extracted 4 times with 5 ml of methylene chloride to remove excess MBS. The aqueous layer (upper) was removed to a new tube and mixed with a solution of reduced BSA. Reduced BSA was prepared by dissolving 10 mg of BSA in 2 ml of 6 M UREA-0.1 M EDTA, and mixing with 20 mg of $NaBH_4$ in 0.2 ml of n-butanol. The $NaBH_4$ solution was added drop by drop to the BSA

solution. The mixture was then incubated at 30°C for 30 min and excess $NaBH_4$ was decomposed by adding 1 ml of 0.1 M sodium phosphate (monobasic) and 0.4 ml of acetone. The two solutions, activated victorin with MBS and reduced BSA, were then incubated at 25°C for 2 hr and the conjugates were purified by microconcentrator (Centricon 30, Amicon). The coupling was determined by measuring the amount of tritium-labeled victorin (5.5 mCi/mmol)(15) bound to the final protein product, and the coupling efficiency was calculated by the difference in M_r between BSA and the BSA-victorin conjugate determined by SDS-PAGE (19). One milligram of victorin-BSA conjugate in 2 ml of saline and Freund's complete adjuvant mixture (1:1) was injected intradermally into female white New Zealand rabbits. Boosts were administered intramuscularly two times at 28 d intervals with 0.5 mg of conjugate in 0.5 ml of saline and Freund's incomplete adjuvant mixture (1:1). Blood was collected 10 to 14 days after each injection. IgG was purified from the serum of each sample by protein A column chromatography using an IgG purification kit of Pierce (Pierce Chemical Co.) with the buffer system of Pierce. The purified polyclonal antibodies were used for ELISA or further purified on an immobilized-BSA column to remove antibody not specific to victorin. The immobilized BSA column was prepared by packing 2 ml of immobilized BSA-agarose into a polystyrene column (8 mm X 102 mm); about 1 mg of antibodies partially purified on the protein A column was loaded on the column and washed with 2 ml of 0.01 M sodium phosphate, pH 7.2, plus 0.5 M NaCl, 6 times (total 12 ml). Each 2 ml fractions was collected and the presence of rabbit IgG in each fraction and protein concentration were

determined by using anti-rabbit IgG-alkaline phosphatase conjugate in indirect ELISA. Briefly, 100 µl of diluted fraction ($1:10^3$ with PBS) was placed in victorin treated microtiter wells (see indirect ELISA) and incubated for 2 hr at 37°C. After the wells were washed with PBS-0.2% Tween (v/v)(PBS-Tween) 4 times, 300 µl of 1% ovalbumin (w/v) in PBS-Tween was added into each well to block remaining sites and incubated for 1 hr at 37°C. The wells were again washed with PBS-Tween 4 times, and 100 µl of 1:500 diluted goat anti-rabbit IgG-alkaline phosphatase was added. After a 30 min incubation at 37°C, the wells were washed 10 times with PBS-Tween and 100 µl of p-nitrophenyl phosphate (1 mg/ml final concentration) in 10 % diethanolamine, pH 9.8, was added to each well as a substrate. The absorbance at 405 nm was determined on a Bio-Tek 308 ELISA plate reader after the addition of 100 µl of 0.1 M EDTA. Protein concentration was determined by the methods described by Bradford (2).

Indirect ELISA

Four micrograms of victorin in 100 µl of PBS (pH 7.2)(16) was added to each well of the microtiter strips (Immulon 2, Dynatech) and incubated overnight at 4°C. Unbound victorin was removed from the plate by washing each well 15 times with PBS containing 0.2 % (v/v) Tween 20 (PBS-Tween). PBS-Tween (300 µl) was added to each well and incubated for 30 min at 37°C to block any remaining binding sites on the well surface. The plate was then washed five times with PBS-Tween, and 100 µl of anti-victorin antibodies (7.9 ng/ml to 1 µg/ml final concentration) in PBS-Tween or a mixture of antibodies (1 µg/ml) and different concentrations of free

victorin (5 pg/ml to 1 μg/ml final concentration) were added to each well and incubated for 1 hr at 37°C. Unbound antibodies were removed by washing 15 times with PBS-Tween, and 100 μl of goat anti-rabbit IgG-alkaline phosphatase conjugate (1:1000 dilution) in PBS-Tween was added to each well. Following incubation for 30 min at 37°C, the plate was again washed 15 times with PBS-Tween. Alkaline phosphatase activity was measured by adding 100 μl of p-nitrophenyl phosphate (1 mg/ml final concentration) in 10 % diethanolamine, pH 9.8, as a substrate. The reaction was stopped after 30 min at room temp. by adding 100 μl of 0.1 M EDTA, and the absorbance at 405 nm was determined on an ELISA reader (Bio-Tek model EL 308).

Direct ELISA

For direct ELISA, victorin was conjugated to horse radish peroxidase (HRP) using a Pierce Activated Peroxidase Kit according to the manufacturer's directions. Briefly, 600 μg of victorin in 50 μl of conjugation buffer (1 M $NaHCO_3$, pH 9.5, 0.9 % NaCl) was mixed with 1 mg of activated HRP in 50 μl of water (HRP solution must be on ice), and incubated overnight at 4°C. The reaction was stopped by adding 30 μl of quench buffer (0.2 M lysine) and incubated for 2 hr at room temp. The solution was then mixed with 350 μl of stabilizing solution (1 % BSA in DW) and dialyzed against PBS. The final conjugate solution (480 μl final volume) was stored at -20°C and used as a stock solution.

The direct ELISA was performed as follows: 100 μl of anti-victorin antibodies in PBS (200 μg of protein A column purified antibodies/ml) was

placed in each well of microtiter strips and incubated overnight at 4°C. Excess antibodies were removed and the plate was washed 15 times with PBS-Tween. Plates were then incubated with 300 µl of the PBS-Tween for 0.5 hr at 37°C to block unbound binding sites and washed with PBS-Tween five times. Different concentrations of Victorin-HRP (diluted $1:10^4$ to $1:10^6$ from the stock solution) or victorin HRP (diluted $1:10^4$ from the stock solution) plus free victorin (100 pg/ml to 1 µg/ml final concentration) were placed in the wells and incubated for 1 hr at 37°C. The plates were then washed 15 times with PBS-Tween, and 100 µl of substrate (0.4 mM 3,3'-5,5'-tetramethylbenzidine with 0.0004 % H_2O_2 in 50 mM sodium acetate buffer, pH 6.0) was added. After 20 min of incubation at room temp., the reaction was stopped with 100 µl of 2 M H_2SO_4 and the absorbance at 450 nm measured on an ELISA reader.

Detection of victorin binding proteins by western blotting

Preparation of victorin binding proteins for western blotting assay.

All protein preparations for western blot assays were modified from the methods described by Wolpert et al. (43).

In vivo victorin binding assay

Nine-d-old oat or wheat leaves (0.5 g) or 3 week old of Arabidopsis leaves (0.5 g) were sliced with a razor blade to 1 mm wide sections and mixed with or without victorin (10 µg/ml final concentration) in 10 ml of

10 mM Mops plus 1% (v/v) sucrose (pH 7.0). The mixture was incubated for 1 h at room temperature with gentle shaking and then washed six times with 500 ml distilled water on filter paper (Whatman No.4). The tissue was either homogenized in 2 ml of isolation buffer (50 mM Tris/HCl, pH 7.0, 0.7 M sucrose, 5 mM DTT, 100 mM KCl, and 5 mM EDTA) with mortar and pestle and immediately mixed with 2 ml of water saturated phenol or homogenized in 2 ml of phenol and 2 ml of the isolation buffer mixture. In each case, the homogenate was transferred to a 15-ml centrifuge tube (Corning Glass Works) and centrifuged at 900g for 5 min. The upper, chlorophyll-containing layer was mixed with 10 ml of 0.1 M ammonium acetate in cold methanol and stored overnight at 4°C. The protein precipitate was collected by centrifugation at 900g for 5 min and washed with 10 ml of methanol three times by centrifugation. The pelleted protein was dried in a vacuum and solubilized in 0.5 ml of resuspension buffer (62.5 mM Tris/HCl, pH 6.8, 2.3%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, and 10%(w/v) sucrose) with a glass homogenizer. The homogenate was stored at -80°C until used.

In vitro victorin binding assay.

Leaves (0.5 g) from 9-d-old S or R oats were homogenized with mortar and pestle in 10 ml of cold buffer containing 50 mM Mops, 2 mM EDTA, 0.4 M sucrose, and 6 mM 2-mercaptoethanol, pH 7.5. The homogenate was filtered through four layers of cheesecloth and two layers of Kimwipe (Kimberly-Clark). The filtrates were centrifuged at 400g for 4 min, and

the supernatants were recentrifuged at 100,000g for 30 min. The pellet was suspended with 0.5 ml of buffer and homogenized in a glass homogenizer. All isolation procedures were done at 4°C. The protein concentration was adjusted to 1 mg/ml with buffer and then different concentrations of victorin added. The mixture was incubated for 1 h at room temperature with occasional stirring and stopped by the addition of the same volume of resuspension buffer (62.5 mM Tris/HCl, pH 6.8, 2.3% (w/v) SDS, 5%(v/v) 2-mercaptoethanol, and 10%(w/v) sucrose). The proteins were stored at -80°C until used.

Western blotting assay.

Plant tissue protein samples prepared for the western blotting assays were analyzed on 6.5 % polyacrylamide gels in the buffer system of Laemmli (20). Forty micrograms of protein from each sample was loaded per lane on the gel. After separation by SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher & Schuell, 0.1 μm pore size) for 4 hr with an electrotransfer unit (LKB2117 multiphor II) at 5 V and 90 mA. Transfer buffer was 25 mM Tris plus 192 mM Glycine. The nitrocellulose filters were blocked with PBS-Tween overnight at room temp. with gentle shaking. The filters were washed several times with PBS-Tween and incubated with anti-victorin antibodies (1 μg/ml final concentration) in PBS-Tween containing 5% BSA (w/v) for 1 hr at room temp. with gentle rocking. The anti-victorin antibodies were purified on immobilized protein A and BSA columns from serum by the methods described above. To determine if antibody was binding specifically to victorin, 1 μg/ml of victorin was

added to the antibody solution as a control. The filters were washed five times with 50 ml of PBS-Tween for 5 min each time and treated with goat anti-rabbit IgG-alkaline phosphatase conjugate at 1:1000 dilution or 5 μCi of goat anti-rabbit IgG labeled with ^{125}I (specific activity 5 to 20 μCi/μg protein) for 30 min at room temp. with gentle shaking. When goat anti-rabbit IgG-alkaline phosphatase was used, the filters were again washed five times with PBS-Tween, and binding was detected with 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium in 10 ml of pH 9.5 buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5) (16) by 30 min incubation at room temp. with gentle rocking. The reaction was stopped by the addition of 2 ml of 0.1 M EDTA. The filter was dried after washing with distilled water. When ^{125}I-conjugated goat anti-rabbit IgG was used, the filter was washed seven times with 50 ml of PBS-Tween buffer for 5 min each time and dried. The filter was exposed to Kodak X-Omat AR film with an intensifying screen at -80°C for 15 hr. The M_r of bands detected on blots were estimated with prestained mol wt standards (M_r range 14,300 to 200,000).

The effect of cycloheximide treatment on victorin binding in oat plants.

Nine day old oat plants (S and R) were pre-treated with 5 μg/ml of cycloheximide solution as described by Gardner and Scheffer (12). Oat plants were grown for 9 days and excised shoots were weighed, and the basal ends inserted in cycloheximide solution (5 μg/ml) or in water (control). Cuttings were allowed to transpire under fluorescent lights (10^3 to 2.2 x 10^3 lx) for 12 hr, thereby infiltrating the tissues; solutes

are known to be distributed quickly through tissues (12). The basal 1 cm of each cutting was then discarded. The cycloheximide or water treated oats were treated with victorin by the methods described in the *in vivo* victorin binding assays. The victorin binding proteins were then determined by western blotting assay.

Protoplast preparation.

Sterile protoplasts were prepared by the methods described by Schaeffer and Walton (32). Briefly, oat leaves were cut from 20 to 25 day old plants grown in the growth chamber (condition described in Plant Materials) and fresh weight was measured. Leaves (500 mg) were sterilized with 10 % bleach, abraded with carborundum, and incubated at 26°C for 3 hr in 2 % (w/v) Cellulysin in SCM (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes, pH 5.5) with gentle shaking. Released protoplasts were filtered through 80 μm nylon screen mesh, washed twice with SCM (pH 5.8), and collected by centrifugation at 500 rpm (100g) for 5 min. The protoplasts were washed by centrifugation two more times with SCM (pH 5.8), and resuspended with SCM (pH 5.8) at a final concentration of 5×10^5 protoplasts/ml for toxicity assays during victorin purification. All procedures were performed under sterile conditions.

RESULTS

Purification of victorin C.

Eighty seven milligrams of victorin was isolated from 40 l of culture filtrates. Victorin appeared at about 65 % ACN in the 20 to 80 % ACN gradient on HPLC system (see Materials and Methods), and the final peak of isolated victorin is indicated in Fig. 2 (A). The molecular mass of the isolated victorin was determined as 796 by mass spectrum analysis (Fig. 2 (B)), and the isolated victorin was confirmed as the major form of the toxin, victorin C (47).

ANTI-VICTORIN POLYCLONAL ANTIBODY PRODUCTION

Preparation of anti-victorin antibodies.

Victorin and BSA were conjugated using the crosslinker N-(m-maleimido benzoyloxy)-succinimide by the methods described by Kitagawa (19). The difference in M_r of non-conjugated BSA and victorin conjugated BSA was about 7800 on SDS-PAGE, and the molar ratio of bound victorin was about seven, calculated according to Kitagawa (19). When a mixture of 0.8 μCi of tritium-labeled victorin plus 3 mg of victorin was conjugated to BSA, about 9 % of tritium-labelled victorin was determined from the

conjugate product. After the first injection and two boosts, antibodies were purified by protein A column chromatography (for ELISA) or by protein A column chromatography and immobilized BSA column chromatography (for western blotting assays). After protein A column chromatography, about 3 mg of antibody (in 3 ml of elution buffer) was obtained from 0.5 ml of serum, which originally had a protein concentration of 90 mg/ml. The antibody solution was stored at -20°C and used as a stock solution for ELISA assays. About 600 μg of antibodies was obtained from 1 mg of the protein A column-purified antibody after passing through the immobilized BSA column. The antibodies were contained in the first 2 ml fraction after washing 6 times with 2 ml washing buffer (Fig. 3). The purified antibodies were stored at -20°C until used for western blotting assays.

Characterization of anti-victorin antibody in ELISA.

The antibodies purified by protein-A were characterized by indirect and direct ELISAs. In the indirect ELISA, since unconjugated victorin bound directly to the wells of the microtiter plate, 40 μg of victorin in 100 μl of PBS was used for coating the plates. As little as 7.9 ng/ml of antibody could detect the bound victorin (Fig. 4). About 1 μg/ml of antibody, which is a 10^{-3} dilution from the stock of antibody after purification by protein A column chromatography, showed an O.D. of 1.2 to 1.3 at A_{405} (Fig. 4), and this concentration was used as a standard concentration for the following competition experiments. The competition experiments in the indirect ELISA were performed using 5 pg/ml to 1 μg/ml

of victorin as a competitor. The binding of anti-victorin antibodies was inhibited by as little as 10 pg/ml of victorin (Fig. 5). Ten ng/ml of victorin inhibited antibody binding by 50% (Fig. 5), and more than 90 % of binding was inhibited by 1 μg/ml of victorin (Fig. 5). The antibodies were also characterized by direct ELISA. Victorin at 1 ng/ml was detectable, and 50% of victorin-HRP binding was inhibited by 25 ng/ml of victorin (Fig. 6). Nearly 100 % of victorin-HRP binding was inhibited by 1 μg/ml of victorin (Fig. 6). In each of the ELISAs, addition of 5 % BSA (w/v) into the anti-victorin antibody solution reduced non-specific binding (data not shown).

DETECTION OF VICTORIN BINDING PROTEINS BY WESTERN BLOTTING

Western blotting analysis of *in vivo* and *in vitro* victorin binding.

For western blots, the anti-victorin antibodies were further purified by immobilized-BSA column after passage through the protein A column. BSA column purification and addition of 5% BSA to the antibody solution significantly reduced the non-specific binding of antibodies recognizing BSA (data not shown). When oat leaf slices were incubated with victorin in the *in vivo* assay, strong binding was detected to proteins of 100 kD and 45 kD in both S cultivar and R cultivar by using a second antibody (goat anti-rabbit IgG) conjugated to [^{125}I] (Fig. 7). These bands disappeared when 1 μg/ml victorin was incubated simultaneously with the

anti-victorin antibody solution (Fig. 7). The binding of victorin to several other S and R oat cultivars was also determined using a second antibody conjugated with alkaline phosphatase (Fig. 8). Victorin bound to a 100 kD protein in all S and R oat cultivars we tested, but the signal from cv. X 424 (R) was consistently less intense than from the S cultivars, or other R cultivars, like Korwood and Old Fulgrain (Fig. 8). Since detection of binding by alkaline phosphatase was less sensitive than [^{125}I], we could not detect 45 kD bands on the blot with alkaline phosphatase. When the tissue was homogenized in phenol and isolation buffer mixture according to the method described by Wolpert *et al.* (43), *in vivo* binding of victorin to the 100 kD protein was still observed in both S and R oat cultivars (Fig. 9). In the *in vivo* experiment, treatment with 10 μg victorin/ml was necessary to detect the binding.

In the *in vitro* assays, we observed victorin binding to 100 kD, 65 kD, and 45 kD proteins in both S and R cultivars with 1 μg victorin/ml (Fig. 10). The 100 kD binding protein was detected with 100 ng victorin/ml, but no binding was observed with less than 10 ng/ml of victorin (Fig. 10). These bands disappeared after addition of 1 μg/ml victorin to the antibody solutions as a competitor (data not shown).

The *in vivo* binding of victorin to non-host plants, wheat and *Arabidopsis*, was also examined by the same methods described above. Victorin bound covalently to a 40 kD protein from wheat, and 55 kD and 41 kD proteins from *Arabidopsis*, respectively (fig. 11).

Effects of cycloheximide pre-treatment on victorin binding.

In vivo binding of victorin was detected by western blotting, after cycloheximide or water treatment of 9 day old excised shoots. In both the cycloheximide and water treatments, 133 kD victorin-binding protein was detected in addition to the 100 kD and 45 kD proteins (Fig. 12).

Densitometer scanning of the autoradiographies indicated a less intense band (about 25% less) of 45 kD binding protein for the cycloheximide treated tissue from the susceptible oat (X469), but not the resistant cultivar (X424). This experiment was performed only once and needs to be repeated for confirmation.

DISCUSSION

Anti-victorin polyclonal antibodies were produced in rabbits immunized with victorin-BSA conjugates. These antibodies showed specificity against victorin in both indirect and direct ELISAs. The antibodies were highly sensitive and could detect as little as 10 pg/ml of victorin in an indirect ELISA (Fig. 3). Lower molecular weight compounds, such as host-specific toxins, are non-immunogenic molecules (34). This study with anti-victorin antibody suggests that other host-specific toxins may also be potential candidates for immunological studies of receptor proteins in host plants.

The anti-victorin antibodies were used to detect victorin that had bound irreversibly to oat tissue proteins by the western blotting assays. The polyclonal anti-victorin antibodies contain a number of idiotypes which recognize the different portions of the victorin molecule as epitopes (16). This polyclonal feature of our antibodies often gives an advantage in the western blotting assays since multiple binding of antibodies to victorin on oat tissue proteins generates a stronger signal (16).

The binding of the antibodies to specific proteins was observed both when victorin was mixed with intact oat tissues (Figs. 7 and 8) and when victorin was mixed with microsomal preparations (Fig. 10). Binding was visualized with both a second antibody conjugated to [^{125}I] (Fig. 7) and

a secondary antibody conjugated to alkaline phosphatase (Fig. 8). The binding was specific because addition of free victorin to the antibody solution eliminated binding (Fig. 7). The molecular masses of victorin-binding proteins were 100 kD and 45 kD in the *in vivo* assay (Fig. 7) and 100 kD, 65 kD, and 45 kD in the *in vitro* assay (Fig. 10), respectively. The intensities of the 65 kD and 45 kD bands varied in different experiments (data not shown). In *in vivo* assays, the apparent intensity of victorin binding to proteins in the R cultivar X424 was consistently less than other R cultivars, like Korwood, Old Fulgrain, and the isogeneic S cultivar X469 (Fig. 8).

Wolpert *et al.* proposed two hypothesises to explain the specificity of victorin based on victorin-receptor interactions; 1) Since the aldehyde group which is known to covalently bind to proteins with biological functions (33), is an essential component for its toxicity of victorin (43), victorin may bind to the receptor irreversibly; 2) covalent binding of victorin to a 100 kD protein was genotype-specific in *in vivo* experiments (43). However, since victorin was bound *in vitro* to a 100 kD proteins in both susceptible and resistant oats, they speculated that the *in vivo* genotype-specific binding was due to the presence of a reducing factor for the victorin binding site directly on the 100 kD protein or in associated proteins near the 100 kD protein of susceptible oats but not of resistant oats (43).

Our results were similar, in that we detected covalent binding of victorin to oat tissue proteins, notably one of 100 kD, in both *in vivo* and *in vitro* experiments. However, our results differ in that we did not

find evidence for host-specific binding of victorin *in vivo*. In our experiments, binding *in vivo* occurred in both S and R oats (Fig. 7 and 8) and also non-host plants (Fig. 11), but not just S oats (43).

Although we followed the protocol of Wolpert *et al.* (43) closely, there were some potentially important differences. Our tissue slices were 1 mm wide instead of 0.5 mm; we used more tissue per experiment (0.5 g as opposed to ten 0.5 mm leaf slices); and we used a higher concentration of victorin (10 μg/ml as opposed to 8.8 ng/ml of the [^{125}I]Bolton-Hunter victorin analog plus 100 ng/ml native victorin) (43). However, the most significant difference is that we used native victorin and specific anti-victorin antibodies to detect the proteins to which it had bound, rather than a radiolabeled derivative of victorin. The Bolton-Hunter victorin conjugate is larger (M_r 945 (44) as opposed to 796 for native victorin), more hydrophobic, and 100-times less active than native victorin (47). Further, Wolpert's experiments required an addition of 100 ng of cold victorin together with [^{125}I]Bolton-Hunter victorin analog only in *in vivo* experiments but not in *in vitro* experiments (only 3 μCi of labeled victorin added without cold victorin in *in vitro* experiments) (43) is difficult to explain, and might explain a potential problem with the victorin analog binding. Since our results showed that the intensity of the 100 kD band of cultivar X424, which Wolpert *et al.* used as their resistant oat cultivar, was less than other cultivars (Fig. 8), 100 ng of cold victorin added with radiolabeled victorin analog probably acted as a competitor to the binding of victorin analog to the 100 kD protein. In our experiments, we used native victorin, but 10 μg/ml of victorin was

required to detect the victorin binding on oat tissues. The higher concentration of victorin (10 μg/ml) necessary in our experiments may result from the polyclonal nature of the anti-victorin antibodies, in which not all clones would produce antibodies capable of binding to available epitopes on victorin which was covalently bound to cell proteins.

A possible explanation for our results is that binding of victorin occurred during extraction; that is, non-specific *in vitro* binding occurred to some extent in our *in vivo* experiments. However, tissues treated with victorin in the *in vivo* assay were extensively washed with 3 L of water before the cells were homogenized. Since a victorin concentration of at least 100 ng/ml was necessary to detect victorin binding in the *in vitro* experiments (Fig. 10), it is unlikely that sufficient free victorin could have remained in the tissues after washing. To avoid any *in vitro* binding of victorin that might have remained in the tissues after washing, the tissue was homogenized in phenol-containing buffer as described by Wolpert *et al.* (43). However, this treatment did not eliminate the binding of victorin to the 100 kD protein from R oats (Fig. 9). Although our results showing no difference in binding to S and R cultivars of oat either *in vivo* or *in vitro* do not exclude a role for the major, 100 kD, victorin binding protein in the action of victorin, they do suggest that host-specificity might be due to some other component in the victorin-transduction pathway after victorin binds to oat tissue proteins. Victorin is the most specific and most toxic of any other host-specific toxins known (40). A mechanism to explain the high

specificity of host-specific toxins was proposed in 1964, and stated that the presence of a receptor site for toxin occurred in only susceptible plants but not in resistant plants (29). Since then, several lines of evidence have indicated indirectly the presence of receptor sites for the host specific toxins; mainly pre-treatment with protein synthesis inhibitors (12, 30, 40) or heat-treatment of host plant tissues (5, 6) which dramatically decrease the sensitivity of susceptible oat tissues for victorin, and the reduction of toxicity of some host specific toxins on its host plant by treatment with toxoids; such as toxoids of HS-toxin converted from HS-toxin by β-galactofuranosidase and sugarcane (23), AF-toxin II which is the isomer of the major form of AF-toxin I and strawberry (27), and a victorin derivative which contains a reduced aldehyde group on the structure and oat (43). These latter examples indicate that the receptor sites were blocked by the toxoids against native toxin binding, and as a result, a reduction of toxicity to the native toxins occurred. However, there has been no evidence to show specific binding of toxin based on the host genotype, except *in vivo* experiments of Wolpert *et al.* (43). Although Strobel reported that radiolabeled HS-toxin specifically binds to the membranes prepared from only susceptible cultivars of sugarcane but not from resistant cultivars (38), later evidence by Scheffer *et al.* (21) pointed out the specific activity of the ^{14}C-HS-toxin derivative used in Strobel's study (7.4 nCi/μmol) was not strong enough to detect binding. The best examined protein which is present only in susceptible plants, is a 13 kD mitochondrial protein in Texas cytoplasmic (Tms-) maize sensitive to HmT-

toxin or PM-toxin (3, 7, 8, 42). The 13 kD protein only exists in mitochondria of Tms-maize and not in normal types of maize (42), and expression in *E. coli* of the mitochondrial gene encoding the 13 kD protein (URF 13) imparts toxin sensitivity to the bacteria (8). Haung *et al.* also demonstrated that the expression of URF 13 in yeast (*Saccharomyces cerevisiae*) mitochondria induced sensitivity to the toxin (17). These data clearly indicate that URF 13 and localization of a 13 kD protein in mitochondria is essential for the sensitivity to HmT-toxin or PM-toxin. However, there is still no direct evidence indicating that the 13 kD protein is the receptor site of these toxins. Frantzen *et al.* first demonstrated the binding of ^{3}H-labeled HmT-toxin and PM-toxin analogs in both Tmc- and normal mitochondria without any differences (9). Braun *et al.* later found the uptake or binding of ^{3}H-labeled analogs on/or into *E. coli* cells is only found in the *E. coli* cells expressing URF 13 (3). In contrast, there was no significant, but only a consistent small difference in the binding of HMT toxin analogs to mitochondria between Tmc- and normal types (3). Although binding or uptake of the analogs to *E. coli* cells and expression of URF 13 is well correlated, there is still no direct evidence for the specific-binding of toxin to the gene product, a 13 kD protein. Based on a receptor hypothesis, the factor(s) which are responsibility for the sensitivity to the host-specific toxins should; (i) be present in only susceptible plants, (ii) be related to the action of toxin in a susceptible plant, (iii) are not necessarily the binding site of the toxin, but may occur anywhere in the transduction-pathway after toxin binding. Our results indicate that there are at least two binding

proteins from *in vivo* experiments and three from *in vitro* experiments for victorin covalent binding sites, but with no difference between susceptible and resistant oats. Therefore, it can be speculated that some other factor exists in susceptible oats that works after victorin binding to the tissues, and is responsible for the specificity. Our binding experiment with cycloheximide treated oats supports this idea. Treatment of cycloheximide dramatically decrease the effect of victorin (12, 30, 40), however, victorin binding was reduced, but not eliminated (Fig. 12). The cycloheximide treatment may be affecting an other component in the victorin transduction pathway which is responsible for victorin specificity. There are alternative ways to explain our results; 1) victorin binds to the same proteins on susceptible and resistant oats, but conformational differences of the victorin-protein complex may affect the subsequent events in the victorin transduction pathways between susceptible and resistant oats, 2) Although the aldehyde group of victorin is essential for its toxicity (45), aldehydes are also known to produce unstable carbinolamines, which may not be a strong enough bond to withstand SDS-PAGE (33). However, the hypothesis that host-specificity might be due to some other component in the victorin-transduction pathway after victorin binds to oat tissue, is the most likely explanation for our data at this point.

In conclusion, since victorin binds to the same proteins in both susceptible and resistant cultivars of oats, and victorin binds irreversibly to the proteins of non-host plants, host-specificity may not

be explained by victorin binding, but rather by some other component in the victorin transduction pathway.

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Fig. 1. The structure of the major form of victorin, victorin C.

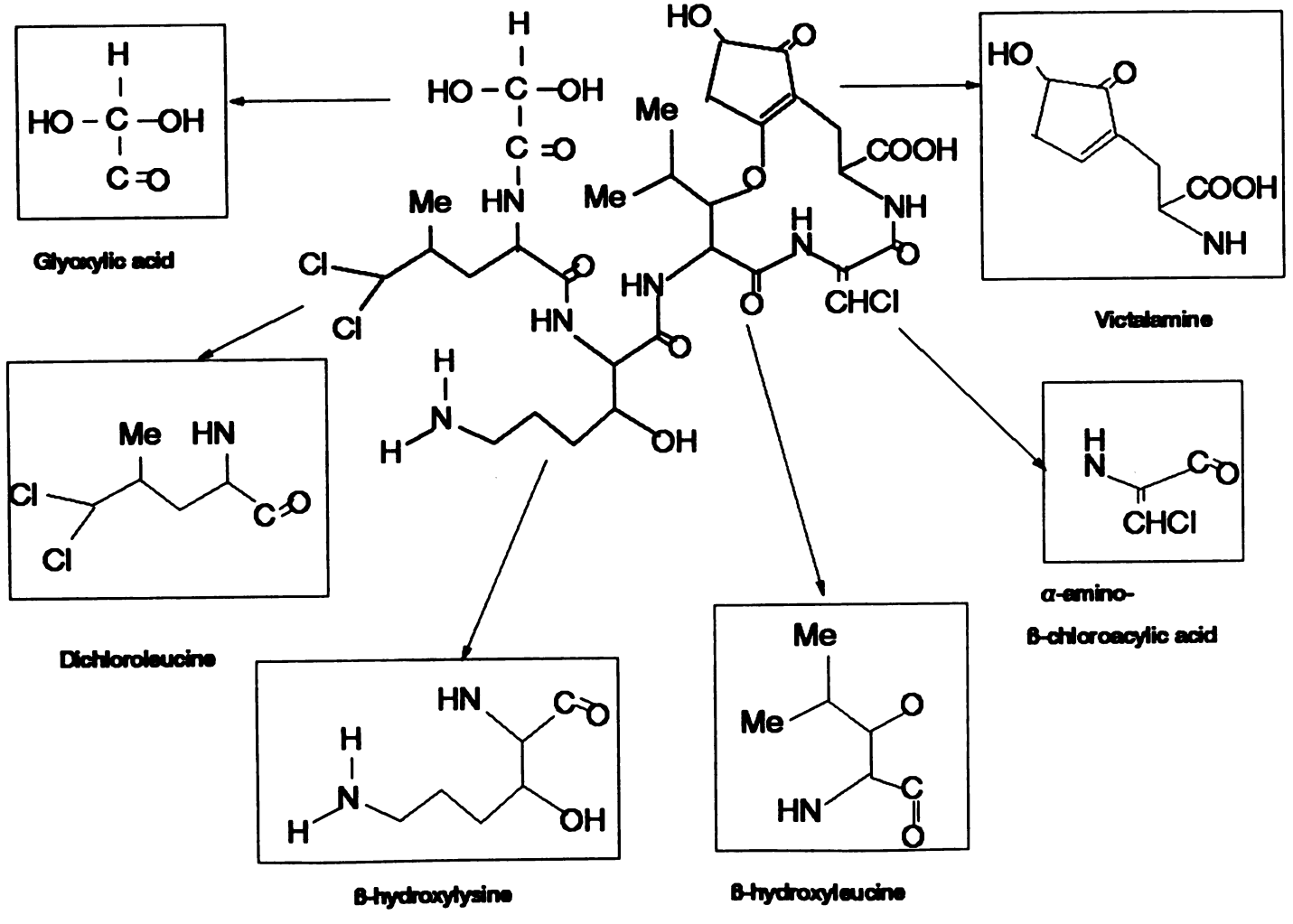

Glyoxylic acid
Victalamine
Dichloroleucine
α-amino-
β-chloroacylic acid
β-hydroxylysine
β-hydroxyleucine

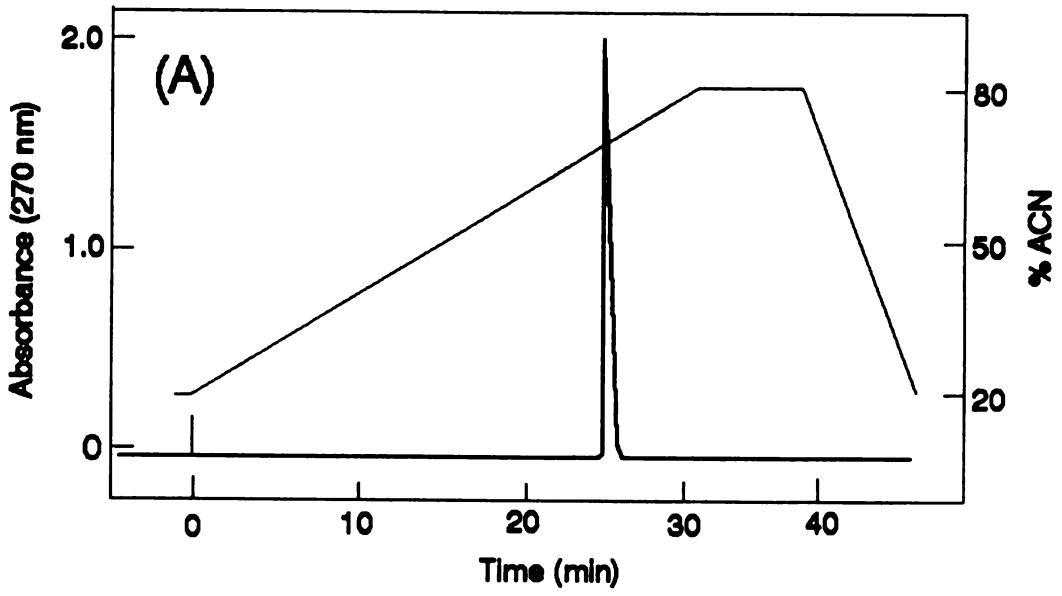
Fig. 2. Isolation of major form of victorin, victorin C.

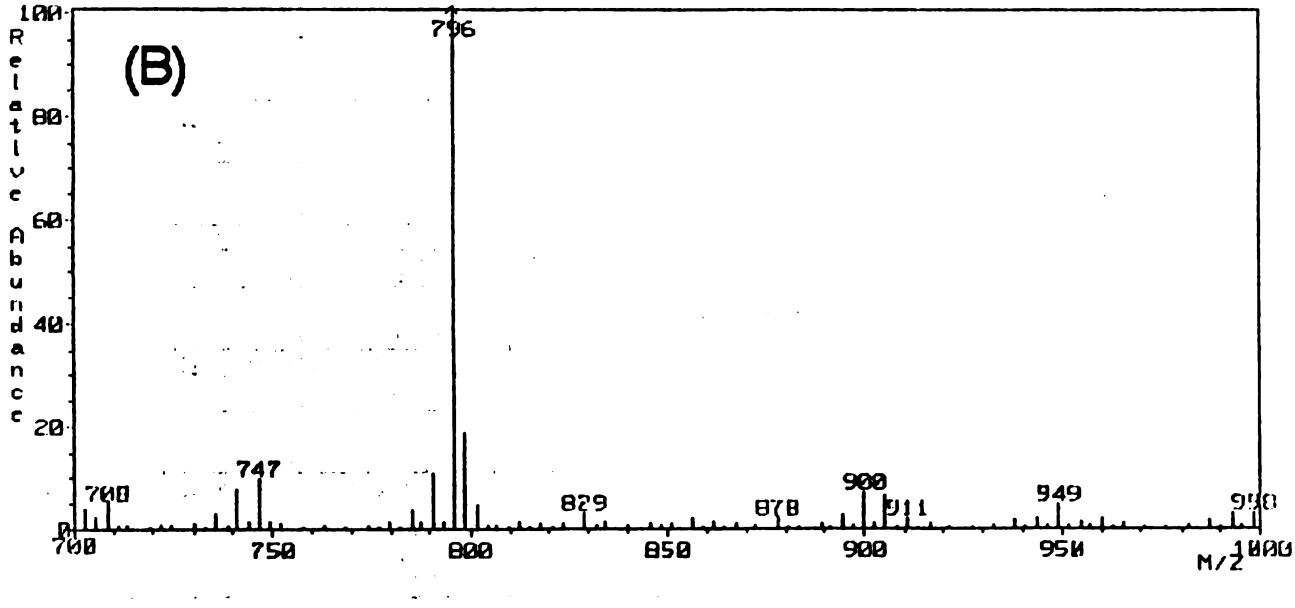
(A) The peak of isolated victorin C on reverse-phase HPLC.

Column:	Chemcosorb 5-ODS-H (20mm X 250mm).
Flow rate:	4 ml/min.
Solvent:	20 to 80 % ACN in water gradient for 30 min.
Detected UV:	270 nm.

(B) The mass spectrum analysis of isolated victorin C.

Sample:	100 μg isolated victorin in MeOH/4 μg in Gly/HCl.
BP:	m/z 185.4871 Int. 84.7565.
Scan Mode:	MF (positive) centroid.
Data File:	W10248801 (Oct/24/88); Mass spectrum facility, MSU.


(A)
Absorbance (270 nm)
2.0
1.0
0
80
50
20
% ACN
0
10
20
30
40
Time (min)


(B)
Relative Abundance
100
80
60
40
20
796
747
700
829
878
900
911
949
950
700
750
800
850
900
950
1000
M/Z

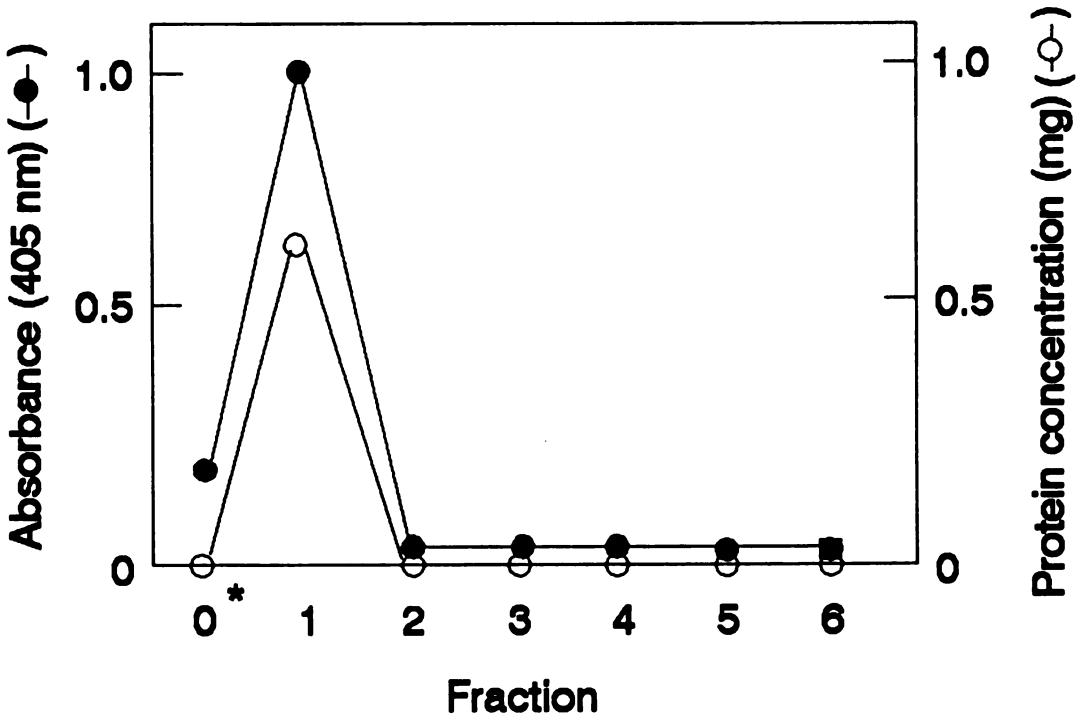


Fig. 3. Purification of anti-victorin IgGs by immobilized BSA column chromatography. About 1 mg/ml of antibody solution purified by protein A column was loaded on the BSA column and were collected as fraction 0*. The column was then washed 6 times with 2 ml each time of 0.01 M sodium phosphate, pH 7.2, plus 0.5 M NaCl, and the elutes were collected as fraction 1 to 6. Left side of the vertical bar indicates the presence of IgGs detected by ELISA with anti-rabbit Ig-alkaline phosphatase conjugate (see Materials and Methods of Chapter 1). Right side of the vertical bar indicates the protein concentration.

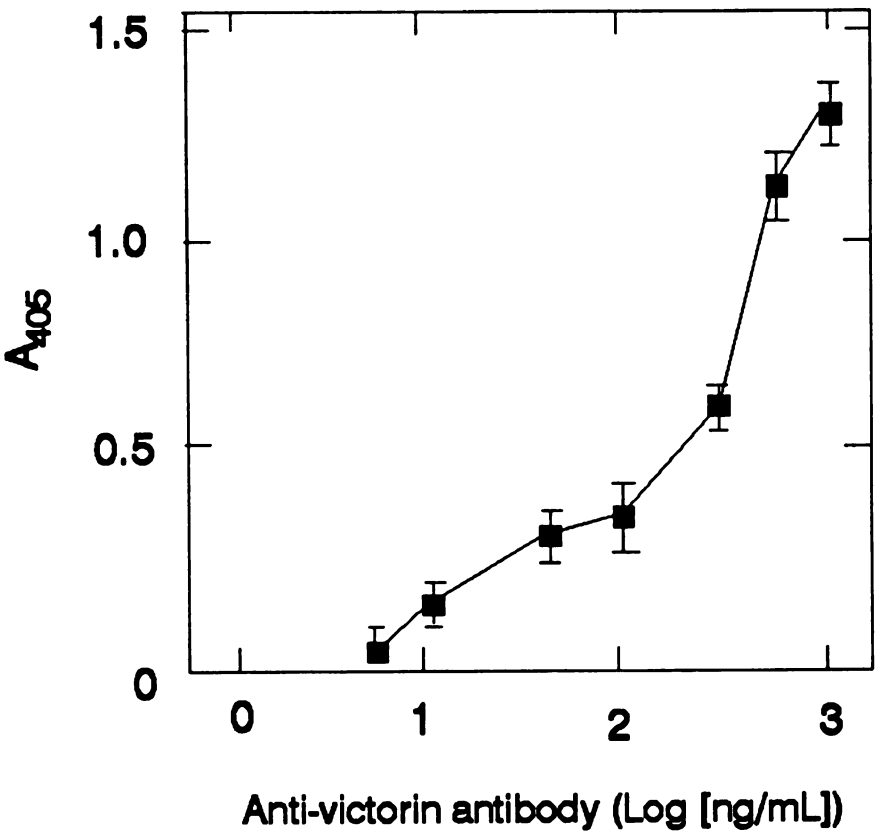


Fig. 4. Sensitivity of anti-victorin antibody to victorin in an indirect ELISA. Different concentrations of anti-victorin antibody (7.9 ng/ml to 1 μg/ml) were added to microtiter wells coated with victorin (see Materials and Methods of Chapter 1) and incubated for 1 hr at 37°C. The antibodies binding to victorin were detected with anti-rabbit IgG conjugated to alkaline phosphatase. Error bars indicate standard deviations of the mean of six repetitions.

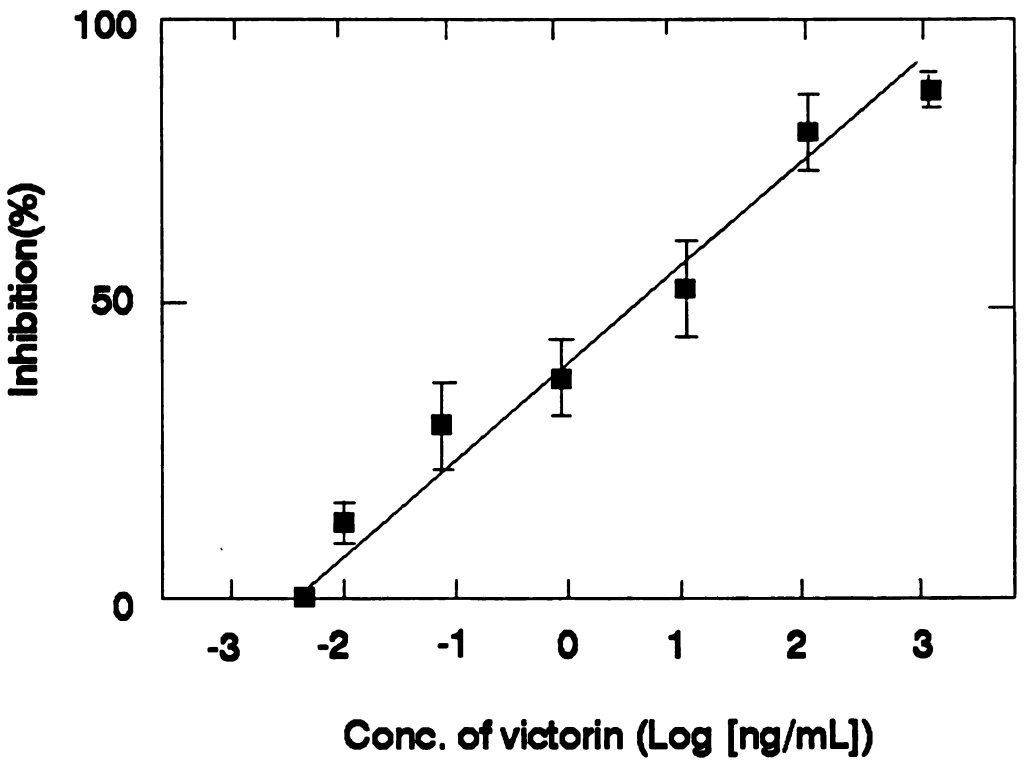


Fig. 5. Sensitivity of anti-victorin antibody in a competitive indirect ELISA. Different concentration of free victorin (5 pg/ml to 1 μg/ml) were mixed with anti-victorin antibody (1 μg/ml) and added to microtiter wells coated with victorin (see Materials and Methods of Chapter 1). The wells were incubated for 1 hr at 37°C, and antibody binding to victorin was determined with anti-rabbit IgG and alkaline phosphatase conjugate. The percentage of inhibition of antibody binding by free victorin compared with control without victorin added was calculated. Error bars indicate standard deviations of the mean of six repetitions.

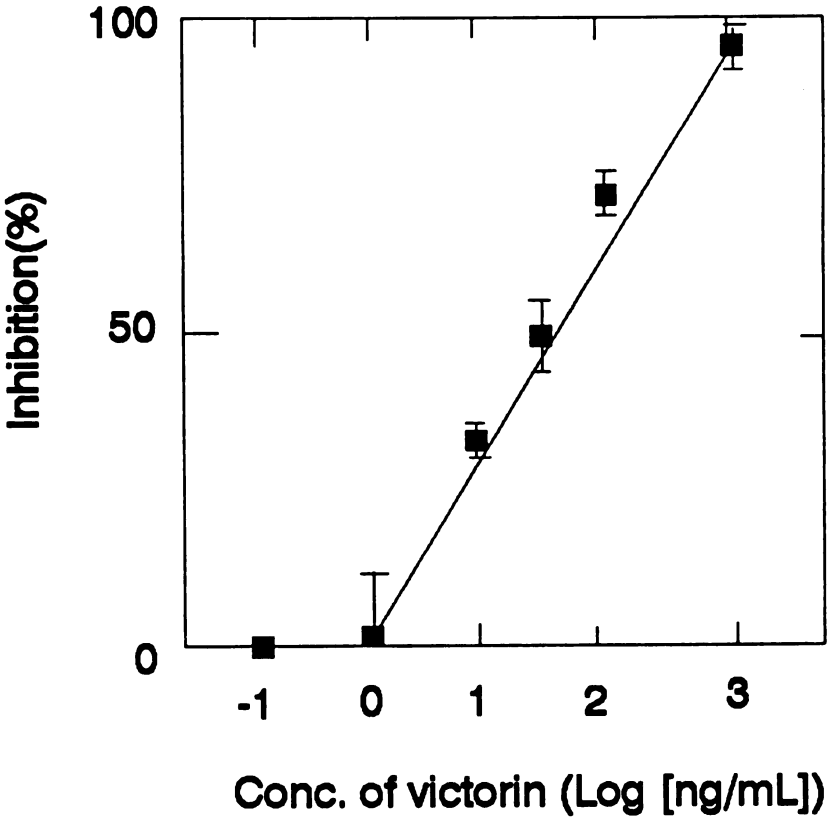


Fig. 6. Sensitivity of anti-victorin antibody in a competitive direct ELISA. Different concentrations of free victorin (100 pg/ml to 1 μg/ml) and victorin-HRP conjugate were mixed and added to microtiter wells coated with anti-victorin antibody (see Materials and Methods of Chapter 1). The A_{450} of each well was read after addition of substrate for HRP, and the percentage of inhibition by free victorin was compared with control without victorin added. Error bars indicate standard deviations of the mean of six repetitions.

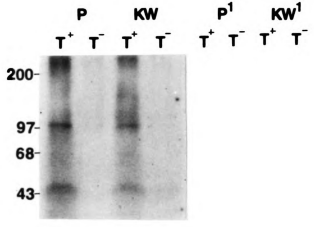


Fig. 7. *In vivo* binding of victorin to proteins from susceptible (P, Park) and resistant (KW, Korwood) oats. The binding of native victorin was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. The samples were prepared from tissues treated with either 10 μg/ml of victorin (T^+) or without victorin (T^-)(see Materials and Methods of Chapter 1). P^1 and KW^1: free victorin (1 μg/ml) was added to the first antibody solution. The values at left are molecular masses of standard protein in kD.

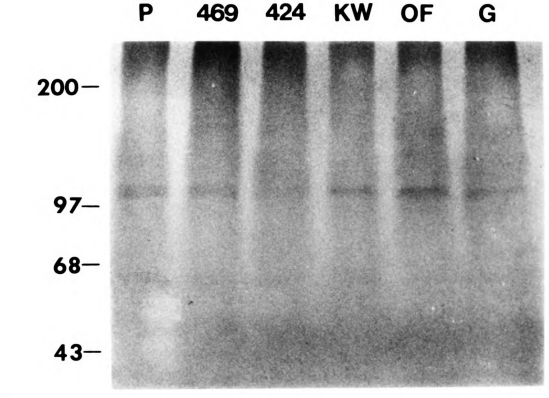


Fig. 8. *In vivo* binding of victorin to susceptible (P, Park; 469, X469) and resistant (424, X424; KW, Korwood; OF, Old Fulgrain; G, Garry) cultivars of oat. The binding of native victorin was visualized on blots with anti-victorin antibody and anti-rabbit IgG conjugated with alkaline phosphatase. The values at left are molecular masses of standard protein in kD.

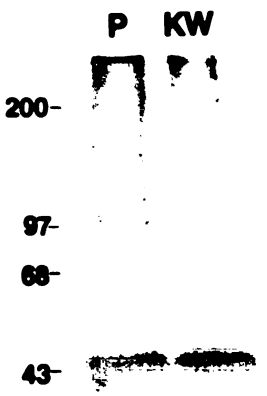


Fig. 9. *In vivo* binding of victorin to proteins from susceptible (P, Park) and resistant (KW, Korwood) oats. Victorin-treated tissue was homogenized with isolation buffer containing phenol to denature the proteins immediately after cells were disrupted (see Materials and Methods in Chapter 1), and the binding was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. The values at left are molecular masses of standard proteins in kD.

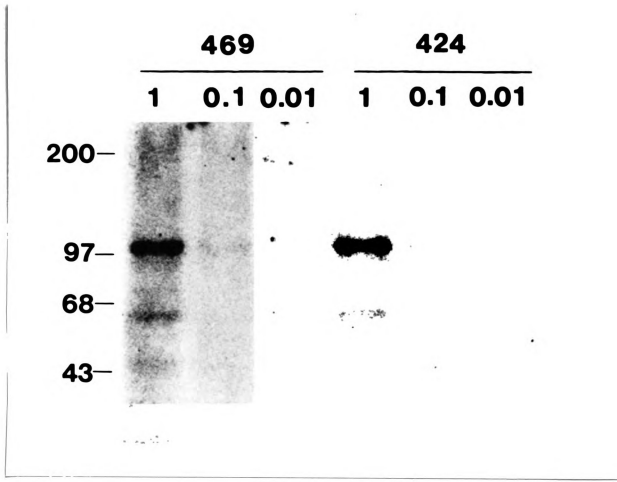


Fig. 10. *In vitro* binding of victorin to proteins from susceptible (X469) and resistant (X424) oats. Concentrations of victorin reacted with microsomal preparations (1 mg/ml) were 1, 0.1, and 0.01 μg/ml, respectively. The binding was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. The values at left are molecular masses of standard protein in kD.

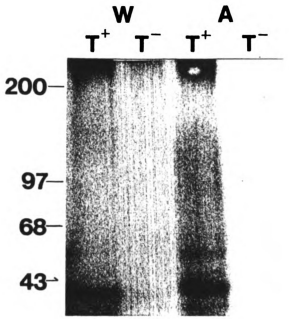


Fig. 11. *In vivo* binding of victorin to proteins from non-host plants. The binding of native victorin was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. The samples were prepared from tissues treated with 10 μg/ml of victorin (see Materials and Methods in Chapter 1). The values at left are molecular masses of standard protein in kD.

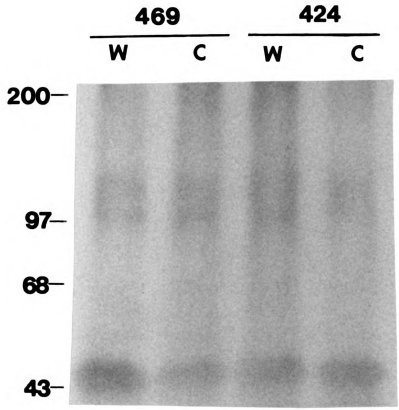


Fig. 12. The effects of cycloheximide treatment on victorin binding to proteins from susceptible (X469) and resistant (X424) oats. After 5 μg/ml of cycloheximide solution (C) or water (W) was transpired into the tissues of excised shoots of oats, the tissues were treated *in vivo* with 10 μg/ml of victorin (see Materials and Methods in Chapter 1). The binding of native victorin was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. The values at left are molecular masses of standard proteins in kD.

CHAPTER 2:

LOCALIZATION OF VICTORIN BINDING IN OAT TISSUES

INTRODUCTION

A hypothesis to explain the specificity of victorin is that the product of the *Vb* gene is a victorin receptor and that the lack of the receptor leads to resistance (27). Since aldehyde residues are known to bind covalently to proteins with biological functions in nature (32), and the aldehyde moiety of victorin is essential for toxicity, covalent binding sites of victorin were speculated as possible receptors of victorin (41, 42). Wolpert and Macko made a radiolabeled victorin derivative with ^{125}I Bolton-Hunter reagent, and first reported (41) that the victorin analog binds covalently to 100 kD protein of only susceptible cultivars of oat *in vivo*, but not to resistant cultivars of oat. ^{125}I Bolton-Hunter victorin also binds to 100 kD protein *in vitro*, but is not genotype-specific because this derivative binds both susceptible and resistant cultivars of oat (41). The mechanism to explain the different pattern of victorin binding between *in vivo* and *in vitro* experiments has not been identified (41).

We generated polyclonal anti-victorin antibody and determined the victorin covalent binding sites of tissue proteins using native victorin and anti-victorin antibody. The results of victorin binding studies described in Chapter 1 differed from that of Wolpert and Macko (41). Victorin binds *in vivo* to 100 kD and 45 kD proteins of both susceptible and resistant cultivars of oat, and to 100 kD, 65 kD, and 45 kD proteins

of susceptible and resistant cultivars of oat *in vitro*. Victorin binds covalently to the same proteins of susceptible and resistant cultivars of oat. Victorin also binds covalently to different molecular weight proteins of non-host plants, wheat and *Arabidopsis*. Based on our data, the specificity of victorin in susceptible and resistant oats may not be explained by victorin binding. Rather, since victorin binds to both susceptible and resistant tissues, the host-specificity of victorin might be determined by another component, the possible product of *Vb* gene, in the victorin transduction pathway.

We speculated that the component responsible for the specificity is in the victorin transduction pathway, but that binding of victorin would be necessary to initiate toxicity. Some evidence indirectly suggests the existence of victorin binding sites. Pre-treatment of susceptible oat tissues with toxoids of victorin, (i.e. a reduced-victorin (42)), in which the glyoxylic acid residue of victorin was converted to glycolic acid, protected cells when the native form of victorin was subsequently added (42). It was concluded that protection occurred because the toxoid mimicked the binding of victorin to receptor (42). Pre-treatment of oat tissue protein with protein synthesis inhibitors, SH-residue blocking reagents, or heat shock reduced the toxic effects of victorin, and were thought to a denature or block the victorin receptor site (6, 8, 9, 28, 37).

Early effects of victorin on susceptible tissue include membrane potential changes (26), permeability changes of the plasma membrane (15), rapid leakage of electrolytes (6, 31), lysis of protoplasts (28), and

extracellular synthesis of callose from protoplasts (37). Depolarization, which is the neutralization of negative charges on plasma membrane potential, is the fastest effect known to occur in susceptible oat cells treated with victorin (26). The membrane potential changes occur 2 to 5 min after victorin treatment (26). Permeability changes determined by ^{86}Rb efflux indicated that 20 min was required for a change of plasma membrane and tonoplast permeability in tissues treated with victorin (15). Whether plasma membrane and tonoplast contain sites equally sensitive to victorin is still unknown. However, they inferred that the effect on the tonoplast was a secondary effect because changes in the plasma membrane appeared faster than the other organelles and because the plasma membrane would be the first membrane to come into contact with victorin when applied exogenously (15). Membrane permeability changes resulted in leakage of electrolytes 1 hr after treatment with victorin (6, 31). Extracellular secretion of callose was observed only from susceptible oats within 2 hr (37). Glucan synthase II, which is a responsible for the production of callose, is located in plasma membrane (24), and is stimulated to produce callose with as little as 6 pg/ml of victorin (37). The stimulation of callose synthesis is the most sensitive reaction of susceptible oat cells to victorin known (37). Victorin first causes a potential change (26), then changes in the permeability properties (6, 15), and finally lysis of the susceptible protoplast plasma membrane within 4 hr of exposure to victorin (28). Our results using anti-victorin anti-idiotypic antibody, which is described in Chapter 3 of this thesis, also indicated that the victorin receptor is located on the

surface of oat cells. Although these data indirectly suggest that the primary site of victorin would be located in the plasma membrane, no direct evidence has been shown.

Several covalent binding sites of victorin in oat tissues were identified (1, 41), but the location of the victorin binding proteins in the cell and the relationship between the toxicity of victorin and covalent binding of victorin still remains an unsolved question.

The objective of this study was to identify the location of covalent victorin binding proteins by western blotting with fractionated oat tissues. Light-grown or dark-grown oat tissues were separated by continuous/discontinuous sucrose gradients, or an aqueous two phase method, mixed with victorin, and the covalent binding proteins of victorin identified. The location of the binding-proteins in the cell was determined by comparison of victorin binding and the examination of each binding fraction by several membrane marker enzyme analyses.

MATERIALS AND METHODS

Plant Materials

Park was used as a *C. victoriae* susceptible and victorin sensitive oat cultivar, and Korwood was used as a *C. victoriae* resistant and victorin insensitive oat cultivar. For aqueous two phase experiments, oats were grown for 9 days in the green house. For continuous and discontinuous sucrose gradients, oat seeds were germinated in sterile conditions on 4 layers of wet cheesecloth in an aluminum box covered with aluminum foil, and grown for 5 to 7 days at room temp. until the height of shoots was about 5 cm.

Victorin C preparation

Victorin C was isolated from 40 L of culture filtrates of *C. victoriae* (HV1146A) by the slightly modified methods described by Mayama et al. (23). Described in detail in Materials and Methods of Chapter 1.

Anti-victorin antibody production

Anti-victorin antibody was produced in rabbits immunized with victorin-BSA conjugate, and purified by immobilized protein A and BSA columns for the western blotting assays by the method described in Materials and Methods of chapter 1.

Detection of victorin binding proteins by western blotting

All protein fractions (50 μl) separated by continuous/or discontinuous sucrose gradient, or aqueous two phase methods, were mixed with 1 μg/ml (final concentration) of victorin, and analyzed on 6.5 % polyacrylamide gels in the buffer systems of Laemmli (19). Forty micrograms of protein from each sample was loaded per lane on the gel. After separation by SDS-PAGE, proteins were transferred to nitrocellulose, blocked with PBS-Tween, incubated with anti-victorin antibodies (1 μg/ml final concentration), and treated with 5 μCi of goat anti-rabbit IgG labeled with ^{125}I by the methods described in chapter 1. The filter was exposed to Kodak X-Omat AR film with an intensifying screen at -80°C for 15 hr. The intensity of bands was measured by a computer densitometer (Image Quant/Molecular Dynamics, Co. Ltd.), and the relative intensity of each band was calculated as a percentage against the bands of the highest sucrose fraction (usually 44% (w/w) sucrose fraction). The M_r of bands detected on blots were estimated with prestained mol wt standards (M_r range 14,300 to 200,000). Protein concentrations were measured by the Bradford method (5) with BSA as a standard.

Continuous sucrose gradient and victorin binding assay.

The procedure used for the continuous gradient was modified from the methods described by Walton *et al* (38, 39).

Fifteen grams of oat shoots (5 cm height) grown in the dark at room temp. were harvested and chilled on ice for 10 to 15 min. The shoots were sliced with a razor blade to 1 mm wide sections then ground at

4°C in a mortar and pestle in 20 ml isolation buffer (50 mM MOPS, pH 7.5, 2 mM EDTA, 0.4 M sucrose, plus 5% (v/v) 2-mercaptoethanol). The homogenate was passed through 4 layers of cheesecloth and two layers of kimwipe and centrifuged 10 min at 1000g. 10 ml each of this supernatant (total 20 ml) was layered onto two different tubes of a 20 ml linear 20 to 45% (w/w) sucrose gradient in gradient medium (50 mM MOPS, pH 7.5, 2 mM EDTA, plus 5 mM DTT), and centrifuged for 3.5 hr at 24,000 rpm (80,000g at r_{av}) in a Beckman SW 27 rotor at 4°C. After centrifugation, a small hole was made by a needle in the bottom of the tubes and 1.5 ml gradient fractions collected. The sucrose percentage (w/w) of each fraction was measured by refractometer. 50 µl of each fraction was transferred to a new tube and mixed with 1 µg/ml (final concentration) of victorin. The tubes were incubated for 1 hr at room temp. with occasional vortexing and stored at -80°C until used. The remaining sample was kept at 4°C for 3 to 4 days for latent IDPase assays or stored at -80°C for other enzyme assays.

Mitochondria preparation and victorin binding assay.

Fifteen grams of dark-grown oat shoots (5 cm height) were harvested, chilled for 10 to 15 min at 4°C, and chopped to 1 mm slices with a razor blade at 4°C. The slices were ground in 60 ml of isolation buffer (0.35 M sorbitol, 30 mM MOPS, pH 7.5, 1 mM EDTA, 0.2 %(w/v) BSA), with a mortar and pestle. The homogenate was passed through 4 layers of cheesecloth and 2 layers of kimwipe and centrifuged at 6,500 rpm (1000g) for 2 min in a Beckman SS 34 rotor. The supernatant was centrifuged again for 5 min

at 12,750 rpm (10,000g) in the same rotor, and the pellet was resuspended gently with 20 ml of washing buffer (0.3 M sorbitol, 20 mM MOPS, pH 7.2, 1 mM EDTA, plus 0.2 %(w/v) BSA). The solution was again centrifuged as described above for two washings, one at 6,500 rpm (1000g) for 2 min and one at 12,750 rpm (10,000g) for 5 min in a Beckman SS 34 rotor, and the pellet was resuspended with 2 ml of suspension buffer (0.25 M sucrose, plus 30 mM MOPS, pH 6.8). The solution was then layered onto the discontinuous sucrose gradient system; 0.6 M, 0.9 M, 1.2M, 1.45 M, and 1.8 M sucrose in 10 mM KH_2PO_4, pH 7.2, plus 1 %(w/v) BSA, and centrifuged for 45 min at 18,000 rpm (40,000g in r_{av}) in a Beckman SW 27 rotor. 1.5 ml fractions were taken from the bottom of the tube and the presence of mitochondria and plasma membrane was determined by cytochrome C oxidase and Glucan synthetase II assays, respectively (see membrane marker enzyme assay in Materials and Methods). For the victorin binding assay, 50 µl of each 1.5 ml fraction was transferred to a new tube and mixed with 1 µg/ml (final concentration) of victorin for 1 hr at room temp. with occasionally stirring. The victorin treated samples and the non-treated samples were stored at -80°C and victorin binding was detected by western blotting as described above.

Aqueous two phase method and victorin binding assay.

The aqueous two-phase method using dextran and polyethylene glycol is based on the methods described by Larsson et al. (18).

Chilled light-grown oat shoots were ground with mortar and pestle in 50 mM Mops, 2mM EDTA, 0.4 M sucrose, and 5 %(v/v) mercaptoethanol, pH 7.5 (1

g tissue/2.5 ml isolation buffer). The homogenate was filtered through 4 layers of cheesecloth and most of the chloroplast and mitochondria were pelleted at 10,000 *g* for 10 min. The supernatant was centrifuged at 50,000 *g* for 30 min and the pellet suspended with 10 ml of 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. Nine g of the suspension was then added onto 27 g of phase mixture to give a 36 g phase system with a final composition of 6.5 % (w/w) dextran T 500, 6.5 %(w/w) polyethylene glycol 3350, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. The phase system was mixed by 30-35 inversions of the tube and centrifuged in a swinging bucket rotor (Beckman SW 27) at 1500 *g* for 5 min. The upper layer was then washed twice with fresh lower phase and diluted with an equal volume of the isolation buffer; and the lower layer of the first tube was also washed twice with fresh upper phase and diluted with 9 volume of the isolation buffer (X10 dilution). Both diluted solutions were centrifuged at 100,000 *g* for 30 min and each pellet resuspended with 0.5 ml to 1 ml of isolation buffer. For the victorin binding assay, the concentration of the preparations was measured by commasie brilliant blue with BSA as the standard (5), immediately mixed with victorin (1 μg victorin/ml final concentration), and incubated for 1 hr with occasionally stirring at room temperature, and then stored in -80°C until used for western blotting. Cytochrome-c oxidase and glucan synthase II assays (see membrane marker enzyme assay in Materials and Methods) were performed with these preparations stored at -80°C. The chlorophyll concentration of each fraction was calculated by the method described by Arnon (3).

Membrane marker enzyme assays.

To determine the location of the victorin-binding proteins, proteins fractionated by continuous/discontinuous sucrose gradients, or aqueous two phase method, were examined with several marker-enzyme assays. 1,3-β-glucan synthase (glucan synthetase II) and vanadate-sensitive ATPase were used as marker enzymes for plasma membrane (29, 36, 40); cytochrome-C oxidase for mitochondria (2, 13); and NADH cytochrome-C reductase for endoplasmic reticulum (13, 18); IDPase (7, 30, 39) for golgi, respectively. After the homogenate was fractionateded by sucrose gradients or aqueous two phase method, the concentration of each fraction was measured by the method using commasie brilliant blue described by Bradford (4) with BSA as a standard, and 10 to 50 μl of each 1.5 ml fraction was used as a protein sample for the following membrane marker enzyme assays.

Vanadate-sensitive H^+-ATPase assay.

The vanadate-sensitive ATPase activity was determined from the slightly modified method described by Widell and Larsson (40).

One to 50 μg of protein in 50 μl of each fractionated sample was mixed with the reaction buffer (3 mM $MgSO_4$, 50 mM KNO_3, 1 mM sodium azide, 0.1 mM sodium molybdate, 50 mM Tris-Mes, pH 6.0, 0.33 M sucrose, 0.1 mM EDTA, 1 mM DTT, 0.05% (v/v) Triton X100), and with or without 0.1 mM sodium orthovanadate; 10 mM sodium orthovanadate solution was freshly prepared by boiling in 50 mM Tris-Mes buffer for 2 min, and adjusting the pH to 6.0 with Mes crystals after boiling, and then added to the reaction

buffer at the final concentration of 0.1 mM. The final volume of the reaction buffer was adjusted to 1 ml. The reaction was initiated by the addition of Tris salted ATP (10) at the final concentration of 3 mM and was run at 37°C for 60 min. The enzyme reaction was stopped by addition of 1 ml of ice-cold ascorbate/molybdate solution[3 % (w/v) ascorbate, 1 % (w/v) SDS, in 0.5 M HCl, mixed 15 min before use with freshly made 8 % (w/v) ammonium molybdate in ratio of 10:1; the reagent should be light-yellow]. Samples were kept on ice for at least 10 min. Excess molybdate was then complexed with 1.5 ml arsenite/ citrate solution [2 %(w/v) sodium citrate dihydrate, 2 %(w/v) sodium arsenite, and 2 %(v/v) glacial acetic acid; the salts were dissolved in water before acetic acid was added]. Samples were then incubated at 37°C for 20 min and the absorbance at 850 nm measured. A phosphate standard curve (0.2, 0.4, and 0.6 μmole phosphate) was made using KH_2PO_4 solution.

1,3-β-glucan synthase (glucan synthase II) assay.

1,3-β-glucan synthase (GS II) was determined by the modified method of Widell and Larsson (40).

The GS II activity was assayed in 100 μl buffer containing 50 mM HEPES-KOH, pH 7.25, 0.33 M sucrose, 0.8 mM spermine, 16 mM cellobiose, 4 mM EGTA/4 mM $CaCl_2$, 1mM DTT, and 0.01 % (w/v) digitonin. 10 μl of sample solution (1 to 10 μg/ml protein) from each fraction was added to 100 μl of buffer, and the reaction was initiated by the addition of 0.025 μCi of UDP-[^{14}C]-glucose [about 0.08 nmole (specific activity 318 mCi/mmole)] and 10 μl of 26 mM UDPG for a final concentration of 2 mM. The reaction

was run for 30 min at room temp. and terminated by adding 95 % EtOH until the final concentration of EtOH was 66 %. The samples were transferred to paper filter (Whatman 3MM) in a millpore vacuum filtration unit (Millipore) and washed twice with 1 ml of 66 % EtOH. The filters were then washed twice in 0.35 M ammonium acetate, pH 3.6, 30 %(v/v) ethanol, for 1 hr each time. The filter background was determined with blanks run without protein. The filters were dried and radioactivity measured in a liquid scintillation counter (Beckman/LS5000TDC).

Cytochrome-c oxidase assay.

The reaction solution contains 10 μl of sample solution (1 to 10 μg protein) from each fraction, 10 μl of 3 % (w/v) digitonin, and 1 ml of reduced cytochrome-C (a few crystals of sodium dithionite were added to 10 ml of 20 μM cytochrome-C, 0.5 mM EDTA, in 50 mM potassium phosphate buffer,pH 7.4). The reaction was started by the addition of reduced cytochrome-C, and the change in optical density was recorded for one minute at 550 nm.

NADH-cytochrome-c reductase assay.

The reaction solution contains 10 μl of 50 mM KCN, 20 μl of 2 mM NADH, 1 ml of cytochrome-C (21 μM cytochrome-c in 50 mM Tris, pH 7.5), and 10 μl protein sample (1 to 10 μg/ml protein) from each fraction. The change in optical density was recorded for one minute at 550 nm. The control was run without NADH addition.

Latent IDPase assay.

13.2 ml of stock solution (66 mM Tris, pH 7.5, 5.5 mM $MgCl_2$, 66 mg IDP), was mixed with either 1.65 ml of water or 1.65 ml of 3 % digitonin. 0.4 ml of the mixture was added to 50 µl of sample solution from each fraction which had been stored at 4°C for 3 to 4 days and vortexed. After 1 hr incubation at room temp., the reaction was stopped with the addition of 1 ml of 14 % TCA. The solution was centrifuged at 1000g for 15 min, and the supernatant transferred to a new tube.

Inorganic phosphorus was determined by Taussky-Shorr reagent (35). Taussky-Shorr reagent was prepared by the following method; 5 g of ferrous sulfate was added to 10 ml of ammonium molybdate stock [10 % (w/v) ammonium molybdate in 10 N sulfuric acid], and the solution was mixed with water until the final volume of the solution was 100 ml. 1 ml of this working reagent was added to the above samples, vortexed, and absorbance at 710 nm was read after 5 to 10 min. KH_2PO_4 was used as a standard of inorganic phosphorus (0.2, 0.4, and 0.6 µmole phosphate).

Effect of victorin on membrane marker enzyme activities.

The most active fraction for each membrane marker enzyme assay in the continuous sucrose gradient fractions was used to examine the effect of victorin on each enzyme activity. The enzyme assays were performed as described above with or without the addition of different concentrations of victorin. Victorin concentrations used for the enzyme preparations of susceptible oat were 100 pg/ml, 1 ng/ml, 10 ng/ml, and 1 µg/ml (final

concentration), and for the enzyme preparations of resistant oat were 10 ng/ml, and 1 μg/ml, respectively. The change in enzyme activity of sample with victorin against control (without victorin) was calculated on a percentage basis and listed on Table 1 and 2.

RESULTS

Location of victorin binding proteins in homogenates of oat tissue separated by continuous sucrose gradient.

The homogenate of dark-grown oat shoots was separated in a 20 to 45 % sucrose gradient (Figs. 13 & 14). There were no significant differences of any membrane marker enzyme analysis between susceptible and resistant cultivars of oats (Figs. 13 & 14). The peaks of several membrane enzyme markers were: plasma membrane at 39 to 41% (w/w) sucrose fractions detected by both glucan synthetase II and vanadate-sensitive H^+-ATPase assays; mitochondria membrane at about 41% (w/w) sucrose fraction examined by cytochrome C oxidase assay; golgi membrane at 28 to 29% (w/w) sucrose fractions detected by IDPase assay; and endoplasmic reticulum membrane at 22 to 24% (w/w) sucrose fractions detected by cytochrome C reductase assay, respectively (Fig. 13 & 14). Each fraction was mixed with 1 μg/ml (final concentration) of victorin, and victorin covalent binding detected by western blotting. The relative intensity of each band was determined by computer densitometer (see Materials and Methods) and compared with the profiles of membrane marker enzyme analysis (Fig. 15 & 16). The 100 kD band appeared in the 36% to 44% (w/w) sucrose fractions containing plasma membrane and mitochondria in both susceptible and resistant cultivars of oats (Figs. 15 & 16), and the intensity of the 100 kD bands was not

significantly different in these fractions (36% to 44%) (Figs. 15 & 16). Golgi and ER membranes were clearly in different fractions from the 100 kD binding protein as determined by marker enzyme assays (Figs. 15 & 16). In contrast, 65 kD and 45 kD proteins were detected in all fractions in both susceptible and resistant cultivars of oats (Fig. 15 & 16). The intensity of the 65 kD band was almost equal in all fractions, but the intensity of the of 45 kD band varied considerably (Fig. 15 & 16).

Effects of victorin on the activities of membrane marker enzymes

The effect of victorin on the activities of each membrane marker enzyme was also examined (Table 1 & 2). The fractions of continuous sucrose gradient containing the highest activity of each enzyme assay were mixed with different concentrations of victorin (100 pg/ml to 1 μg/ml final concentration), and the enzyme activities compared with activity without victorin. As indicated in Tables 1 & 2, victorin had no effect on the activities of marker enzymes in both susceptible and resistant cultivar of oats.

Victorin binding proteins on mitochondria isolated from oat tissues.

Mitochondria were isolated from oat homogenate by discontinuous sucrose gradient. Mitochondria appeared as a band between the 1.8 M and 1.45 M sucrose interface and were identified by cytochrome C oxidase assay (see Materials and Methods). 50 μl of each fraction of the discontinuous

sucrose gradient was taken and mixed with 1 μg/ml (final concentration) of victorin. Covalent binding of victorin was detected by western blotting with anti-victorin antibody and second antibody conjugated with ^{125}I (Fig. 17). The 65 kD band was detected from fractions of the mitochondria of both susceptible and resistant cultivars of oats (Fig. 17). However, the 100 kD and 45 kD bands were not detected (Fig. 17).

Victorin binding proteins on the plasma membrane of oat isolated by aqueous two phase method.

The 100 kD victorin covalent binding protein was detected in the fractions containing plasma membrane and mitochondria in continuous sucrose gradient experiments (Fig. 15 & 16). Since the 100 kD protein was not detected in mitochondria (Fig. 17), plasma membrane was isolated from oat tissue by the aqueous two phase method and the binding of victorin was examined. The procedure of Larsson (18) was followed for the two phase separation of plasma membrane from light-grown oat leaves. The plasma membrane fraction was the upper layer of the two phases and all other membranes were in the lower phase. There was no cytochrome C oxidase activity in the plasma membrane fraction (Table 3) and the ratio of 4.2 for upper phase/lower phase in the glucan synthase II assay (Table 3) was similar to that reported by Larsson (18). The preparations were then mixed with 1 μg/ml (final concentration) of victorin and the binding of victorin was detected by western blotting with anti-victorin antibody and second antibody conjugated with ^{125}I. The 100 kD protein was

detected in the lower phase preparation, but not in the upper phase which contained plasma membrane from both susceptible and resistant cultivar of oats (Fig. 18), indicating that the location of 100 kD protein is not in the plasma membrane of oat cells. In contrast, the 65 kD and 45 kD proteins were identified in upper plasma membrane fractions and lower fractions of both susceptible and resistant cultivar of oats (Fig. 18).

DISCUSSION

The concept of plasma membrane receptors is well established in animal systems. A specific protein receptor located in the plasma membrane is assumed to initiate a sequence of stimulus-receptor-transduction-responses (11). The example of mammalian hormones are the best examined systems in regard to the receptor concept. The hormone glycoprotein receptors are localized in the plasma membrane (16). The binding of hormones on the out-side of the plasma membrane triggers a second message to the inside of the membrane by G protein (10). This is followed by the activation of adenylate cyclase, which produces cAMP from ATP, which in turn activates specific cAMP-dependent protein kinases which phosphorylate certain enzymes, and induce the physiological responses (34). Steroid hormones also might interact with the receptor in a plasma membrane, but here the function is probably to transport the hormones across the membrane (17).

A similar concept has been suggested in plant systems. Cell surface proteins are believed to be involved in the recognition of pollen\stigma interactions for self-incompatibility (11), of *Rhizobium* to root hair interactions (4), and plant hormones (16). For the plant and microorganism interactions, the existence of cell surface receptors to factors produced by microorganisms, such as elicitor or toxins, has also been hypothesized (16).

As discussed in the Chapter 1, the receptor of victorin is thought to exist in the plasma membrane of oat cells. Some victorin covalent binding proteins were identified (1, 41), but the location of these proteins in oat cells were not examined.

In this study, oat homogenates were separated by continuous/ discontinuous sucrose gradients, and an aqueous two phase method, and victorin binding to each fraction was examined by western blotting. In the continuous sucrose gradient experiment, the membrane marker enzyme analysis indicated that oat homogenates were well separated (Figs. 13 & 14). Leonard *et al.* indicated (20, 21) that oat plasma membranes and golgi are not like maize, and have clearly different densities. They observed the highest activity of plasma membrane marker enzyme at 40 to 41%, and golgi at 27 to 29 % sucrose fractions in a continuous sucrose gradient using oat (20). Their observation of the highest activity of NADH cytochrome C reductase which is a marker enzyme of ER, was 23 to 24 % sucrose fraction (14, 20). Hodges *et al.* (14) also indicated that since the density of mitochondria is only slightly higher than the plasma membrane (1.18-1.22 for mitochondria and 1.14 to 1.17g/cm^3) in oats, plasma membrane and mitochondria appeared in the same fractions in a continuous sucrose gradient (20). The membrane marker enzyme analysis of fractions of continuous sucrose gradient in this study (Figs. 13 & 14) were exactly the same as the results of these previous experiments (14, 20, 21).

Each fraction of continuous sucrose gradient was mixed with 1 μg/ml of victorin and covalent binding was detected by western blotting with anti-victorin antibody and second antibody conjugated with ^{125}I. 100 kD

victorin binding protein was detected from 44% to 37% sucrose (w/w) fractions, and 65 kD and 45 kD victorin-binding proteins were detected from all fractions of the continuous gradient (Figs. 15 & 16). The binding of victorin was detected from the same fractions in both susceptible and resistant cultivars of oats (Figs, 15 & 16).

In Chapter 1, the *in vivo* and *in vitro victorin* binding experiments indicated that victorin binds covalently at the same sites in both susceptible and resistant cultivars of oats (Figs. 7 & 8 & 10). One possible explanation for the specificity of victorin in oats could be that victorin binds to the same proteins but the location of the proteins in susceptible and resistant oat cells are different. However, our results indicated that victorin binding proteins were located at the same sites in both susceptible and resistant oat cells (Figs. 15 & 16).

Interestingly, 65 kD and 45 kD proteins were detected from all fractions of continuous sucrose gradients in both susceptible and resistant oats (Figs. 15 & 16). The results indicate that victorin can bind covalently throughout the oat cell, including to the plasma membrane, mitochondria, ER, and golgi. The intensity of the 65 kD band of victorin binding protein was constant throughout the fractions of continuous sucrose gradient (Figs. 15 & 16), which may indicate that victorin can bind equally to the protein throughout the oat cell.
The intensity of the 45 kD band of victorin binding protein varied considerably (Figs. 15 & 16). In fact, 45 kD binding was not detected in mitochondria (Fig. 17). But victorin binding to a 45 kD protein was observed in the fractions containing mitochondria in a continuous sucrose

gradient, because the fraction also contained plasma membrane (Figs. 15 & 16). These results suggest that the 45 kD victorin binding protein may not be located equally throughout the oat cell.

Both 65 kD and 45 kD proteins were located in the plasma membrane of oats (Fig. 18). The *in vivo* binding experiment in Chapter 1 indicated that victorin bound *in vivo* to 100 kD and 45 kD proteins of both susceptible and resistant oat tissues, but not bound to 65 kD protein (Fig. 7). Previous reports discussed in Chapter 1 (6, 15, 26, 28, 37) hypothesized that a receptor of victorin could be located in the plasma membrane of oat cells. Based on this hypothesis, the 45 kD victorin binding protein found in the plasma membrane (Fig. 18) and victorin bound *in vivo* (Fig. 7) could be a candidate for the receptor site of victorin. The 65 kD protein was also located in the plasma membrane (Fig. 18), and did bind victorin *in vitro* (Fig. 10), but it was not detected in *in vivo* binding experiments (Fig. 7). Although there could be a mechanism to explain the *in vivo* binding of victorin, ie., the 65 kD may be located in the inside of the plasma membrane and not exposed to victorin in *in vivo* binding assay, no evidence to support this conclusion was observed.

The effects of victorin on several membrane marker enzymes was tested, including glucan synthetase II for plasma membrane (29, 36, 40), NADH-cytochrome C reductase for ER (13, 18), cytochrome C oxidase for mitochondria (2, 13), and IDPase for golgi (7, 30, 39). There were no obvious changes on the enzyme activities between victorin treated enzyme preparations and non-treated preparations (Table 1 & 2). Victorin did bind to a 65 kD protein in all of these organelles (Figs 15 & 16), but

the role of binding on the toxicity of victorin is still unknown.

The 100 kD protein was detected in the higher fractions in a continuous sucrose gradient (Figs. 15 & 16), and attempts were made to further localize the 100 kD victorin binding protein. These fractions in the sucrose gradient contained plasma membrane and mitochondria but were clearly separated from the ER and golgi as determined by several enzyme analyses (Figs. 15 & 16). In the second experiment, a pure plasma membrane was isolated by aqueous two phase method, mitochondria were purified by discontinuous sucrose gradient methods, and victorin covalent binding was examined (Figs. 17 & 18). The 100 kD victorin binding protein was located on neither the plasma membrane nor the mitochondria (Figs. 17 & 18). Differential centrifugation experiments reported by Wolpert *et al.* (41) indicated that the 100 kD victorin binding protein was located only in the high centrifugation fraction (pellet of 100,000*g* for 30 min) but not the low centrifugation fraction (pellet of 4,000 *g* for 4 min) nor supernatant. These results suggested that the 100 kD protein was not in the cell wall or a soluble protein. The 100 KD victorin binding protein seems like a unique protein. It has a high density, like plasma membrane or mitochondria, as indicated by continuous sucrose gradient results (Figs, 15 & 16), but it was not in the plasma membrane (Fig. 18) nor the mitochondria (Fig. 17), and not in cell wall (41). The role of the 100 kD victorin binding protein for the toxicity of victorin is not solved yet.

However, if the hypothesis that victorin toxicity is related to its binding to a plasma membrane receptor is true, then clearly the 100 kD protein is not involved.

Although we found that victorin binds to 65 kD and 45 kD proteins in the plasma membrane, it is not clear if victorin directly causes damage to susceptible oat plasma membranes or if damage occurs through other interactions which affect the function of the plasma membrane. The ultrastructural study in 1968 (12) indicated that victorin-induced changes include: early changes in the plasma membrane, formation of enlarged, densely stained vesicles by the golgi apparatus, organization of the ER into roughly parallel profiles, and swollen mitochondria after cells were damaged heavily by victorin. Since victorin had no effect on succinate oxidation of isolated mitochondria (33), the TCA cycle seems to be intact and oxidatively generated energy may not be affected by victorin. In the ultrastructural study, the swelling of mitochondria was detected in only heavily damaged cells (12), indicating that it was probably not caused by an ionophore effect of victorin on mitochondria. In this study, victorin did not show any effects on marker enzymes of organelle membranes. However, since victorin binding proteins of 65 kD and 45 kD were also located not only in plasma membrane but also in other cell fractions, additional studies of victorin effects on each organelle may be necessary to identify the effects of victorin in oats.

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Table 1. The effect of victorin on the activities of marker enzymes in susceptible oat tissues (Park).

Enzyme (organelle)**	Change in marker enzyme activity* victorin concentration (ng/ml)				
	0.1	1	10	1000	Control***
Glucan synthetase II (PM)	6788 (99)	7337 (107)	7131 (104)	6377 (93)	6857 (100)
NADH-Cytochrome C reductase (ER)	0.123 (102)	0.123 (102)	0.125 (103)	0.117 (97)	0.121 (100)
Cytochrome C oxidase (Mt)	0.119 (108)	0.11 (100)	0.107 (97)	0.107 (97)	0.11 (100)
IDPase (Golgi)	0.34 (100)	0.347 (102)	0.323 (94)	0.333 (98)	0.34 (100)

*Number in parenthesis indicates percent change of enzyme activity from control.

**PM, plasma membrane; ER, endoplasmic reticulum; Mt, mitochondria. Enzyme activities were indicated as following; GS II, cpm; NADH-cyt C reductase and cyt C oxidase, OD/min; IDPase, μmole phosphate. For the assays of each enzyme, see Materials and Methods in Chapter 2.

***Water was treated instead of the different concentrations of victorin as a control.

Table 2. The effect of victorin on the activities of marker enzymes in resistant oat tissues (Korwood).

Enzyme (organelle)**	Change in marker enzyme activity* Victorin concentration (ng/ml) 10	1000	Control***
Glucan synthetase II (PM)	4095 (98)	4054 (97)	4179 (100)
NADH-cytochrome C reductase (ER)	0.25 (100)	0.245 (98)	0.25 (100)
Cytochrome C oxidase (Mt)	0.297 (99)	0.294 (98)	0.3 (100)
IDPase (Golgi)	0.473 (105)	0.459 (102)	0.45 (100)

*Number in parenthesis indicates percent change of enzyme activity from control.

**PM, plasma membrane; ER, endoplasmic reticulum; Mt, Mitochondria. Enzyme activities were indicated as following; GS II, cpm; NADH dependent reductase and oxidase, OD/min; IDPase, μmole phosphate. For the assays of each enzyme, see Materials and Methods of Chapter 2.

***Water was treated instead of the different concentrations of victorin as a control.

Table 3. Enzyme activity profiles of the plasma membrane from susceptible and resistant oat tissues isolated by aqueous two phase methods.

Enzyme (organella)	MF[1]		U[2]		L[3]		U/L	
	S[4]	R[4]	S[4]	R[4]	S[4]	R[4]	S[4]	R[4]
Glucan synthetase II[5] (Plasma membrane)	8648	8654	7532	5496	1830	1324	4.1	4.2
Cytochrome C oxidase[6] (mitochondria)	0.03	0.024	0	0	0.03	0.024	-	-

[1]MF: microsomal fraction.
[2]U: Upper layer of the two phases.
[3]L: Lower layer of the two phases.
[4]S: susceptible oat (cv. Park); R: resistant oat (cv. Korwood).
[5]Incorporation of ^{14}C-UDPG per 10 μl of each sample (cpm) was listed. The method of the assay was described in Materials and Methods of Chapter 2.
[6]The change of O.D. at absorbance 550 nm in one minute after the substrate was added to 10 μl of each sample. The method of the assays described in Materials and Methods of Chapter 2.

Fig. 13. Enzyme activity profiles from sucrose gradients of oat seedling homogenates (cv. Park). Oat tissue homogenate was separated in a 20 to 45 % sucrose continuous gradient, and the activities of several membrane marker enzymes in each fraction were measured (see Materials and Methods of Chapter 2 for the methods of each enzyme assays). (A) Glucan synthetase II, (B) vanadate sensitive H^+-ATPase, (C) NADH cytochrome C reductase and cytochrome C oxidase, (D) latent IDPase, respectively.

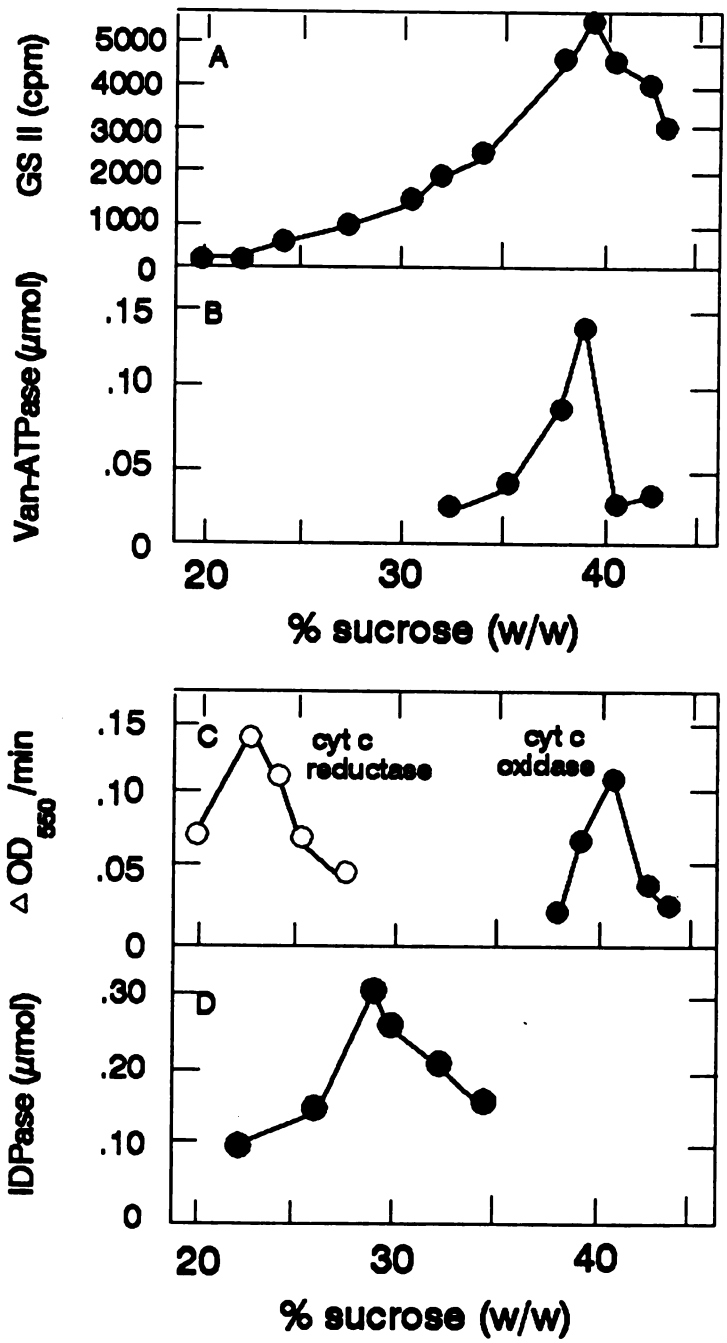

GS II (cpm)
5000
4000
3000
2000
1000
0
A
Van-ATPase (µmol)
.15
.10
.05
0
B
20
30
40
% sucrose (w/w)
Δ OD850 /min
.15
.10
.05
0
C
cyt c reductase
cyt c oxidase
IDPase (µmol)
.30
.20
.10
0
D
20
30
40
% sucrose (w/w)

Fig. 14. Enzyme activity profiles from sucrose gradients of oat seedling homogenates (cv. Korwood). Oat tissues homogenate was separated in a 20 to 45 % sucrose continuous gradient, and the activities of several membrane marker enzymes in each fraction were measured (see Materials and Methods of Chapter 2 for the methods of each enzyme assays).
(A) Glucan synthetase II, (B) vanadate sensitive H^+-ATPase, (C) NADH cytochrome C reductase and cytochrome C oxidase, (D) latent IDPase, respectively.

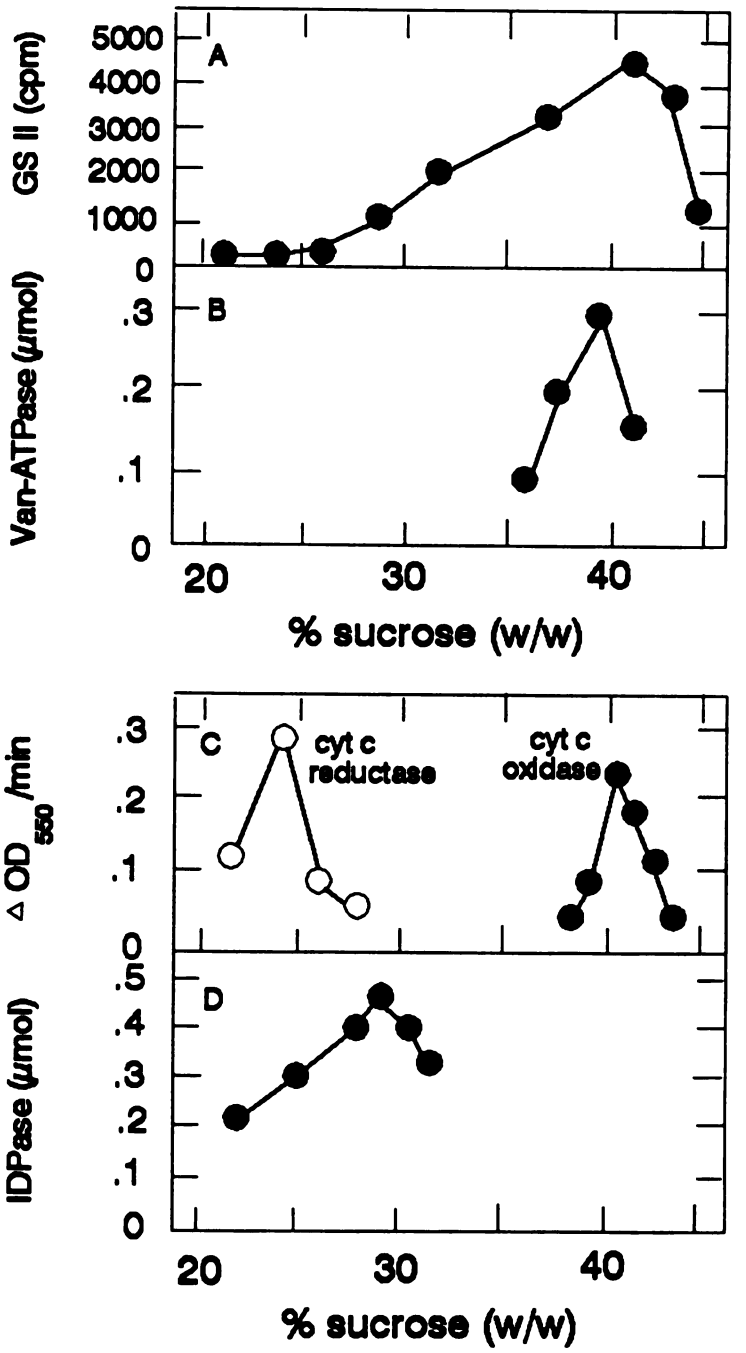

GS II (cpm)
5000
4000
3000
2000
1000
0
A
Van-ATPase (μmol)
.3
.2
.1
0
B
20
30
40
% sucrose (w/w)
Δ OD550 /min
.3
.2
.1
0
C
cyt c reductase
cyt c oxidase
IDPase (μmol)
.5
.4
.3
.2
.1
0
D
20
30
40
% sucrose (w/w)

Fig. 15. Victorin binding intensity and enzyme activity profiles from sucrose gradients of susceptible oat seedlings (cv. Park). The enzyme profiles are the same as Fig. 12. Each fraction after separation in a 20 to 45 % sucrose continuous gradient was mixed with 1 μg/ml of victorin. The binding of victorin was detected with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody (see Materials and Methods of Chapter 2). The intensity of bands (100, 65, and 45 kD) was determined by measuring each band area with ImageQuant (Molecular Dynamics Co. Ltd.). Relative intensity of each band was calculated as a percentage against the intensity of the band at the highest sucrose fraction (about 44 % sucrose fraction).

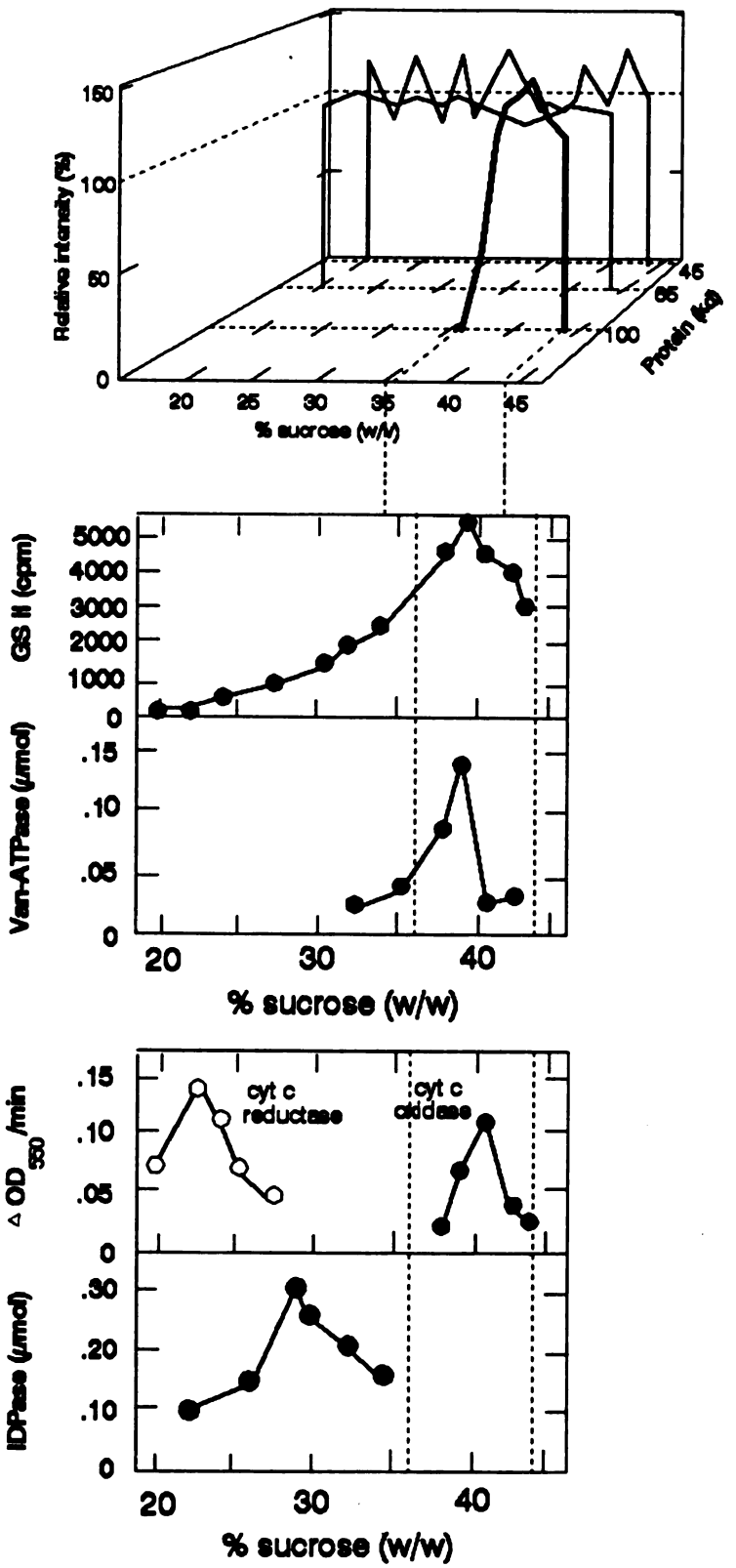
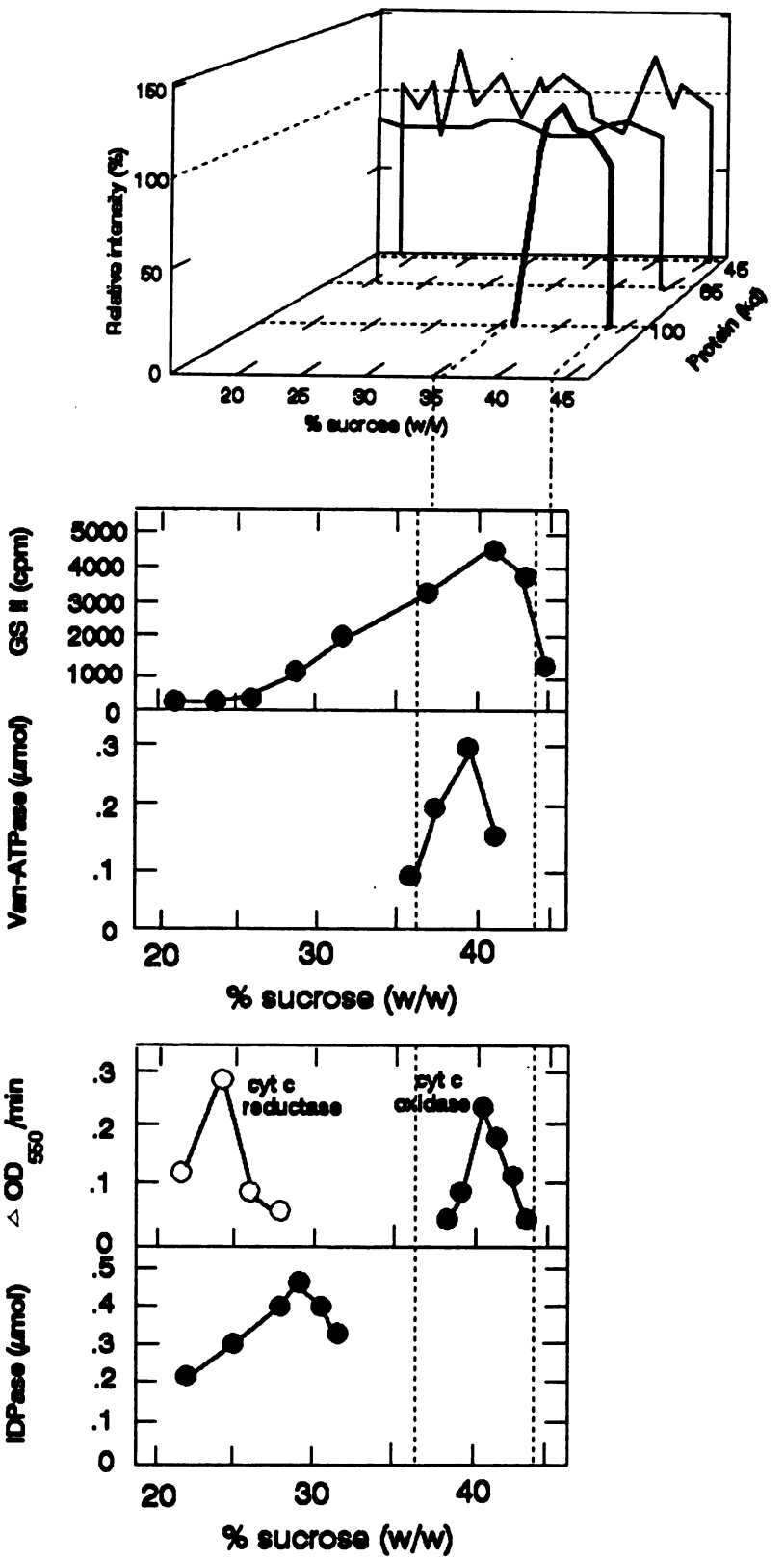

Relative Intensity (%)
150
100
50
0
20
25
30
35
40
45
% sucrose (w/v)
45
55
100
Protein (kd)
GS II (cpm)
5000
4000
3000
2000
1000
0
Van-ATPase (μmol)
.15
.10
.05
0
20
30
40
% sucrose (w/w)
Δ OD550/min
cyt c reductase
cyt c oxidase
.15
.10
.05
0
IDPase (μmol)
.30
.20
.10
0
20
30
40
% sucrose (w/w)

Fig. 16. Victorin binding intensity and enzyme activity profiles from sucrose gradients of resistant oat seedlings (cv. Korwood). The enzyme profiles are the same as Fig. 13. Each fraction after separation in a 20 to 45 % sucrose continuous gradient was mixed with 1 μg/ml of victorin. The binding of victorin was detected with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody (see Materials and Methods of Chapter 2). The intensity of bands (100, 65, and 45 kD) was determined by measuring each band area with ImageQuant (Molecular Dynamics Co. Ltd.). Relative intensity of each band was calculated as a percentage against the intensity of the band at the highest sucrose fraction (about 44 % sucrose fraction).


Relative Intensity (%)
150
100
50
0
20
25
30
35
40
45
% sucrose (w/w)
45
65
100
Protein (kg)
GS II (cpm)
5000
4000
3000
2000
1000
0
Van-ATPase (µmol)
.3
.2
.1
0
20
30
40
% sucrose (w/w)
cyt c reductase
cyt c oxidase
Δ OD550/min
.3
.2
.1
0
IDPase (µmol)
.50
.4
.3
.2
.1
0
20
30
40
% sucrose (w/w)

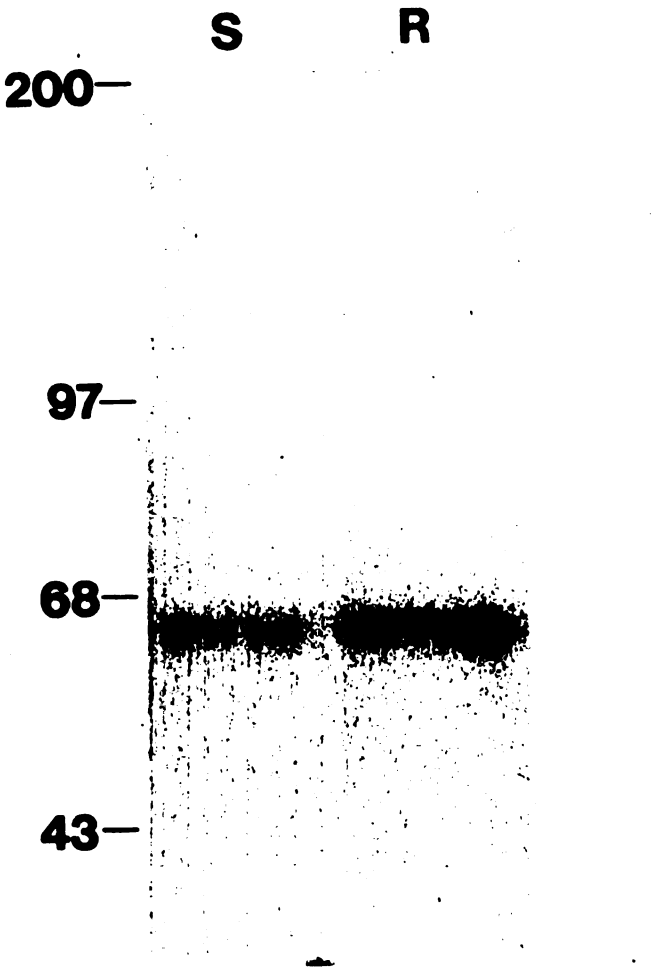


Fig. 17. Victorin binding to purified mitochondria from susceptible (P, Park) and resistant (KW, Korwood) oat tissues. Mitochondria were purified by a discontinuous sucrose gradient from oat tissues (see Materials and methods of Chapter 2). The mitochondria were mixed with 1 μg/ml of victorin and the binding of victorin was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. Values at left are molecular masses of standard protein in kD.

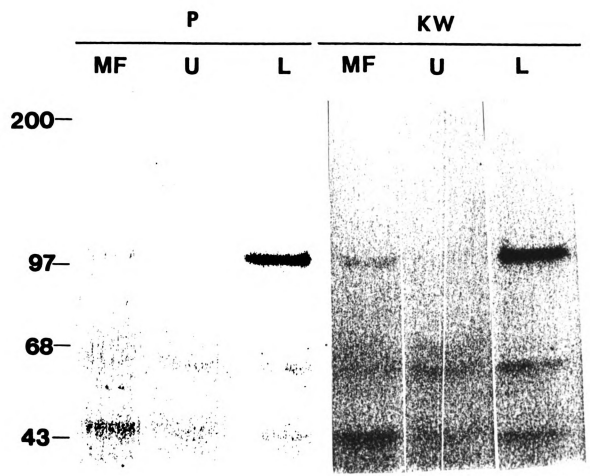


Fig. 18. Victorin binding to plasma membrane from susceptible (P, Park) and resistant (KW, Korwood) oat tissues isolated by an aqueous two phase method. The isolated plasma membrane (see Materials and Methods of Chapter 2 for isolation procedures and Table 3 for the marker enzyme profiles of the isolated plasma membrane) was mixed with 1 μg/ml of victorin and the binding of victorin was visualized on blots with anti-victorin antibody and ^{125}I-conjugated anti-rabbit IgG as a second antibody. MF indicates microsomal fraction; U indicates upper phase fraction of two phase which contains the pure plasma membrane; and L indicates lower phase fraction of two phase. Values at left are molecular masses of standard protein in kD.

CHAPTER 3:

PRODUCTION OF ANTI-VICTORIN ANTI-IDIOTYPIC ANTIBODY

INTRODUCTION

Anti-victorin antibody has been used in studies reported in this dissertation to identify 1) the covalent binding sites of victorin in oat tissues (Chapter 1)(2), and 2) the localization of the victorin binding proteins in oat cells (Chapter 2), by using western blotting. The uniqueness of our attempts using an antibody to study victorin binding sites is that the technique allows for the use of native forms of victorin to detect the binding proteins rather than victorin analogs, such as radiolabelled victorin (22, 23). The anti-victorin antibody was highly sensitive and as little as 10 pg/ml of victorin was detected in an indirect ELISA (Fig. 4). Using this anti-victorin antibody as an immunogen, we generated the second antibody, so called anti-idiotypic antibody, for further examination of the victorin receptor, again using an immunological approach.

Epitopes associated with antigen-binding sites in the variable region of antibody are called idiotype. Antibody against the idiotype is called anti-idiotypic antibody (20). Anti-idiotypic antibody raised against the idiotpe of antibody to biologically important ligands have been used as cell-surface probes in various systems (19, 20). The strategy of using anti-idiotypic antibody for the receptor study is; 1) first make primary antibody against ligand epitope, which specifically binds to the receptor

site, and 2) the primary antibody is used as a immunogen to make a second antibody, called anti-idiotypic antibody (20). Since the internal image of anti-idiotypic antibody and ligand epitope against the receptor will be identical or very similar, the anti-idiotypic antibody can recognize the ligand binding site of the receptor. The production of anti-idiotypic antibody can be considered as a production of the antibody to a receptor without having to isolate the receptor protein. Since the antibody molecule is too large to enter the cell, anti-idiotypic antibodies are often used as probes for the determination of cell surface receptor sites (19, 20). This technique has been used successfully in such varied systems as the insulin receptor (19), B-adrenergic receptor (6, 18), the receptor of nicotine (1), diphtheria toxin receptor (15), and other animal hormones, neurotransmitters, and lymphotrophic viruses or factors (20).

As discussed in detail in Chapter 1 and 2, several lines of evidence indirectly suggest the existence of victorin receptor site(s) (3, 4, 5, 14, 21, 23), and the receptor could be located on the surface of the plasma membrane of susceptible oat cells (3, 7, 9 , 12, 14, 21).

We found that 65 kD and 45 kD victorin covalent binding proteins were located in the plasma membrane of oats (Chapter 2)(Fig. 18). Since covalent binding may be mediated by the aldehyde group, which may be biologically functional in nature (17) and is essential for the toxicity of victorin (23), these covalent binding proteins could be the receptor of victorin. Since the 45 kD victorin covalent binding protein was detected in *the in vivo* binding assay (Fig. 7), whereas the 65 kD protein not detected, the 45 kD protein may be the binding site associated with

victorin toxicity. However, the relationship between the covalent binding of victorin to a protein and its toxicity is still unknown. Therefore, proof indicating the importance of a cell surface binding protein of victorin for its toxicity is desirable.

In this study, we generated anti-victorin anti-idiotypic polyclonal antibodies from the polyclonal anti-victorin antibody-OVA conjugate. The anti-victorin anti-idiotypic antibodies were added to oat protoplasts with or without victorin, and the biological activity of the anti-idiotypic antibodies or biological function of anti-idiotypic antibodies against victorin activity was examined.

MATERIALS AND METHODS

Plant Materials

Park was used as a *C. victoriae* susceptible and victorin sensitive oat cultivar, and Korwood was used as a *C. victoriae* resistant and victorin insensitive oat cultivar. Oats were grown 20 to 25 days in the growth chamber under fluorescent and incandescent lamps (fluence rate: 140 μmol/m/sec) for 12 hr photo-period at 18°C, for the protoplast preparation.

Victorin C preparation

Victorin C was isolated from 40 L of culture filtrates of *C. victoriae* (HV1146A) by the slightly modified methods described by Mayama et al. (11). The procedures in detail was described in Materials and Methods of Chapter 1.

Anti-victorin antibody production

Anti-victorin antibody was produced in rabbits immunized with victorin-BSA conjugate by the method described in Materials and Methods of Chapter 1. The anti-victorin polyclonal antibodies were purified by immobilized protein A and BSA columns for the preparation of anti-victorin antibody-OVA conjugation.

Production of anti-victorin anti-idiotypic antibody

Immunogen preparation.

Anti-victorin antibodies were purified by immobilized protein A and BSA columns by the methods described in Materials and Method of Chapter 1. The purified antibodies were conjugated with ovalbumin in PBS, pH 7.2, with glutaraldehyde by the methods described by Langone *et al*. (10). 1 mg of the antibodies were dissolved in PBS, pH 7.2, and mixed with 1 mg of ovalbumin. 80 µl of glutaraldehyde (25 % commercial solution from Sigma) was added to the solution and incubated for 45 min at room temp. The reaction was stopped by the addition of 200 µl of 2 M lysine and the solution was dialyzed against PBS (PBS was changed 4 times) overnight at 4°C.

Immunization.

0.5 mg of the conjugate was dissolved in 1 ml of PBS and mixed with Freund's complete adjuvant (0.5 mg conjugate/2 ml PBS-adjuvant mixture (1:1)/rabbit). The mixture was injected intradermally into 3 female white New Zealand rabbits, and boosted 3 times intramuscularly at 28 day intervals with 0.5 mg conjugates/2 ml PBS-Freund's incomplete adjuvant mixture (1:1)/rabbit. Blood was taken from rabbits 12 days after each injection, and serum was isolated (8). The serum was purified by protein A column chromatography (Pierce/ImmunoPure IgG Purification kit), and the presence of anti-victorin anti-idiotypic antibodies was determined as follows.

Screening of anti-victorin anti-idiotypic antibody.

The anti-victorin anti-idiotypic antibody was screened by the inhibition of victorin-HRP binding against anti-victorin antibodies in direct ELISA (20). Production of victorin-HRP was described in Materials and Methods of Chapter 1. 20 μg of anti-victorin antibody purified by both immobilized protein A and BSA columns in 100 μl PBS (200 μg/ml) was placed in wells of a microtiter plate, and incubated overnight at 4°C. The antibody solution was removed (reused at least 5 times if the solution contained 0.02 % (w/v) NaN_3), and the well was washed with PBS-Tween 15 times. The plate was then incubated with 300 μl of PBS-Tween for 0.5 hr at 37°C to block unbound binding sites and washed with PBS-Tween 5 times. Victorin-HRP (diluted $1:10^4$ from the stock solution (see Materials and Methods in Chapter 1)) or victorin-HRP plus different concentrations of sample serum (purified by protein A column) or victorin-HRP plus different concentrations of victorin, in 1 ml of PBS-Tween were placed on each well and incubated for 1 hr at 37°C. The plate was then washed 15 times with PBS-Tween, and 100 μl of substrate (0.4 mM tetramethylbenzidine, 0.004 % H_2O_2, in 50 mM sodium acetate, pH 6.0) for HRP was added. After 30 min incubation at room temp., the reaction was stopped by addition of 2 M H_2SO_4 and absorbance at 450 nm was read by a Bio-Tec ELISA reader.

Protoplast preparation.

Sterile protoplasts were prepared by the methods described by Schaeffer and Walton (16). Briefly, oat leaves were cut from 20 to 25 day old plants grown in the growth chamber (conditions described in Plant

Materials) and fresh weights measured. Leaves (500 mg) were sterilized with 10 % bleach, abraded with carborundum, and incubated at 26°C for 3 hr in 2 % (w/v) Cellulysin in sorbitol/calcium/mes buffer (SCM) (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes, pH 5.5) with gentle shaking. Released protoplasts were filtered through 80 μm nylon screen mesh, washed twice with SCM (pH 5.8), and collected by centrifugation at 500 rpm (100g) for 5 min. The protoplasts were washed by centrifugation two more times with SCM (pH 5.8), and resuspended with SCM (pH 5.8) at a final concentration of 4 X 10^4 protoplasts/ml. All procedures were performed under sterile conditions.

Anti-victorin anti-idiotypic antibody treatment of oat protoplasts.

0.5 ml of 4 X 10^4 protoplasts/ml SCM (pH 5.8) were placed in wells of 24 well Falcon multiwell plates (Becton-Dickinson), and mixed with either 60 pg/ml final concentration of victorin, 60 pg/ml of victorin plus 1:20 diluted serum containing anti-idiotypic antibodies, 60 pg/ml of victorin plus 1:20 diluted pre-serum, 1:20 diluted serum containing anti-idiotypic antibodies, or 1:20 diluted pre-serum. 20 μg/ml of BSA or 20 μg/ml of human IgG (protein standard from Sigma) was also mixed with 60 pg/ml of victorin and added to susceptible oat protoplasts as a control. The mixtures were incubated for various periods at room temp. in the dark, and callose production was observed by staining with 1:1 mixture of 0.1% Calcofluor white and SCM (pH 5.8) and viewing under a Zeiss epifluorescence microscope (filter set G 365) at a final magnification of X400.

^{14}C-glucose incorporation

^{14}C-glucose incorporation into callose produced from susceptible oat protoplasts treated with 60 pg/ml of victorin or 60 pg/ml of victorin plus 20 times diluted pre-serum, or 60 pg/ml of victorin plus 20 times diluted anti-idiotypic antibody (3rd boost) for various periods, was kindly measured by Ms. Holly Schaefer, DOE-PRL, Michigan State University, by the method described by Schaeffer *et al.* (16).

RESULTS

Screening of anti-victorin anti-idiotypic antibody.

The presence of anti-victorin anti-idiotypic antibodies in the serum was screened by the inhibition of victorin-HRP binding against anti-victorin antibody in a direct ELISA. When victorin was used as a competitor of victorin-HRP binding against anti-victorin antibody in the direct ELISA system, 25 ng/ml of victorin inhibited 50 % of victorin-HRP binding and 1 μg/ml of victorin inhibited 97.5 % of victorin-HRP binding (Fig. 6). When different concentrations of protein A column purified serum collected after the 3rd boost were mixed with victorin-HRP, as high as 84 % of victorin-HRP binding was inhibited by the addition of 1×10^{-1} diluted serum and at least 4×10^{-3} diluted serum still inhibited 33 % of binding (Fig. 19). There was no significant inhibition when pre-serum was used (Fig. 19). The increase of anti-idiotypic antibody production in serum was correlated with the number of boosts (Fig. 20).

Effects of anti-victorin anti-idiotypic antibody on oat protoplasts.

Victorin induces callose synthesis from susceptible oat protoplasts (21). About 70 % of susceptible oat protoplasts produced callose extracellarlly after 8 hr incubation with 60 pg victorin/ml, and the callose secretion continued to increase for 24 hr (Figs. 21 (A) & 23).

The secretion is host-specific since no induction occurred in resistant oat protoplasts by the same treatment (Figs. 21 (B) & 23). The twenty times diluted serum (3rd boost) containing anti-victorin anti-idiotypic antibodies induced callose synthesis from 10 % of the susceptible oat protoplasts, but not from resistant oat protoplasts (Figs. 21 (C) & 22). The amount of callose secreted from susceptible oat cells treated with anti-idiotypic antibodies was similar to that induced by 6 pg/ml of victorin (Figs. 21 (C) & (D)). Treatment with pre-serum did not show the induction of callose synthesis on either susceptible or resistant oat protoplasts (Fig. 22). When the same concentration of serum containing anti-idiotypic antibodies and 60 pg/ml of victorin was simultaneously added to oat protoplast, inhibition of callose synthesis was observed on susceptible protoplast (Fig. 23). The highest inhibition was observed after 5 hr incubation, and the inhibition rate of callose synthesis was 68 % (Fig. 23). When the same concentration of pre-serum and victorin was added to oat protoplasts, no significant differences on the percentage of the number of protoplast which producing callose per total protoplasts observed (Fig. 23). However, the incorporation of ^{14}C-glucose experiment showed a 60% inhibition of incorporation by the pre-serum fraction, and about 75% inhibition in anti-idiotypic antibody fraction (Fig. 24). 20 μg/ml of BSA or human Ig did not have any effect on the callose secretion induced by 60 pg/ml of victorin (Table 4).

DISCUSSION

Anti-victorin anti-idiotypic antibodies were produced in rabbits immunized with anti-victorin antibody-OVA conjugate (Fig. 19). The anti-idiotypic antibody has been used as immunological indirect proof of the existence of cell surface receptors (19, 20). Although a large number of anti-idiotypic antibodies have been prepared (20), immunogens used to detect their receptors were mainly hormones, neurotransmitters, lymphotropic virus, factors, and lymphokines (20), and this technique is rarely used in plant biology or to study plant-microorganism interactions.

The idea of producing anti-victorin anti-idiotypic antibody is to make polyclonal antibody against idiotypes (variable region) of anti-victorin polyclonal antibody recognizing various victorin epitopes. If the anti-victorin polyclonal antibodies can recognize the receptor-binding part of the victorin molecule as their epitopes, the anti-idiotypic antibody generated from the antibodies will have the identical or very similar "internal image" to the receptor-binding part of victorin molecule, and should also react with the receptor (Fig. 25)(19, 20).

There are four different methods to detect the receptor by using anti-idiotypic antibody; 1) fluorescent or stain-detection of anti-idiotypic antibody binding on cells by microscope studies, 2) construction of a receptor affinity column with anti-idiotypic antibody, 3) inhibition of binding of ligand to receptor by addition of anti-idiotypic antibody, and

4) inhibition or stimulation of biological activities (20).

In this study, we examined the effect of anti-victorin anti-idiotypic antibody on the physiological production of callose from oat cells. The anti-idiotypic antibody acted weakly as an agonist of victorin and also as an antagonist against the effects of victorin in susceptible oat cells. The addition of anti-idiotypic antibody induced callose synthesis from about 10 % of susceptible cells (Fig. 22). The amount of callose production from the protoplasts stimulated by anti-idiotypic antibody was similar to that stimulated by 6 pg/ml of victorin treated protoplast (Fig. 21 (A) and (D)). When the callose production was examined by the number of the cells producing callose, anti-idiotypic antibody also inhibited the callose secretion induced by 60 pg/ml of victorin addition and no significant inhibition was observed by the pre-serum treatment (Fig. 23). However, inhibition of ^{14}C-glucose incorporation induced by 60 pg/ml of victorin was about 75% when anti-idiotypic antibody and 60 pg/ml of victorin simultaneously treated, and about 60% of incorporation was also inhibited when pre-serum was added with 60 pg/ml of victorin (Fig. 24). The differences between microscopic data and ^{14}C-glucose incorporation data may result from the difference of these two assays. Although the ^{14}C-glucose incorporation reflected the total amount of callose production from all of protoplasts, this comparison of the total amount of callose from protoplasts was impossible by the microscopic observation study. The microscopic study determined whether callose secretion was initiated, and not how much callose was produced. The results may indicate that the quantity of total callose production was decreased by the pre-serum

treatment, even though the number of the cells producing callose were not changed between victorin treated protoplasts and victorin plus pre-serum treated protoplasts.

These results using anti-victorin anti-idiotypic antibody indirectly suggest that there may be a victorin binding site on the surface of the plasma membrane of susceptible oat cells, which is related to victorin toxicity, and the evidence supports previous reports hypothesizing the existence of a victorin receptor site on the plasma membrane of susceptible oats (3, 4, 5, 9, 12, 13, 14, 21, 23). As discussed in Chapter 2, we found that victorin bound to a 45 kD protein *in vivo (Fig. 7)*, and the protein was located in the plasma membrane of oat cells (Fig. 18). The 65 kD binding protein was not detected in *in vivo* victorin binding assay (Fig. 7), but was also located in the plasma membrane (Fig. 18). Thus, whether the anti-victorin idiotypic antibody reacted with the these membrane protein was examined. Attempts were made to observe the binding of the anti-idiotypic antibody to the oat microsomal proteins, which were separated by SDS-PAGE and transferred to nitrocellulose filter, but neither anti-idiotypic antibodies nor victorin reacted with those proteins which were denatured through the procedures of western blotting (data not shown).

The anti-idiotypic antibodies worked weakly as an agonist on susceptible oat protoplasts, but also worked as an antagonist to the action of victorin. These results may be explained by the polyclonal nature of these anti-idiotypic antibodies which may consist of several "internal images" of the binding part of the victorin molecule to

receptor. Several of the "internal images" carried by the anti-idiotypic antibodies might lead to more than a single type of interaction with receptor; some anti-idiotypic antibodies could act like victorin to induce callose synthesis, but other anti-idiotypic antibodies would only block victorin binding to the receptor. Moreover, the large size of the antibodies, compared to the small victorin molecule, might affect more than a single interaction in the complex receptor-callose synthesis signal transmission. In fact, similar observations were reported in the study of β-adrenergic receptor using polyclonal anti-idiotypic antibodies (6, 18). The group studying β-adrenergic receptor speculated that multiple types of interactions of anti-idiotypic polyclonal antibodies to the receptor (18), and later found a monoclonal anti-idiotypic antibody which had only agonist properties of the physiological function of the original ligand (6).

These studies on the effects of antisera on callose induction from oat protoplasts with or without victorin treatment were possible because of the high sensitivity of the assay system. Extracellular secretion of callose from susceptible oat protoplasts is the most sensitive reaction of oat cells treated victorin known (21). 60 pg/ml of victorin initiated the secretion of callose from 70 % of cells after 5 hr (Fig. 23) and as little as 6 pg/ml of victorin stimulated callose secretion (Fig. 21 (D))(21). Since such a sensitive assay was not possible in these other examples of anti-idiotypic antibody studies, the anti-idiotypic antisera usually reacted only at high concentrations in the examination of biological effects modulated by cell surface receptors (19, 20). However, in this

study, antisera diluted twenty times (about 0.02 mg/ml final concentration) still elicited a physiological responses (Fig. 22 & 23).

These data suggest that anti-victorin anti-idiotypic antibody may be a useful tool to examine the cell surface receptor(s) of victorin. However, the production of monoclonal anti-idiotypic antibody will probably be necessary to examine the receptor-anti-idiotypic antibody interactions in detail.

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Table 4. Effect of BSA and human Igs on callose synthesis from victorin treated oat protoplast.

Treatment[1]	% of callose producing cell/total cell[2]	
60 pg/ml victorin	48.9	(100)
60 pg/ml victorin plus 20 μg/ml BSA	55.3	(113)
60 pg/ml victorin plus human Igs	49.3	(101)

[1]All of the concentrations listed were final concentration in 0.5 ml of susceptible protoplast in SCM (4 x 10^4 protoplasts/ml). The method described in Materials and Methods of Chapter 3.

[2]The number of cells which secreted callose was counted and percentage in total cells (average 100 protoplasts were counted per fraction) was indicated. Number in parenthesis indicates percent change from control (60 pg/ml victorin alone treatment).

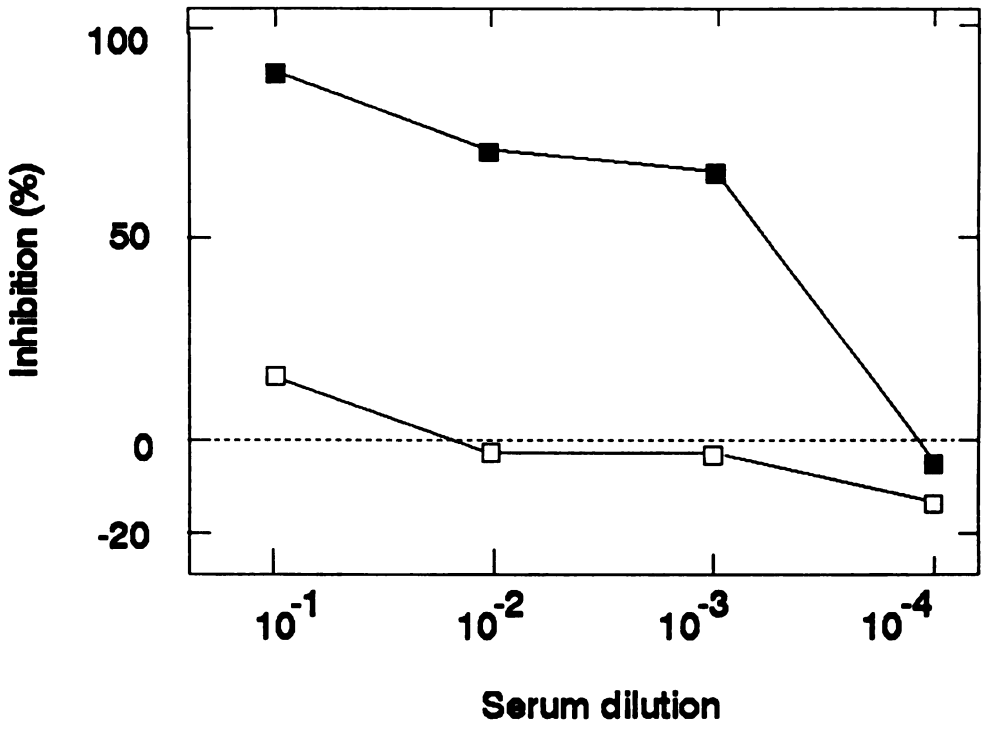


Fig. 19. Anti-victorin anti-idiotypic antibody inhibition of victorin-HRP binding to anti-victorin antibody in direct ELISA. Sera after 3rd boost with anti-victorin antibody-OVA conjugates or pre-sera was purified with the protein A column, diluted with PBS to the different concentrations (10^{-1} to 10^{-4}), and mixed with victorin HRP. The solution was placed in microtiter wells coated with anti-victorin antibody, and the binding of victorin-HRP was monitored by reading A_{450} after addition of HRP-substrate (see Materials and Methods). Inhibition of victorin-HRP binding to the coated antibody by the addition of sample serum was compared with the binding of control well which was treated with only victorin-HRP, and indicated by percentage of inhibition.

-■- antisera immunized with anti-victorin antibody-OVA
-□- pre-sera

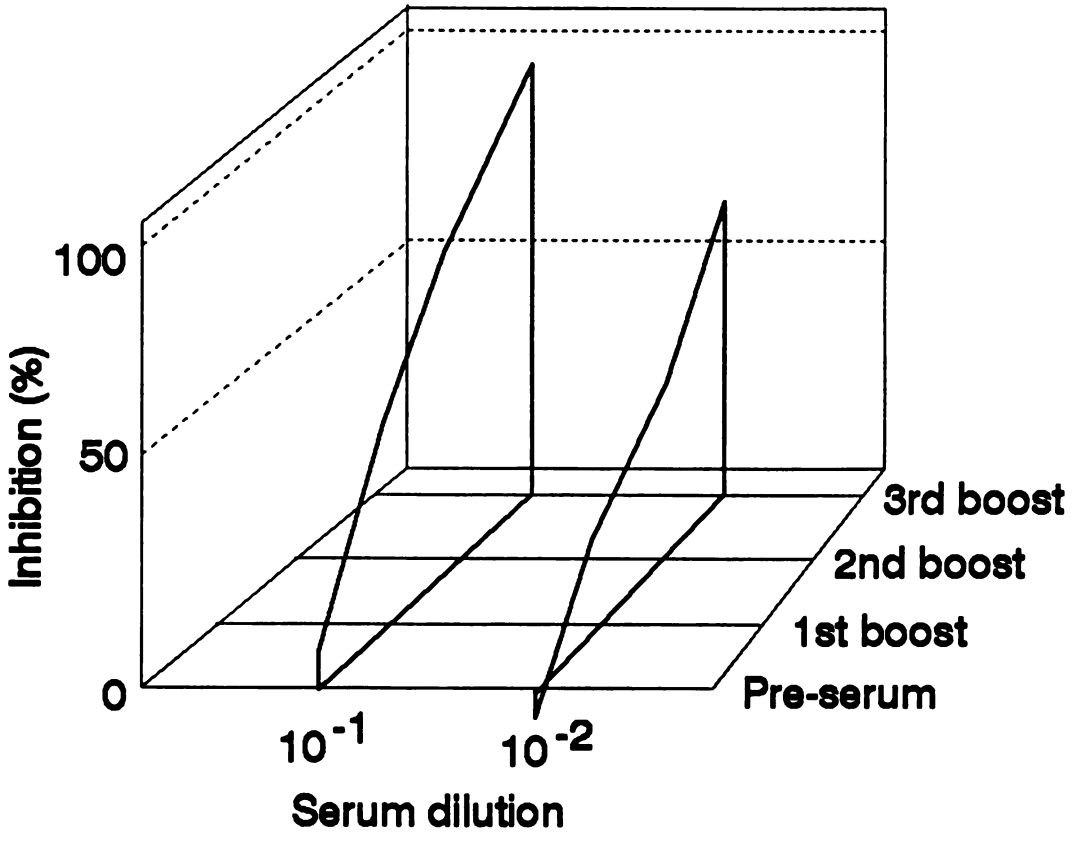


Fig. 20. Effect of number of injections on anti-victorin anti-idiotypic antibody production in sera. The existence of anti-victorin anti-idiotype was examined by the inhibition of victorin-HRP binding to anti-victorin antibody on direct ELISA (see Fig. 19 and Materials and Methods). Sera after each boost or pre-sera was diluted 10^{-1} and 10^{-2} after the protein A purification, and the presence of anti-idiotype was indicated by the inhibition rates against victorin-HRP binding.

Fig. 21. The production of callose from oat protoplasts induced by victorin or anti-victorin anti-idiotypic antibody.

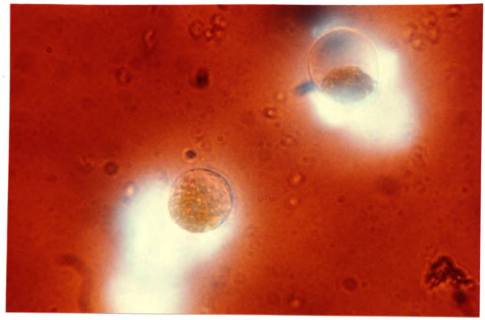
(A) callose secretion from susceptible oat protoplasts (Park) induced by 60 pg/ml of victorin after 18 hr incubation (X400).

(B) resistant oat protoplasts (Korwood) incubated for 18 hr with 60 pg/ml of victorin (X400).

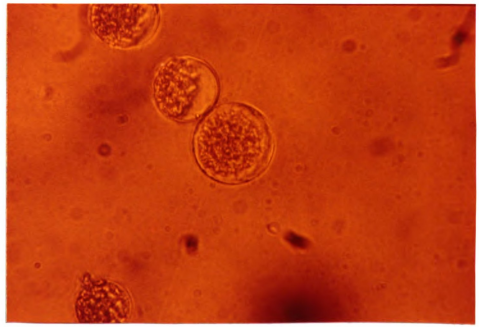
(C) callose secretion from susceptible oat protoplasts (Park) induced by twenty times diluted sera containing anti-victorin anti-idiotype after 18 hr incubation (X400).

(D) callose secretion from susceptible oat protoplasts (Park) induced by 6 pg/ml of victorin after 18 hr incubation (X400).

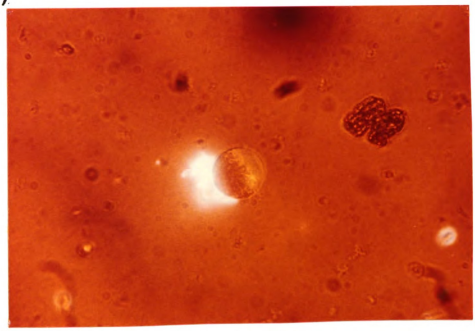
(A)



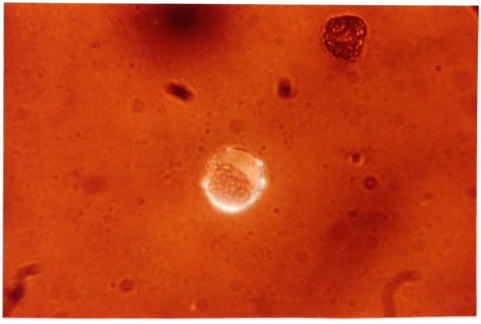
(B)



(C)



(D)



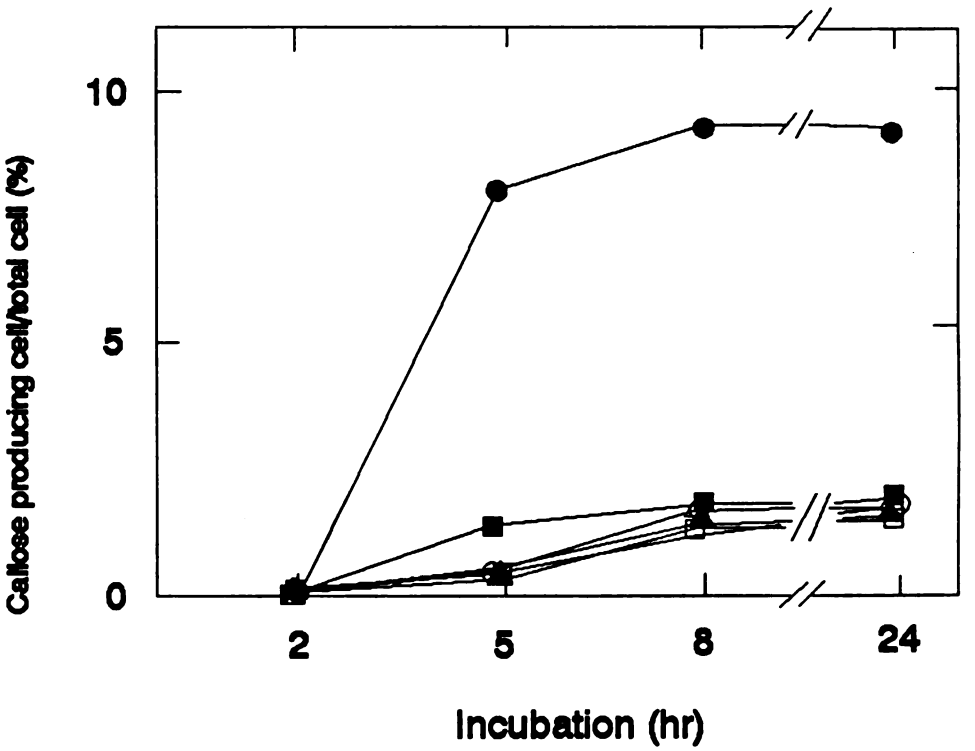


Fig. 22. Induction of callose secretion from oat protoplasts by the addition of anti-victorin anti-idiotypic antibody. Twenty times diluted sera after 3 boosts or the same concentration of pre-sera was mixed with 0.5 ml of protoplast in SCM (4 X 10^4 protoplast/ml), and incubated for various periods. The umber of the cells which secreted callose were counted and the percentage of callose producing cells in total cell (total about 100 protoplasts were counted per fraction per experiment) was indicated (see Materials and Methods).

-o- 20^{-1} diluted anti-idiotype to susceptible protoplast.
-o- " to resistant protoplast.
-■- 20^{-1} diluted pre-sera to susceptible protoplast.
-□- " to resistant protoplast.
-▲- susceptible protoplast without any treatment.
-△- resistant protoplast without any treatment.

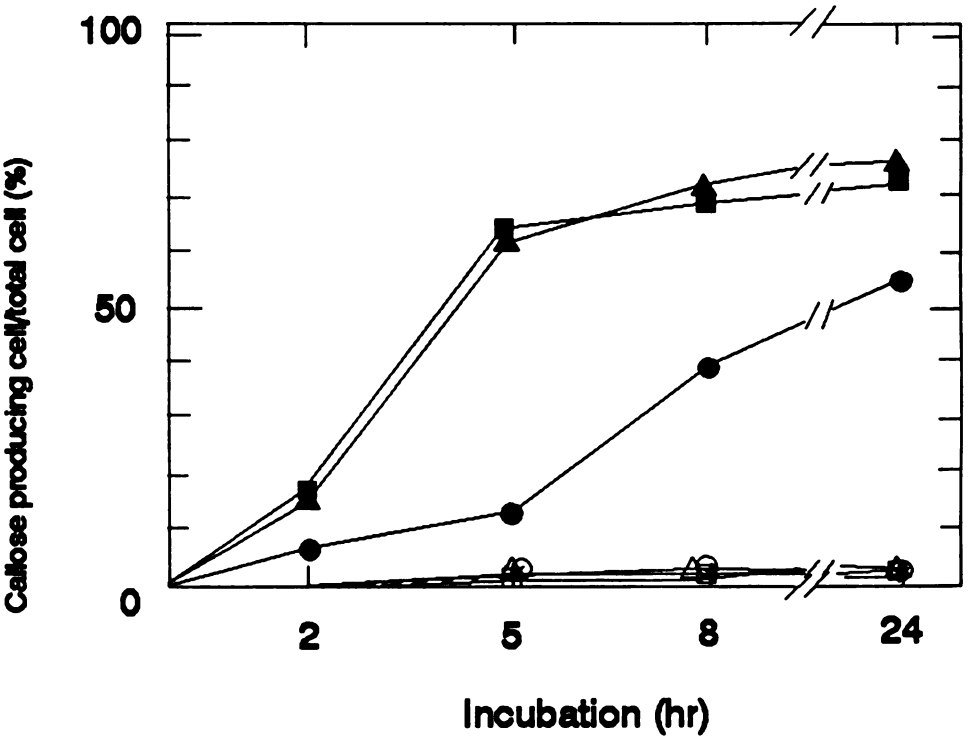


Fig. 23. Inhibition of callose synthesis by anti-victorin anti-idiotypic antibody from victorin treated susceptible oat protoplasts. Twenty times diluted sera after the 3rd boost with anti-victorin antibody-OVA conjugates or pre-sera was mixed with 0.5 ml of oat protoplasts in SCM (4 X 10^4 protoplast/ml) plus 60 pg/ml of victorin, and incubated for various periods. The number of cells which secreted callose was counted and the percentage of the callose secreting cells in total cells was determined (about 100 cells were counted pre fraction pre experiment)(see Materials and Methods).

-o- victorin plus anti-idiotype to susceptible protoplasts.
-o- " to resistant protoplasts.
-▲- victorin plus pre-sera to susceptible protoplasts.
-△- " to resistant protoplasts.
-■- victorin to susceptible protoplasts.
-□- victorin to resistant protoplasts.

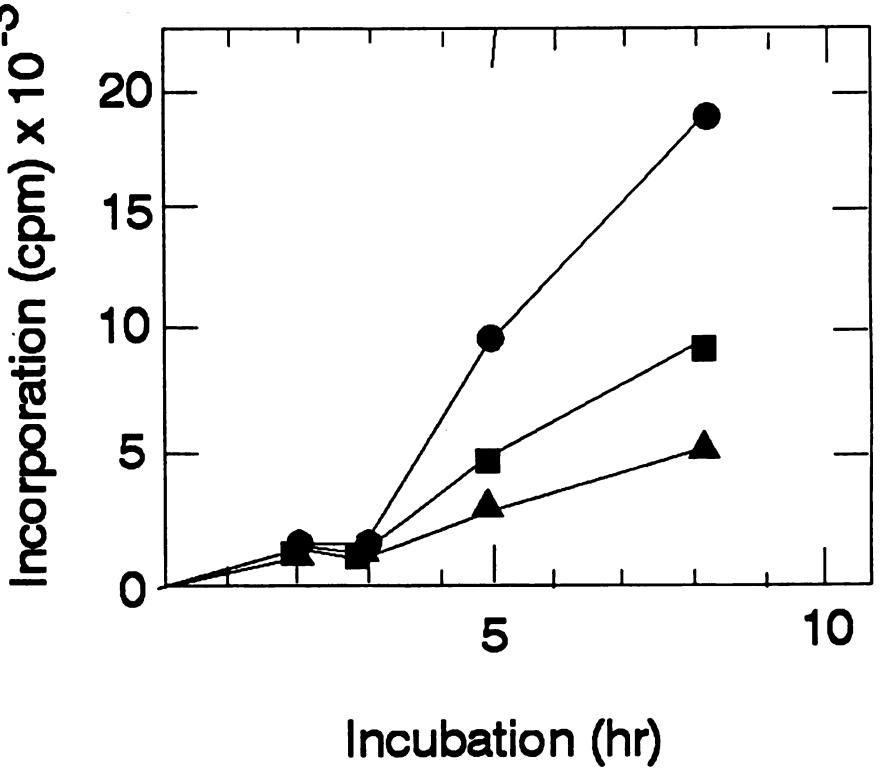


Fig. 24. ^{14}C-glucose incorporation to callose produced from oat protoplasts treated with victorin, victorin plus anti-idiotypic antibody, victorin plus pre-serum. Protoplasts (2 x 10^4) were incubated for several different periods with 60 pg/ml of victorin, or 60 pg/ml of victorin plus 20 fold diluted anti-idiotypic antibody, or 60 pg/ml of victorin plus 20 hold diluted pre-serum, with 0.25 μCi ^{14}C-glucose. Material insoluble in 70% EtOH was collected by filtration, and the incorporation of labeled glucose was measured by liquid scintillation counter.

-●- victorin
-▲- victorin plus anti-idiotypic antibody
-■- victorin plus pre-serum

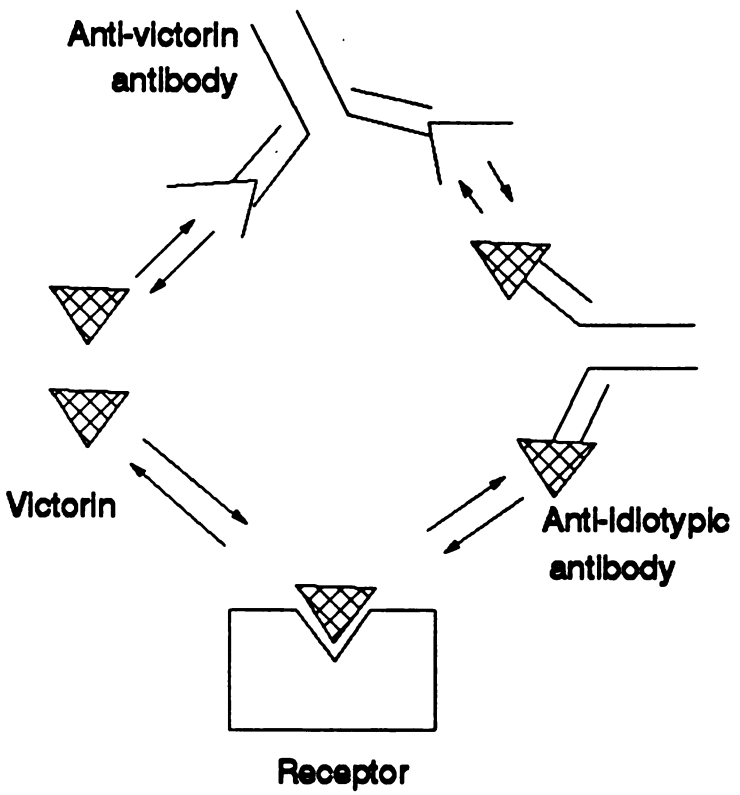


Fig. 25. Schematic representation of the relationship between victorin, anti-victorin antibody, anti-victorin anti-idiotypic antibody, and receptor of victorin.

SUMMARY OF THESIS AND FUTURE PROSPECTS

Anti-victorin polyclonal antibody and anti-victorin polyclonal anti-idiotypic antibody were used to study victorin receptors in oat cells. Data found using these antibodies were:

1) victorin bound covalently to the same oat tissue proteins from susceptible and resistant cultivars *in vivo* and *in vitro*. The molecular masses of the binding proteins detected were 100 kD, and 45 kD in *in vivo* experiments, and 100 kD, 65 kD, and 45 kD in *in vitro*.
2) victorin also bound covalently to different molecular weights of tissue proteins of non-host plants, wheat and *Arabidopsis*.
3) cycloheximide pre-treatment may have slightly reduced the covalent binding of victorin in susceptible, but not resistant oat tissues.
4) The 100 kD victorin binding protein in oat cells appeared to have a high density, like plasma membrane or mitochondria membrane, in a continuous sucrose gradient. However, the 100 kD protein was not located in either isolated plasma or mitochondria membranes as determined by additional experiments including two phase separation, and discontinuous sucrose gradient, respectively.
5) The 65 kD and 45 kD victorin binding proteins were located in all fractions of separated oat tissues, except the 45 kD protein was not detected in isolated mitochondria.

6) Victorin did not affect the activities of membrane marker enzymes, Glucan synthetase II, cytochrome C oxidase, NADH cytochrome C reductase, and IDPase, of susceptible and resistant oats.
7) Anti-victorin anti-idiotypic antibodies acted as an agonist of victorin and also acted as an antagonist to the action of victorin in susceptible oat protoplasts.

There were three hypotheses to explain the mechanism of specificity and the mode of action of victorin. The first hypothesis was the receptor hypothesis stated in Chapter 1 and 2, which was the product of the *Vb* gene may be the receptor of victorin, and the lack of the receptor leads to resistance. The second was that the location of the receptor may be on the plasma membrane. As discussed in Chapter 2, several lines of indirect evidence support this hypothesis. The third hypothesis was that covalent binding of victorin may be essential for sensitivity of oat because the aldehyde group of the victorin molecule is necessary for toxicity.

Victorin binding studies with immunological methods indicated there was no difference in victorin covalent binding between susceptible and resistant cultivars of oats. Since victorin binds covalently to non-host plants, that are insensitive to the toxin, it can not be firmly established that covalent binding in a sensitive oat line is a requirement for toxicity. However, anti-victorin anti-idiotypic antibody mimicked some victorin actions on susceptible protoplasts, suggesting that the receptor of victorin could be located on the surface of susceptible oat cells. Victorin covalent binding sites found in the plasma membrane were

65 kD and 45 kD proteins, but these were also located in other cell organelles. The 45 kD protein was detected when victorin was treated *in vivo* to oat tissues but not the 65 kD protein, indicating that the 45 kD protein could be a possible candidate as the receptor of victorin. However, if the 45 kD protein was the receptor of victorin, the apparently equal binding of victorin to the 45 kD protein in both susceptible and resistant oats suggests that specificity can not be explained by binding, but may another factor located in the victorin transduction pathway. The 100 kD binding protein identified by Wolpert and Macko, does not appear to be located in the plasma membrane of oat cells, and victorin binding to this protein was also detected in both susceptible and resistant oats. If the 100 kD protein was essential for specificity, these data would suggest a very complex mechanism to explain since all the evidence points to a receptor on the oat cell surface.

Based on the findings reported in this dissertation, it appears that victorin interacts with a cell surface receptor, and if covalent binding is required for toxicity, then the receptor itself does not appear to be genotype specific. Furthermore, the 45 kD protein, which is found in the plasma membrane and other organelles, would be the most likely candidate as the victorin receptor. The possibility that binding to the 45 kD protein, 65 kD protein and/or 100 kD protein enhances or contributes to toxicity by some other mechanisms can not be dismissed. Further studies may address this issue by building on the immunological approach used in this study. Use of anti-idiotypic antibody that is a toxoid of victorin, might be an avenue for further understanding of the relationship

between cell surface and internal receptors in regard to overall toxicity. If the *Vb* gene product is not a cell surface protein, then an immunological approach will probably do little to answer the question of specificity.

APPENDIXES

Additional experiments not reported in Chapters 1 to 3.

The production of biotinylated victorin and its application

a. The production of biotinylated victorin.

Victorin and biocytin (Biotinylated-L-lysine) were conjugated by the following method. First, the amino group of victorin was blocked by methylation with formaldehyde (1). Five milligrams of victorin C, 5 μl of 37% formaldehyde (v/v)(final concentration of 63 mM), and 2 mg of recrystaled sodium cyanoborohydrate (2) were mixed in 1 ml of 0.2 M sodium phosphate buffer, pH 8.5, and incubated for 4 hr at 37°C. The products was purified by HPLC as described for victorin C purification (see Chapter 1). The highest peak (at around 66% ACN) was taken (Fig. 26 (A)) and the methylation was confirmed by Dr. R. Hollingsworth with the appearance of a peak at about 2.8 on a NMR spectrum without any other changes (Fig. 26 (B)). The biological activity of the methylated victorin was determined by the dilution end-point for toxicity in the root growth inhibition assay (see Chapter 1), and was 100 to 150 pg methylated victorin/ml. About 2 to 3 times less active than native victorin (the dilution endpoint of native victorin was 50 pg/ml).

The methylated victorin (5 mg) was then mixed with 20 mg of 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (CDI) and 10 mg of biocytin in 1 ml

of water. The mixture was incubated for 2 hr at room temp. at pH 4.5 to 5.2 adjusted with 0.01 N HCl throughout the incubation. The products were purified by HPLC as described for victorin C purification (see Chapter 1). All major peaks were taken and the biological active of each peak was examined (Fig. 27) by protoplast death assay and root growth inhibition assay (see Chapter 1). The activity of the biotinylated victorin was about 100 times less active than native victorin. The dilution end point for the toxicity was about 8 ng/ml (Fig. 27).

b. Applications of biotinylated victorin.

Attempts to observe the binding of biotinylated victorin to oat protoplasts was performed with anti-biotin IgG-FITC conjugate or Avidin-FITC conjugate as second markers. Protoplasts were isolated from oat leaves (see Chapter 1) and adjusted to a final concentration to 2.5 x 10^5 protoplasts/ml SCM. One μg/ml (final concentration) of biotinylated victorin was mixed with the protoplasts and incubated for 2 hr at room temp. Either anti-biotin IgG-FITC (20 μg/ml) or Avidin-FITC (20 μg/ml) was then added, incubated for 1 hr, and the protoplast solution was washed 3 times by centrifugation. The protoplasts were observed by fluorescent microscopy with a Leitz filter (BP450-490). Avidin-FITC alone strongly bound non-specifically to both susceptible and resistant oat protoplasts. Anti-biotin IgG-FITC did not bind non-specifically to oat protoplasts if the cells were intact. In both treatments, anti-biotin IgG-FITC or Avidin-FITC, the protoplasts in which the plasma membrane appeared broken

by the isolation procedures or the effect of victorin, showed strong fluorescent even without the addition of biotinylated victorin. When anti-biotin IgG-FITC was used as a second antibody, the detection of biotinylated victorin was possible, and the binding was observed on both susceptible and resistant oat protoplasts. However, the signal was very weak, and much stronger signal or more specific filter system for the detection of FITC should be used for clear a detection.

Biotinylated victorin was also used for western blotting (see Chapter 1). However, all of second markers used, including anti-biotin IgG-alkaline phosphatase (AP), avidin-AP, and strept-avidin-AP, bound non-specifically to oat proteins and specific binding to biotinylated victorin was impossible to detect.

<REFERENCE>

1) Dottavio-Martin, D., and Ravel, J. M. (1978) Radiolabeling of proteins by reductive alkylation with ^{14}C-formaldehyde and sodium cyanoborohydride. Anal Biochem 87: 562-565.

2) Jentoft, N., and Dearborn, D. G. (1979) Labeling of proteins by reductive methylation using sodium cyanoborohydride. J Biol Chem 254: 4359-4365

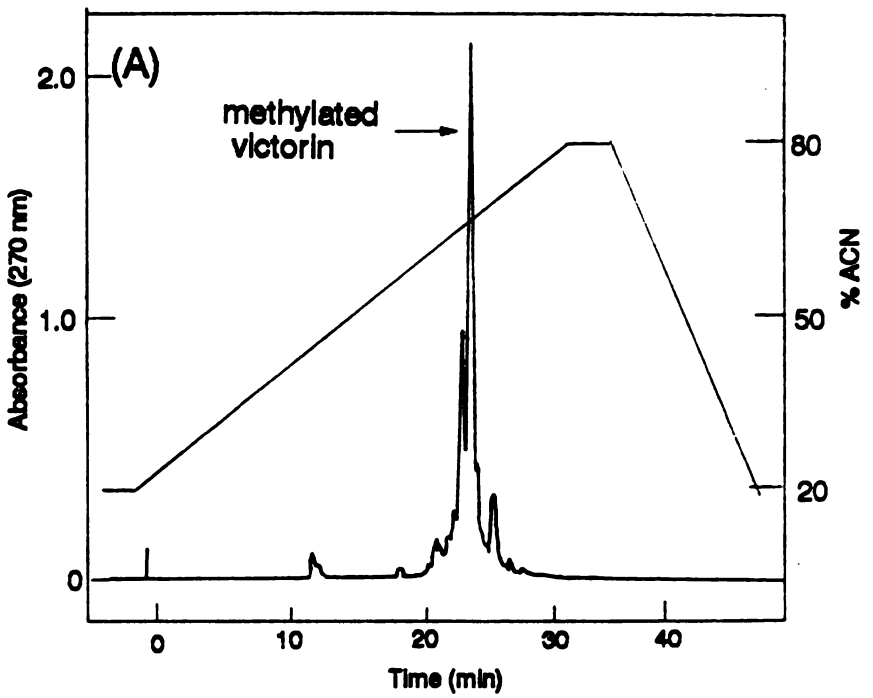
Fig. 26. Reductive methylation of victorin.

(A) The peak of methylated victorin on reverse-phase HPLC.

Column:	Chemcosorb 5-ODS-H (20mm X 250mm).
Flow rate:	4 ml/min.
Solvent:	20 to 80% ACN in water gradient for 30 min.
Detected UV:	270 nm.

(B) NMR analysis of methylated victorin.

(1) 5 mg victorin in D_2O/0.01% TFA
(2) 5 mg methylated victorin in D_2O/0.01% TFA


(A)
methylated victorin
2.0
1.0
0
Absorbance (270 nm)
80
50
20
% ACN
0
10
20
30
40
Time (min)

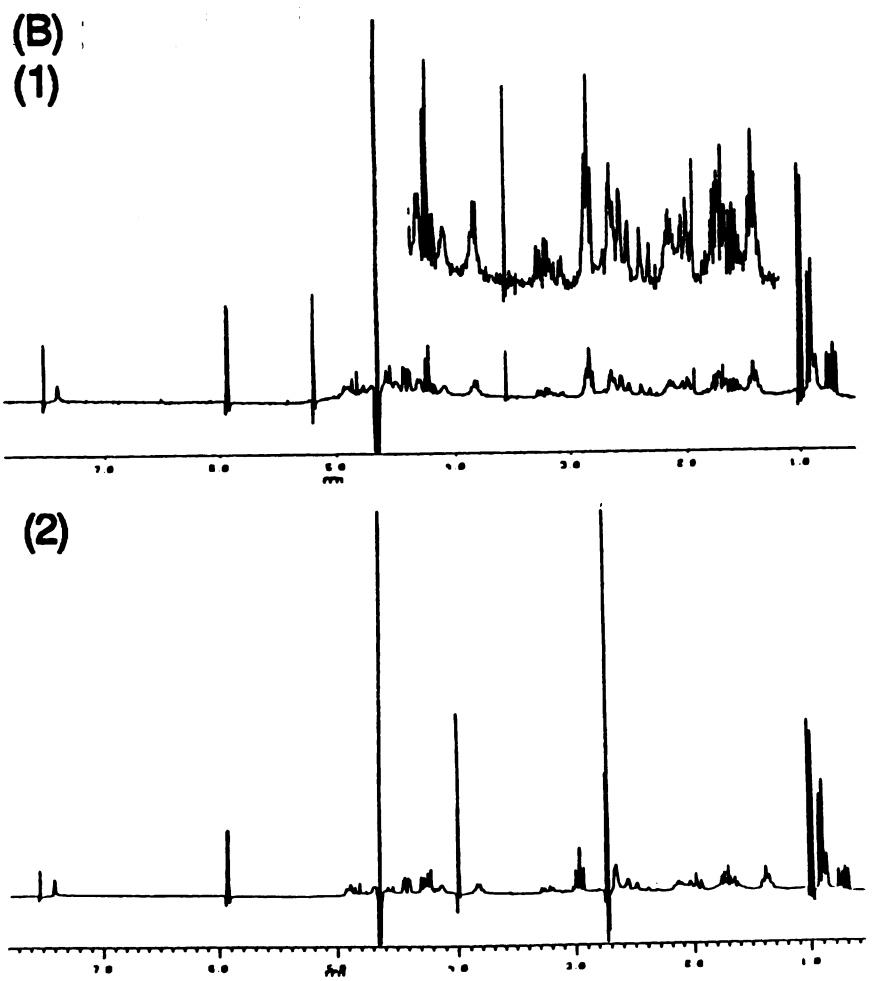
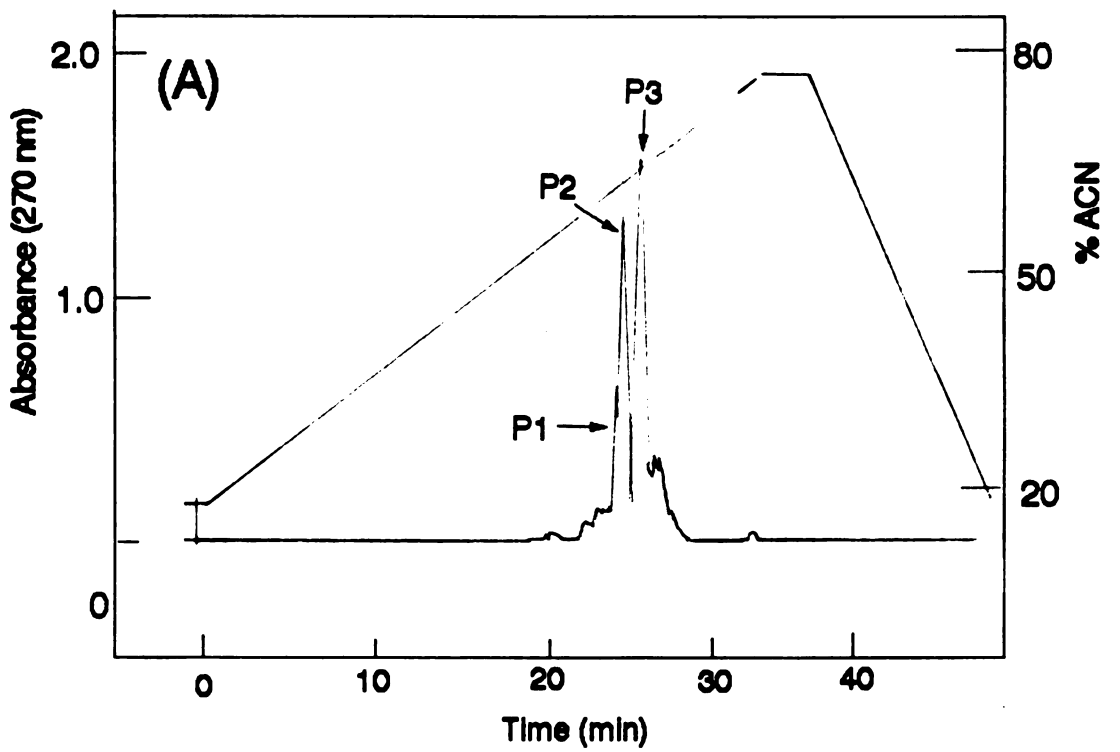

(B)
(1)
(2)

Fig. 27. Purification and biological activity of biotinylated victorin.

The peak of biotinylated victorin on reverse-phase HPLC (A).

Column:	Chemcosorb 5-ODS-H (20mm X 250mm)
Flow rate:	4 ml/min.
Solvent:	20 to 80% ACN in water gradient for 30 min.
Detected UV:	270 nm

Each major peak (P1, P2, and P3) was taken, and the biological activities of the fractions were measured (B).



(B) Biological activities of peaks detected on above HPLC system.

Assays	Dilution end point for the toxicity			
	P1	P2	P3	victorin
Root growt inhibition	>2 μg/ml	>2 μg/ml	8 ng/ml	75 pg/ml

Detection of victorin on the germination tubes of *C. victoriae* by using anti-victorin antibody and second antibody-FITC conjugate

Fifty μl of spore solution (5 x 10^5 spores/ml water) of *C. victoriae* was placed on a glass plate, and incubated for various periods in a moist chamber. After the incubation, the water was almost dried (leave it for 10 min at room temp) and 50 μl of anti-victorin antibody solution (1 μg/ml) was added onto the glass where spores were germinating. After 1 hr incubation in a moist chamber, the plate was again dried and 50 μl of anti-rabbit IgG-FITC (1:100 diluted from stock of sigma) was added. The plate was then observed by fluorescent microscopy with a Leitz I2 filter.

The spores germinated after 1 hr, and more than 90 % of the spores had germinated after 6 hr (Fig. 28 (A)). Fluorescence due to specific anti-victorin antibody binding was observed on germ tubes of the spores as soon as 6 hr after the spores were mixed with water (Fig. 28 (A)). About 15% of the germ tubes showed fluorescence on their after 13 hr (Fig 28 (A) & (B)), and the fluorescence was limited to the germ tubes and not on the spore itself. Anti-victorin antibody binding was specific because the fluorescence disappeared when 1 μg/ml of free victorin was added simultaneously with anti-victorin solution (data not shown). The binding (ie. florescence) was not observed when anti-victorin antibody was mixed with spores of *C. carbonum* (2R15(mat a))(data not shown). The number of germ tubes of *C. victoriae* with fluorescence increased significantly after 15 hr (Fig. 28 (A)). However, this increased fluorescence was non-specific binding of second antibody to the germ tubes since fluorescence

was observed without the addition of anti-victorin antibody (data not shown). To detect only victorin specific binding without non-specific binding of the second antibody, observations should be made less than 13 hr after incubation started, and stronger marker systems, such as gold labeling and EM observation, may be required for greater detail.

Fig. 28. Detection of victorin on the germination tubes of *C. victoriae* using anti-victorin antibody and second antibody-FITC conjugate.

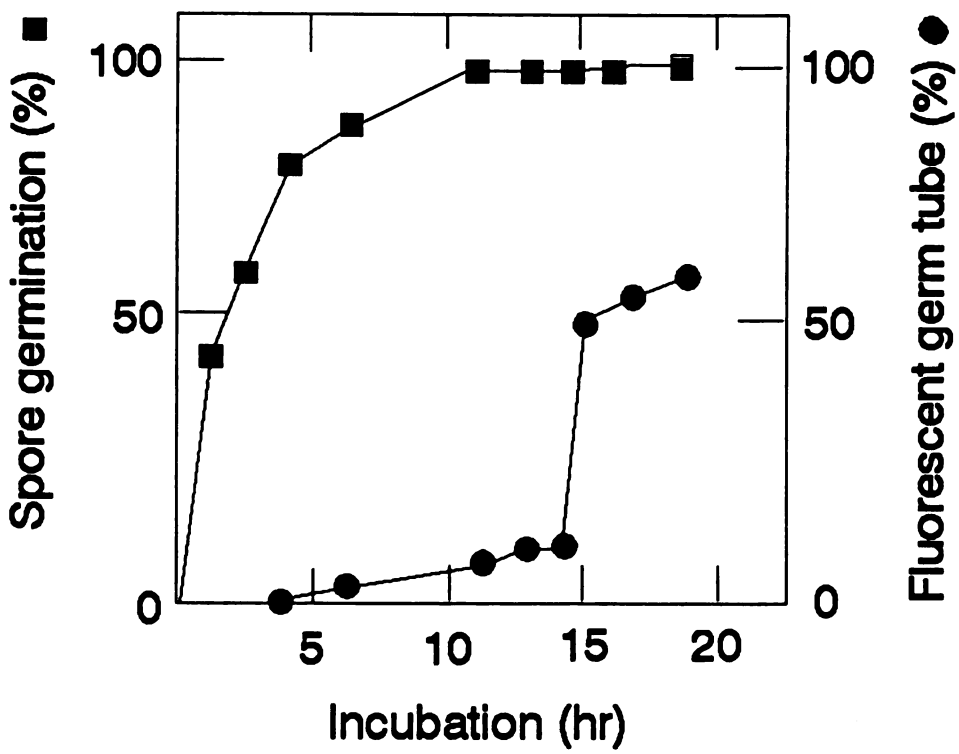
(A) Time course of fungal germination and growth, and detection of victorin. Spore solution (5×10^{5}/ml water) was placed onto the glass plate, and the spores were germinated for various periods. Victorin produced during germination was detected on the germ tubes using anti-victorin antibody and second antibody-FITC conjugate. Methods in detail indicated in Appendixes.

(B) Fluorescent detection of victorin on germination tube.

(1) Light microscopic observation of germinating spores of *C. victoriae*. X400.

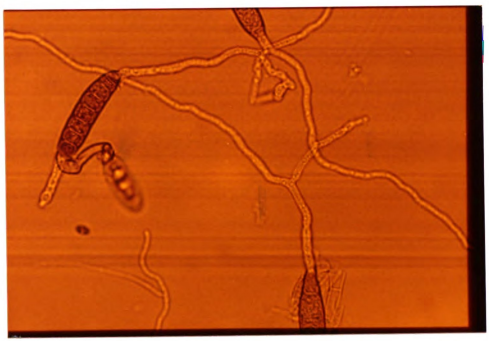
(2) Fluorescent microscopic observation with Leitz I2 filter of (1). Germinating spores were treated with anti-victorin antibody and second antibody-FITC conjugate (see Appendixes was for detail). X400.

(A)

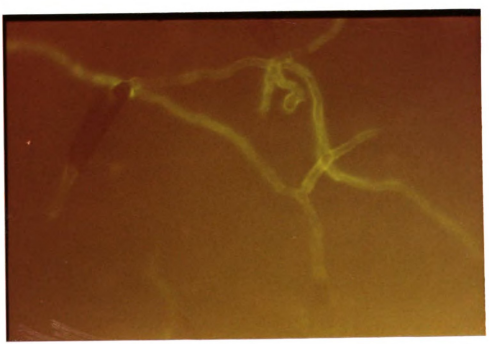

Spore germination (%) ■
100
50
0
5
10
15
20
Incubation (hr)
100
50
0
Fluorescent germ tube (%) ●

(B)

(1)



(2)



Monoclonal anti-victorin antibody production using Ig gene cloning method

a. Immunogen preparation:

Victorin was conjugated to cholera toxin through the carboxyl group of victorin with the crosslinker EDC. The amino group of victorin was first blocked by methylation as described for the production of biotinylated victorin. The methylated victorin was then conjugated to cholera toxin with EDC by the protocol of Pierce. Victorin was also conjugated to cholera toxin through the amino group by a glutaraldehyde method. Cholera toxin in 870 μl of PBS (1 mg/ml) and 500 μg victorin in 1050 μl of PBS were mixed with 60 μl of 25% glutaraldehyde stock solution (Sigma). The mixture (total 2 ml) was incubated for 45 min at room temp, and 200 μl of 2 M lysine (final 0.2 M) added to stop the reaction. The conjugate was dialyzed against PBS, and concentrated by microcencentrator (amicon). The concentration of the solution was adjusted to 35 μg/ml, and used for the immunization.

2. Immunization:

7.5 μg victorin-cholera toxin conjugate/200 μl PBS was injected intravenously to female mice (B6C3F1) and boosted 4 times. No adjuvants were used. Serum was collected 6 days after each injection and the presence of anti-victorin antibodies was screened by indirect ELISA (see Chapter 1). After the 3rd boost, the titer of serum against victorin was 3 X 10^3 and 250 ng/ml of victorin inhibited 50% of binding in indirect

competitive ELISA (Fig. 29).

c. cDNA library of Ig gene construction:

All protocols are described in detail by Stratacyte Ig gene cloning kit, and the materials required for the procedure were provided in the kit.

Briefly, after the 4th boost, the spleens were taken, total RNA purified from the tissue homogenate, mRNA isolated, and first strand DNA synthesized. With the primer set provided by the Stratacyte kit, the genes for heavy chain and light chain of IgG were amplified separately from the single strand cDNA by PCR. The isolated IgG gene was digested, ligated with ImmunoZap arms, and packed into the lamda phage. A light chain library and a heavy chain library were constructed in this way. Heavy and light chains were combined in a single library using the protocols described in the stratacyte manual.

d. Screening:

The phages were plated, plaque lifted, treated with IPTG, and the positive clone isolated by using victorin, anti-victorin polyclonal antibody, and second antibody conjugated to ^{125}I. The nitrocellulose filter lifted plaques were first blocked with 0.2% tween in PBS, and treated with 10 μg/ml of victorin in PBS. If a positive clone was present, victorin bound to the plaque and the bound victorin was detected by the addition of anti-victorin antibody and second antibody. By this method, 3 positive clones from heavy and light chain combinationally

library, 2 positive clones from heavy chain library, and 6 positive clones from light chain library, were isolated.

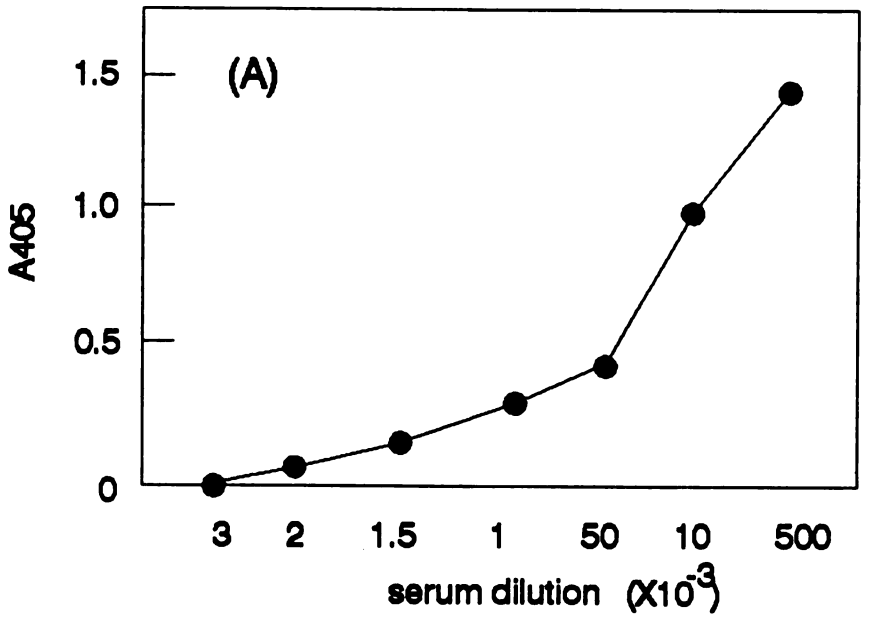
f. Analysis of Ig fragments:

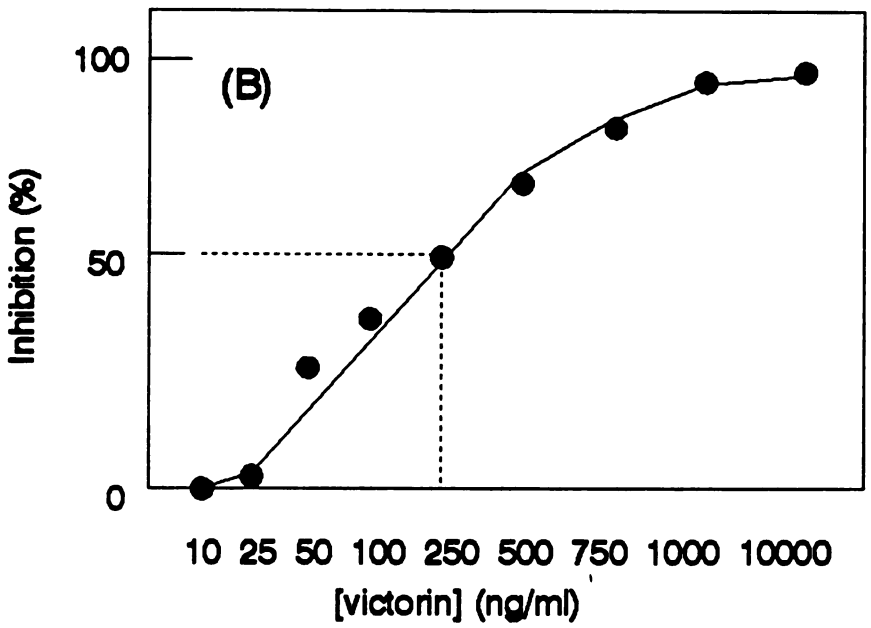
After the In vivo phagemid excision from positive clone to *E. coli* cells, IgG fragments were produced and secreted from the *E. coli* cells grown in the presence of IPTG. These IgG fragments were then analyzed by ELISA, and immuno-blottings. Several IgG fragments produced from positive clones did bind to victorin coated on ELISA plate wells. However, none of them showed inhibition when free victorin was mixed with the IgG fragments.

Fig. 29. Immunization of mouse with victorin-cholera toxin conjugate for the production of monoclonal anti-victorin antibody.
Indirect ELISA and competition indirect ELISA were used to examine screen titers of mice immunized with the victorin-cholera toxin conjugate. Procedures for ELISAs were described in Materials and Methods of Chapter 1.

(A) Indirect ELISA

(B) Indirect competition ELISA


(A)
1.5
1.0
0.5
0
A405
3
2
1.5
1
50
10
500
serum dilution (X10-3)


(B)
100
50
0
Inhibition (%)
10 25 50 100 250 500 750 1000 10000
[victorin] (ng/ml)