Mode of action of maculosin, a host-specific phytotoxin, produced by Alternaria alternata on spotted knapweed (Centaurea maculosa L.)
by Sang Ho Park

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Maculosin [diketopiperazine, cyclo (L-Pro-L-Tyr)] is a host-specific phytotoxin produced by Alternaria alternata on spotted knapweed (Centaurea maculosa L.). This study involves its putative receptor, cellular effects and its fate in the host plant. Initially, 14C-maculosin was synthesized by organic methods. The purity and structure were identified by HPLC, NMR and MS.

Host leaves possessed maculosin-binding activity in the cytosolic and membrane fraction and most of the binding activity was recovered from the cytosolic fraction. The binding component was identified as a protein because of its heat-lability and sensitivity to proteases. A 16-fold purification of toxin-binding protein was carried out by ammonium sulfate fractionation, Sephadex G-200 column, and maculosin-affinity chromatography. The affinity column was prepared with epoxy activated Sepharose 6B to which the phenolic group of maculosin was attached. Furthermore, the &aplha;-amino group of tyrosine was coupled to CNBr-activated Sepharose 4B. However, the tyrosine affinity column did not bind any cytosolic proteins. The molecular weight of maculosin-binding protein was estimated as 604 kdaltons and the receptor contained more than one binding protein. One of the maculosin-binding proteins was identified as ribulose 1,5-biphosphate carboxylase.

Metabolites of maculosin were analyzed 5 days after treatment of 14C-maculosin on detached leaves. Maculosin was converted to three polar compounds (M-l, M-2, and M-3). The major metabolite (M-2) was purified and identified as maculosin β-O-D-glucoside. M-3 metabolite was surmised as one of the dipeptides (L-Pro-L-Tyr-OH or L-Tyr-L-Pro-OH), the hydrolyzed products of diketopiperazine (maculosin). The conversion mechanism was enzymatically mediated. The formation of maculosin β-O-glucoside was identified as a biologically inactive intermediate compound which is converted to a methanol insoluble complex in the leaves of spotted knapweed. In oats (monocot), the metabolism of maculosin was the same as in spotted knapweed.

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Sang Ho Park

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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CHAPTER I

INTRODUCTION

The plant host-parasite interaction resulting in disease development is the result of the interplay of many factors induced or produced by pathogen and the host. Many pathogens causing necrosis of plants often produce disease symptoms by elaborating one or more toxic compounds which are called phytotoxins. Phytotoxins which are involved in disease are classically subdivided into two classes, host-specific and host non-specific.

The term, "host non-specific toxin" implies that the susceptibility of the host plant to a pathogen does not parallel its sensitivity to the toxin. The toxin probably does not play a role in the establishment of the pathogen in the host. However, it is able to induce some characteristics of the disease syndrome and is also known as a virulence factor or secondary determinant of pathogenicity. A great number of non-specific phytotoxins are known which do not reproduce the patterns of resistance and susceptibility of the host to the pathogen. They include all the bacterial phytotoxins known so far and the majority of the fungal toxins (19,40,59).

At present, the host specificity exhibited by a pathogen is the most intriguing problem in plant pathology. It is assumed that many complex physiological and biochemical mechanisms are involved in host specificity. In some cases, the causal fungi recognize their host plants and invade tissues by exuding highly biologically potent chemicals, namely one or more "host-specific toxins." These compounds cause specific cell toxicity in the initial stages of the infection process, but only to the normal hosts of the fungus. It is well known that such toxic substances participate in establishing disease development as a primary determinant of pathogenicity. There are at least fourteen examples in which host specific phytotoxins appear to play a significant role in pathogenicity (52,59,75). Some specific examples are Victoria blight of oats caused by Cochliobolus victoriae in 1947 (39) and Southern leaf blight of corn by Cochliobolus heterostrophus in early 1970 in the USA (54). Commonly, these phytotoxins have been isolated from the fungal genera Alternaria and Helminthosporium (Cochliobolus, Bipolaris) and a few others (31,45).

Along with this important role in disease development in various crops, phytotoxins have been helpful in establishing the molecular basis of disease production and in gaining an understanding of the normal physiology and biochemistry of plants (59).

Fusicoccin is one of the best known phytotoxins which has been used as a molecular probe. It is the major phytotoxic metabolite of Fusicoccum amygdali, the causative agent of peach and almond canker. It acts directly and primarily at the plasma membrane level, where it selectively activates the H^+-ATPase responsible for electrogenic proton extrusion, with consequent influence on the activity of a number of metabolic and physiological processes (36). Furthermore, evidence has been produced that the stimulation of proton extrusion is triggered by a signal that originates from the interaction of fusicoccin with specific receptors in plasma membrane-enriched fractions of a large number of higher plants, and located at the apoplastic side of the plasma membrane (10,16,36).

Green islands, localized areas of chlorophyll retention, are common symptoms in plant tissues infected with certain biotropic fungi. Some phytotoxins can induce such symptoms (49). Gigantenone, a new eremophilane diepoxide, produced by Drechslera gigantea causes the formation of green islands on many graminaceous species tested (29). However, it generally causes necrotic lesions on dicotyledonous species. Gigantenone also induces root formation in mung bean hypocotyls and shows a high level of activity in several plant tissue culture systems (29).

Conventional plant breeding for resistance to pathogens, in many cases, is still too slow to keep pace with the

adaptation of pathogens. Phytotoxins, because of their role in disease development and host selectivity have been proposed as tools for in vitro selection (4,35).

Although most well described phytopathogenic microorganisms are parasitic on crop plants, weeds also suffer from various pathogens (43,60,70). There is considerable current interest in the use of plant pathogenic microbes as agents for the control of certain economically important weeds (66). Numerous plant pathogens are being studied throughout the world for possible use as mycoherbicides or biological control agents. Phytophthora palmivora and Colletotrichum gloeopsorioides f. sp. aeschynomens have been developed, registrated as De Vine and Collego, respectively and marketed for the control of strangler (milkweed) vine and northern jointvetch in the United States (66).

In many cases, it is virtually impossible to conceive of the use of a weed pathogen in the field. The application of weedy pathogens may have to do with the longevity of inoculum, the regulatory restriction on the use of the pathogen, the means of application of the pathogen, the favorability of environmental conditions for epidemic spread of pathogens, and the inherent heterogenicity of weeds (7,66,74).

The potential of phytotoxins as herbicides or as a model for new herbicides has been recently suggested (14,18,60). Higher plants and their pathogens have, in all likelihood,

evolved together and in the process may have had considerable biochemical changes. In the recent past, considerable effort has been expended in finding novel and selective phytotoxins with potential use for the control of weeds.

The use of plant phytotoxins in controlling weeds may have several advantages over the use of weed pathogens. The possible use of phytotoxins to create novel models for more effective herbicides is a very promising area of research and may result in herbicides that might enhance effectiveness and environmental safety. For these reasons, phytotoxins may constitute an efficient method of weed control, although to date, none are in commercial use.

Our laboratory has focused on weed pathogens with the hope of finding both novel and more selective chemical control agents. This has led to the isolation of several phytotoxins from weed pathogens (28,60). Some examples of this are tryptophol from Drechslera nodulosum, a pathogen of goose-grass (62); bipolaroxin, a host selective phytotoxin from Bipolaris cynodontis, a pathogen of Bermuda grass (63); and Dihydropyrenophorin from Drechslera avenae, a pathogen of both wild and cultivated oats (61).

This type of research was further extended to the study of spotted knapweed (Centaurea maculosa L.), a member of the family compositae, which is one of the most serious weed problems in Montana. As a fast spreading rangeland weed, this plant was introduced from Europe in the early 1900's and

has widely spread in the northwestern area of the United States and southwest Canada. Since first reported in Montana in 1927, this weed now infests over 4.7 million acres of rangeland, pasture, and roadside (32).

The perennial growth habit, profuse seed production and aggressiveness of spotted knapweed results in its rapid establishment and spread. Initial infestations occur in disturbed areas such as roadsides, trails, construction sites, overgrazed land and waterways (71). Once established it is very competitive, displacing native grasses and forbs, resulting in near-monoculture stands of spotted knapweed. Another factor contributing to its success in North America is the lack of natural enemies. In Europe, its center of origin, spotted knapweed evolved with host-specific insects and pathogens which keep the plant frequency and density at low levels. In North America most of these agents are not present.

After an intensive search through several counties in southern Montana and an examination of thousands of plants in three counties, a fungus was found that causes black necrotic lesions on spotted knapweed. This fungus was further identified as Alternaria alternata. From a cultural filtrate a host specific phytotoxin, maculosin, was isolated (Figure 1) and its structure was confirmed by organic synthesis (56).

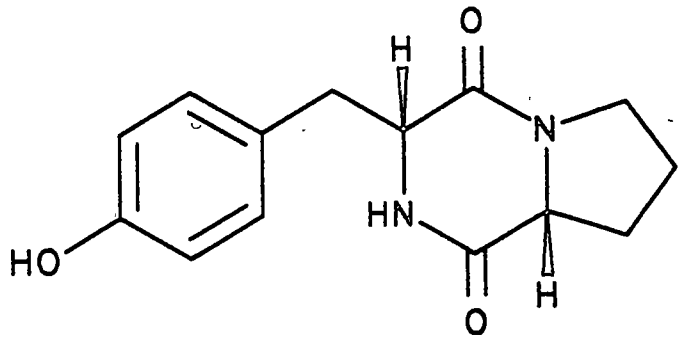


Figure 1. Structure of maculosin, cyclo (L-Pro-L-Tyro).

Fungi belonging to the genus Alternaria are commonly found causing leaf spots and some are known saprophytes (20). Host specific toxins isolated from A. alternata are shown in Table 1. These fungi are not distinguishable based on conidial morphology, but are characterized by the production of a toxin that appears to be responsible for the host range of the fungus and its pathogenicity. Each of these organisms is referred to as a distinct pathotype of A. alternata (Table 1) (44,45).

In the case of A. alternata on spotted knapweed, it produces numerous, non-toxic diketopiperazines (56). However, one termed maculosin [cyclo (L-Pro-L-Tyr)] caused black necrotic lesions on spotted knapweed at 10 μM. Even at 1 mM, maculosin did not affect 19 other grasses and dicots that were tested (56). Thus it is host specific toxin of the diketopiperazines isolated from A. alternata. Maculosin, the most active substance, possesses a phenolic moiety, not

Table 1. Host-specific toxins from A. alternata.

Disease	Pathogen (previous name)	Toxin	Host (pathotype)
Alternaria blotch of apple	A. mali	AM-toxin I, II ,III	Apple
Alternaria leaf spot of Pigeonpea	A. tenuissima	ACT-toxin	Pigeonpea
Alternaria stem canker of tomato	A. alternata f.sp. lycopersici	AL (AAL)-toxin I, II	Tomato
Black leaf spot of strawberry	A. alternata	AF-toxin I, II, III	Strawberry
Black spot of Japanese pear	A. kikuchiana	AK-toxin I, II	Japanese pear
Black spot of rough lemon	A. citri	ACR(L)-toxin	Rough lemon
Brown spot of tangerine	A. citri	ACT-toxin ACTG-toxin	Tangerine
Brown spot of tobacco	A. longipes	AT-toxin	Tobacco

uncommon in phytotoxins. The minor activity of cyclo (L-Pro-L-Tyr), compared to the inactivity of its diastereomer, suggested the importance of structural conformation in its bioactivity.

Although the exact chemistry and host range of maculosin had been established, at the set of this study we had no

information on the factor(s) in the susceptible plant that interact with maculosin and thus no understanding of its mode of action.

In general, studies on the mode of action of the host-specific toxins will play an important role in elucidating the mechanism of host selectivity, providing tools for probing the normal functions of plants. Thus, having the ability to synthesize radiolabelled maculosin, we were in an excellent position to carry out a comprehensive study on its cellular effects, its putative plant receptor(s) and its fate in its host plant. This thesis is a report of such studies.

CHAPTER II

CELLULAR EFFECTS OF MACULOSIN

Introduction

The general biochemical and molecular mechanisms underlying the mode of action of a toxin is of particular interest especially in those cases in which the toxin is specific to a limited host range of certain animals or plants. The hypothesis generally employed to understand this specificity is one of site specific recognition.

Thus the toxin is the ligand and its interaction with a specific receptor is a first site of interaction. This may be on the surface or within the cells such as cytosolic organelles and membranes. For instance, in mammalian cells, workers have shown the value and power of toxins as probes of normal cell function using bacterial toxins in the identification and purification of cellular receptors (69).

In parallel with mammalian toxins, there are also reports of the interaction of phytotoxins with receptors in plant cells (37,59,73). This came with the experimental demonstration of helminthosporoside binding protein in sugar cane in early 1970's (58). Also, fusicoccin, the major phytotoxic metabolite of Fusicoccum amygdali binds to the

plasma membrane of a large number of higher plants and it selectively activates the H^+-ATPase responsible for electrogenic proton extrusion (36). The binding of zinniol, a toxin produced by the Alternaria group, to the membranes and chloroplasts stimulates the entry of calcium into plant protoplasts in zinniol sensitive carrot cells (67). Furthermore, tentoxin, a host non-specific phytotoxin, inhibits photophosphorylation on binding to the chloroplast coupling factor CF1 (55).

In the case of maculosin, a host specific toxin of spotted knapweed, if the host plant possesses a specific receptor which has a normal role in the maintenance of cellular function, the binding of the phytotoxin may result in a site specific effect which may modify one or more metabolic processes in the cell. This process would eventually lead to the development of symptoms in knapweed plants affected by the toxin. Also, the interaction of receptor and maculosin may reveal an association of a receptor with the target organelles.

Therefore, the availability of the purified receptor would be crucial in the understanding of host specificity, receptor function, and in characterizing the molecular events occurring at the reactive site in the tissues of spotted knapweed. Thus, in order to assay for a receptor it is critical to have radiolabelled maculosin on hand.

In addition, affinity chromatography has been helpful in the isolation and characterization of receptors from animals and plants (64). After introduction of a ligand to a solid matrix by specific coupling methods, the application of this bioaffinity method is an effective tool in isolating and purifying not only specific receptor molecules but also cell organelles and even cells themselves. For example, the fusicoccin (FC) binding protein was purified by affinity chromatography using FC-linked adipic acid dihydrazide agarose (16), and the nicotine binding protein from rat brain was purified by conjugation of (R,S)-6-(2-hydroxyethyl) nicotine to epoxy-activated Sepharose (1).

This chapter, therefore, elaborates a procedure which allows immobilization of maculosin to a solid matrix. Subsequently, this matrix was used to isolate and characterize those receptors in spotted knapweed which may serve as the binding site for maculosin. Basically two types of affinity columns were constructed. First, epoxy activated Sepharose 6B to which a phenolic group of synthetic maculosin was attached. Secondly, the α-amino group of tyrosine was coupled to Sepharose 4B via CNBr activation to expose the phenoxy group free in the space.

Materials and Methods

Plant Material

Spotted knapweed (*Centaurea maculosa* L.) were individually grown in plastic pots containing a mixture of pasteurized sand, top soil, and peat (1:1:1) in the university plant growth facility with 12 hour light and dark period.

Synthesis of ^{14}C-maculosin

The synthesis of ^{14}C-maculosin followed a two step procedure that involved protection of one of the amino acids with BOC-ON, by the method of Itoh et al. (27). The BOC-protected amino acid was reacted with the methyl ester of the second amino acid, following the method of Nitecki et al., which is known to proceed without racemization (46).

L-tyrosine, 0.23 mmole, mixed with L-[U-^{14}C] tyrosine (75 μCi, specific activity 250 μCi/μmole) was reacted with 0.27 mmole of BOC-ON [2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile], which was stirred at 25°C in 7 ml aqueous acetone and 50 μl triethylamine. After two hours the mixture was evaporated and washed with ethyl acetate. The aqueous layer was acidified with 1 N Hcl, then extracted with methylene chloride.

The crude t-boc-L-tyrosine was dissolved in an equimolar mixture of methylene chloride and triethylamine. The L-proline methyl ester hydrochloride was added (0.23 mM),

followed by the addition of ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.23 mM). To prepare the L-proline methyl ester hydrochloride, a L-proline mixture (L-[U-^{14}C] proline 75 μCi, specific activity 250 μCi/μmole) was refluxed with a mixture of thionyl chloride and methanol. The mixture was left at -5°C overnight, then reduced in vacuo. The crude dipeptide was sequentially washed with water, citric acid (1 N), sodium bicarbonate (5 %), water, then evaporated to dryness.

The t-boc-L-tyrosine-L-proline methyl ester was dissolved in 8 ml of formic acid and stirred at room temperature for 2 hours. After removal of excess formic acid, the residue was dissolved in 10 ml of sec-butanol and toluene (vol/vol, 1:1) and refluxed for 2 hours. The structure and purity of the product, maculosin, was confirmed by HPLC, NMR and MS (56). The specific activity of maculosin was 0.11 μCi/μmole.

Localization and Translocation of Maculosin

Spotted knapweed plants, grown for 4 weeks in the green house, were removed from soil, and the roots washed with water to remove debris. Each intact plant was kept in a small vial containing water. ^{14}C-maculosin (0.02 μmole) was overlayered on the leaf with and without a small puncture with a 10 μl Hamilton syringe. For root treatment, ^{14}C-maculosin was adjusted with water to 4.5 mM in a small vial

and the root was dipped into that solution. Each treated plant was maintained in a moist chamber to prevent the evaporation of applied toxin from the leaf. After three days, the droplet of applied ^{14}C-maculosin was carefully removed from the leaf with a syringe and the root was extensively washed with distilled water. The plant was dried with several paper towels and mounted on a sheet of chromatography paper. The distribution of radioactivity in the plant was ascertained by exposure of the plant to autoradiographic film (Kodak Omat) at -70°C for 2 weeks.

To determine the localization of radioactivity, two to three month-old leaves of spotted knapweed were excised and placed in a moist chamber. Wounds were made in the center of each half of the leaf with a 10 μl Hamilton syringe. Each wound was overlayered with 5 μl of 2% ethanol containing 1900 dpm (8 nmole) of ^{14}C-maculosin (specific activity 0.11 μCi/μmole). The treated leaves were kept for three days at 28°C under 12 hours light and dark period. After incubation the leaves were cut into three equal portions; base, middle, and tip, and placed in separate glass tubes. The leaves were then digested with 0.5 ml perchloric acid: hydrogen peroxide (1:2, v/v) and heated at 100°C for 30 min. The digests were solubilized with 10 ml Aquasol and the radioactivity determined.

Fractionation of Maculosin Binding Material

The procedure to isolate the membranes and cytosolic fraction of spotted knapweed was carried out at 0 to 5°C. The leaves (50 g) of three to four month old plants were homogenized in 200 ml of 25 mM Tris (hydroxymethyl)-aminomethane-HCl buffer (pH 7.5) containing 1 mM dithiotreitol (DTT). The homogenate was filtered and strained through 6 layers of cheesecloth and centrifuged at 3000 x g for 20 min. The supernatant was further centrifuged at 100,000 x g for 1 hour. Both the pellet (membrane) and cytosolic fraction were kept at 4°C until used for the binding assay.

Detergent Solubilization of Membrane Components

The membrane fraction was diluted with the suspension buffer containing 25 mM Tris buffer (pH 7.5) to a protein concentration of 2.2 mg/ml. Each detergent was adjusted to give a final concentration of 0.5% and the membrane proteins extracted under mild conditions with a hand glass homogenizer and the solution kept at 4°C for 2 hours. The solution was centrifuged at 100,000 x g for 1 hour. The supernatant was saved and exhaustively dialyzed in the same buffer whereas the Triton X-100 and digitonin treated fractions were passed through Sephadex G-25 (30 x 1.5 cm) because of their inability to be dialyzed.

Characterization of the Radioactive Component Bound to the Protein

The radioactive fraction of the 10,000 x g supernatant in Sephadex G-25 size exclusion chromatography was pooled and reduced to dryness in vacuo at 35°C. The residue was dissolved in 20 ml of ethanol, further concentrated in vacuo and dissolved in 0.5 ml of ethanol. This was streaked on a normal phase silica gel plate (20 x 5 cm) and developed using a solvent (chloroform:methanol:acetic acid = 14:2:1, v/v/v). The relative R_f region of intact maculosin was scraped and the radioactivity determined.

Preparation of a Biospecific Adsorbent for Maculosin Receptors

Maculosin Affinity Chromatography. Maculosin-Sepharose was prepared by coupling maculosin to epoxy-activated Sepharose 6B (64). Epoxy-activated Sepharose 6B was prepared by reacting it with 1,4-bis-(2,3 epoxy-propyl)-butane giving an uncharged ether linkage between the spacer, gel, and free oxirane group (64). In order to carry out this reaction, Sepharose 6B was washed with distilled water, and the excess water removed by filtration through glass wool. The wet gel (10 ml) was resuspended in 20 ml of 1 M NaOH containing $NaBH_4$ (40 mg) and 10 ml of 1,4-butandiol-diglycidylether. The solution was rotated at 25°C for 10 hours and the reaction mixture was washed with distilled water.

The activated Sepharose was then reacted with 500 mg of

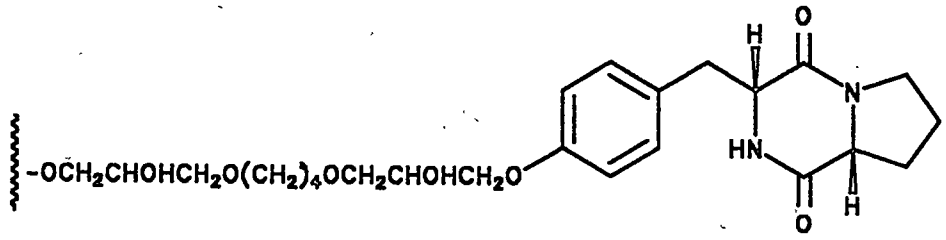
maculosin in NaOH (0.5 M) for 20 hours at 35°C with gentle shaking. The gel was then washed exhaustively with distilled water, followed with bicarbonate buffer (0.1 M, pH 8.0) and acetate buffer (0.1 M, pH 4.0). Then the residual active groups of gel were blocked with 1.0 M ethanolamine overnight. The gel was suspended and washed extensively with 500 ml of 10 mM Tris-HCl buffer pH 7.5 (Figure 2).

Tyrosine Affinity Chromatography. Tyrosine-Sepharose was synthesized by the method of Chan and Takahashi (8). Sepharose 4B was washed with distilled water and decanted. CNBr 1 g, freshly dissolved in 15 ml of distilled water, was added and the pH was adjusted to and maintained at 11.0 by adding 4 M NaOH with continuous stirring. Then 2 g of L-tyrosine was added as a fine powder and the pH was readjusted to 11.0 with 4 M NaOH. The mixture was stirred gently for 16 hours at 4°C. After the reaction was complete, the residual active groups of gel were blocked with 0.2 M glycine. Then the gel was washed with water and equilibrated with 10 mM Tris buffer pH 7.5 (Figure 2).

Preparation of Cytosolic Proteins

The leaves (50 g) of three to four month old plants were homogenized in 200 ml of 25 mM Tris (hydroxymethy)-aminomethane)-HCl buffer pH 7.5 containing 1 mM DTT. The homogenate was filtered through 4 layers of cheesecloth and

COUPLING OF MACULOSIN TO EPOXY-ACTIVATED SEPHAROSE 6B



COUPLING OF TYROSINE TO CNBR-ACTIVATED SEPHAROSE 4B

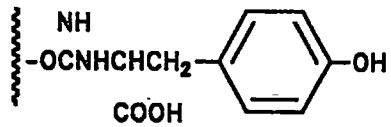


Figure 2. Construction of maculosin-affinity support gel.

centrifuged at 12,000 x g for 30 min and the supernatant was again centrifuged at 100,000 x g for 1 hour. The final supernatant was saved for further purification.

Purification of Cytosolic Maculosin Binding Protein

Solid ammonium sulfate was added slowly to the high-speed supernatant to a 20% concentration. The mixture was stirred for 1 hour and then centrifuged at 12,000 x g for 30 min. The resultant 20% ammonium sulfate supernatant was brought to a final 60% concentration by the addition of solid ammonium sulfate and then stirred for 1 hour. The mixture was again centrifuged at 12,000 x g for 30 min and the precipitate was saved. The precipitate was then resuspended in 10 ml of 10 mM Tris buffer pH 7.5 containing 0.5 mM DTT, and dialyzed against of 4 liters of the same buffer for 12 hours. The dialyzate was centrifuged at 12,000 x g for 30 min and supernatant was applied to a column of Sephadex G-200 (60 x 1.4 cm) pre-equilibrated with 10 mM Tris buffer pH 7.5. The column was eluted with 10 mM Tris buffer at a rate of 15 ml/hour. The eluate was collected in 1.5 ml fractions and monitored at 280 nm with a Beckman spectrophotometer. Fractions were collected at the void volume which contained protein and exhibited binding activity. This pooled void volume fraction was applied to the maculosin affinity column (10 x 1.0 cm) pre-equilibrated with 10 mM Tris buffer. After washing the column extensively with 10 mM Tris buffer, the

protein was eluted with a stepwise gradient (each 30 ml) of 0.0, 0.15, 0.3, 0.6 and 0.9 M NaCl in 10 mM Tris buffer. The eluted proteins were combined, concentrated appropriately with ultrafiltration (Amicon ultrafilter, molecular cutoff 10,000), and dialyzed in 10 mM Tris buffer pH 7.5. The tyrosine affinity column (10 x 1.0 cm) was eluted with a stepwise gradient of salt (NaCl up to 0.9 M) as described above, and a pH stepwise gradient using 30 ml each of 50 mM citrate buffer (pH 3, 4.5, 6) and 50 mM Tris buffer (pH 7, 9).

The purification scheme is presented in Figure 3.

Equilibrium Dialysis

Aliquots (0.5 ml) of the proteins were dialyzed in an Kontron equilibrium dialysis system (a Kontron-Diapak dialysis unit) against 0.5 ml of binding buffer containing a range of different concentrations of ^{14}C-maculosin (30). The dialysis membrane (Spectra/Por, Spectrum, Inc.) had a molecular weight exclusion limit of 8,000. Equilibrium was reached after 10 hours of incubation at 28°C by gentle turning of the dialysis cells. Radioactivity was determined on the contents of each half cell. The total amount of maculosin bound was equal to the difference in radioactivity between each pair of half cells.

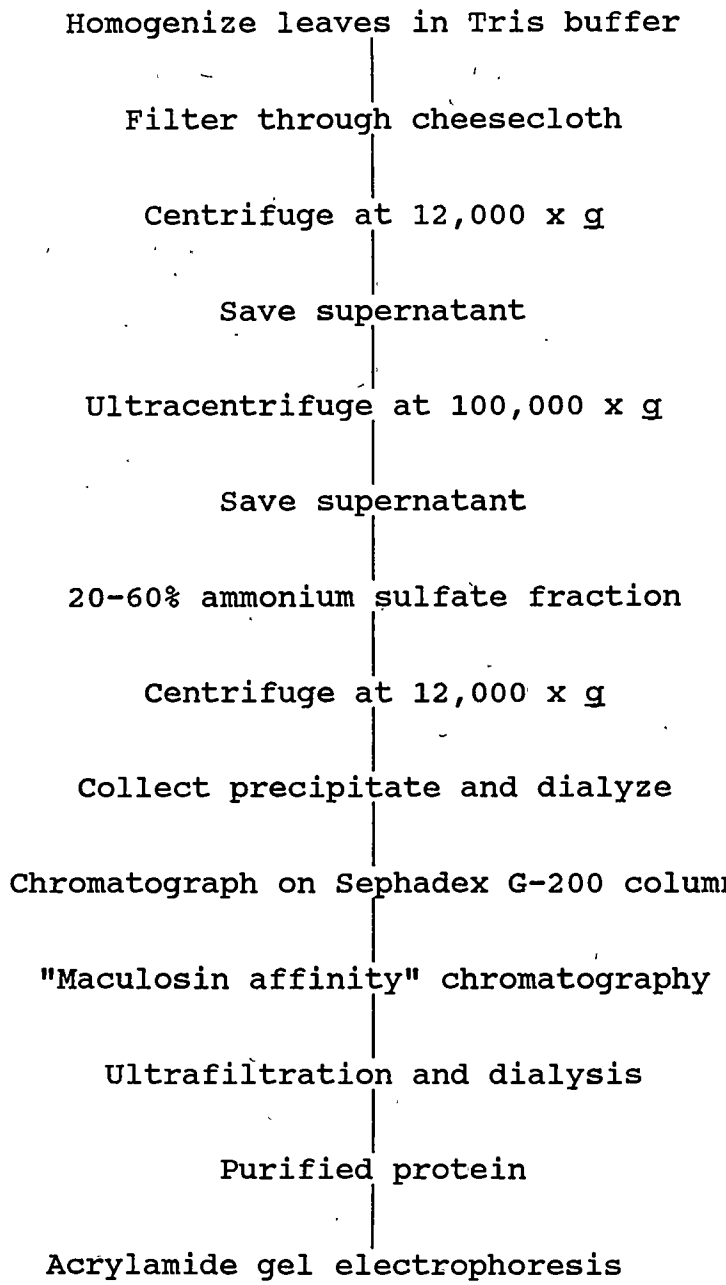


Figure 3. Purification scheme of cytosolic maculosin binding protein.

Protein Determination

Protein was determined by the method of Bradford (3), using Bio-Rad Protein Dye Reagent (Bio-Rad). Bovine serum albumin was used as the standard.

Estimation of Molecular Weight

The molecular weight of maculosin binding protein was determined using a Sepharose 6B column (60 x 1.4 cm) which was calibrated using the following protein standards: thyroglobulin (669,000), apoferritin (443,000), α-amylase (200,000) and bovine serum albumin (66,000).

Radioactivity Determination

All samples were mixed with 10 ml of Aquasol (New England Nuclear Corp.) before counting. Radioactivity measurements were made on a Packard liquid scintillation spectrometer model 2200CA and counts were corrected to disintegrations per minute (dpm).

Electrophoresis

Native Polyacrylamide Gel. Electrophoresis was performed on 7.5% gel according to Davis (15).

Discontinuous Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel. Electrophoresis was carried out in a 10% gel by the method of Laemmli (33) with modifications. The separating gel contained 10% acrylamide, 0.26% bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8, 0.033% ammonium

persulfate, 0.033% (V/V) N'N'N'N' tetramethyl-ethylenediamine (TEMED). The stacking gel contained 4.0% acrylamide, 0.1% bisacrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8, 0.008% ammonium persulfate, 0.005% TEMED. Samples were mixed at a 1:1 volume ratio with sample buffer containing 125 mM Tris-HCl, pH 6.8, 4.0% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue and 30% sucrose. Samples were heated for two minutes at 100°C, and then 50 μl aliquots of the protein preparation (35 μg) were applied to the gel. Electrode buffer containing 19 mM glycine, 25 mM Tris-HCl, pH 8.5 and 0.1% SDS was used in both electrode chambers. Electrophoresis was conducted at 150 volts until the bromophenol blue tracking dye reached the bottom of the gel.

Protein was stained with Coomassie Blue (CB) following the procedures of Howard and Traut (25). Gels were soaked 4-10 hours in solution of 50% methanol, 10% acetic acid and 0.25% CB. The gel was then destained with several rinses of a 50% methanol and 10% acetic acid solution.

Double Immunodiffusion

A 1.0% gel was prepared on a glass plate by the method of Ouchterlony (47). The agarose gel was prepared in 50 mM phosphate buffer, pH 7.4 containing 0.03% sodium azide. The plate was kept in a moist chamber at 25°C. The antiserum (20 μl) was pipetted in a center well and cytosolic maculosin binding protein (20 μl, 43 μg) was pipetted into an adjacent

well. The control well contained phosphate buffer. Evidence for a cross reaction was investigated at 36 hours. Antiserum (polyclonal) against ribulose-1,5-biphosphate carboxylase from tobacco plants was provided by Dr. Jensen, University of Arizona.

Measurement of Ribulose-1,5-Biphosphate Carboxylase (RuBPCase) Activity

RuBPCase was prepared and assayed by the method of Chu and Bassham (9) with some modification.

Enzyme Isolation. RuBPCase was extracted from the leaves (10 g) ground in a Sorvall Omnimixer for 5 x 30 seconds with 40 ml of 0.1 M Tris buffer, pH 7.5 containing 1 mM ethylenediaminotetraacetic acid (EDTA), and 10 mM DTT. The homogenate was squeezed through cheesecloth and centrifuged at 12,000 x g for 30 min. The soluble supernatant was fractionated with ammonium sulfate. The protein which precipitated between 35 and 55% saturation with ammonium sulfate was collected by centrifugation and dissolved in a minimum volume of 0.1 M Tris buffer, pH 7.5. The protein solution was dialyzed overnight against 0.1 M Tris buffer, pH 7.5. The dialyzate was centrifuged at 12,000 x g to remove any insoluble material. The solution was layered on a Sephadex G-200 column (60 x 1.4 cm) which had been previously equilibrated with 0.1 M Tris buffer, pH 7.5 containing 5 mM $MgCl_2$. The protein was eluted from the

column with the same buffer and collected from the column in the void volume.

Enzyme Assay. In a final volume of 0.25 ml, the reaction mixture contained the following components at concentrations noted: Tris-HCl, 175 mM; $MgCl_2$, 10 mM; pH 7.8; Ribulose biphosphate (RuBP) 0.08 mM. The concentration of $NaH^{14}CO_3$, maculosin, and enzymes are shown in Table 7 in the results. The assay was incubated at 30°C in a stoppered glass vial and was started by the addition of RuBP. After 10 min incubation in the water bath at 30°C, 0.1 ml of 6 N HCl was added to stop the reaction. The solution was dried at 90°C and the residue was dissolved in 0.2 ml of water. Scintillation fluid (5 ml, Aquasol) was added and the radioactivity determined.

Results

Localization of Maculosin

When ^{14}C-maculosin was applied "in vivo" onto the leaves without a wound, most of the radioactivity was localized at the site of application (Figure 4), whereas some radioactivity was mobilized to the tip of the leaf in which maculosin had been applied to a wound. However, when the root of the plant was dipped into the maculosin solution, most of the radioactivity was acropetally translocated (Figure 4).

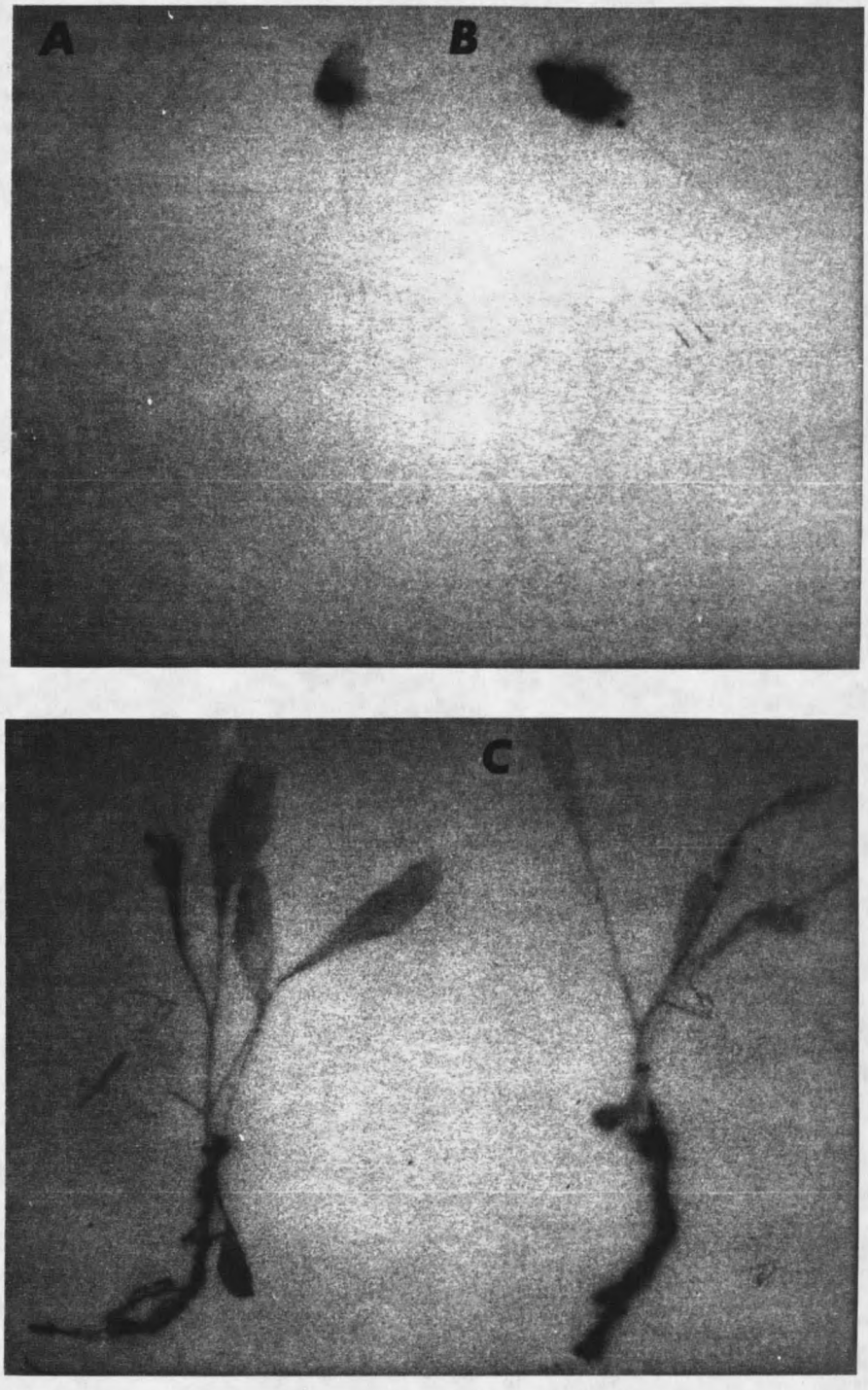


Figure 4. Distribution and translocation of ^{14}C-maculosin on spotted knapweed. Toxin was treated on a leaf without (A) and with (B) a wound, and the root (C).

On detached leaves, ^{14}C-maculosin was applied onto a wound made in the center of each half of the detached leaf (ca. 5.5 cm in length). The leaves were incubated in a moist chamber under intermittent light (6-8 $\mu E/m^2/s$, 12 hr/day) for three days. After incubation, the leaves were segmented into three parts, tip, middle, and base. In the middle part (which was inoculated), there appeared a black necrotic lesion while both the tip and base remained symptomless. The leaf segments were digested and the radioactivity determined. As seen in Table 2, most of radioactivity remained in the center of leaf, in an area approximately 1.8 cm^2. There was very little radioactivity found outside the necrotic area. These data indicated that maculosin applied on the leaves was not mobilized to induce symptom expression.

Table 2. Translocation of maculosin in detached leaves of spotted knapweed. ^{14}C-maculosin (0.02 μmole) was applied to each leaf on a puncture wound. Intermittent light was equivalent to 6-8 $\mu E/m^2/sec$ for 12 hr/day. Values represent the average of three replicates. Plus/minus values represent one S.D.

Leaf segment	DPM
Tip	172 ± 133
Middle	2569 ± 194
Base	46 ± 9

Nature of Toxin Binding Activity

A number of experiments were conducted in order to ascertain the nature of toxin binding activity. The leaf homogenate was centrifuged at 10,000 x g for 30 min and the supernatant was saved. Then 1.5 ml of supernatant (equivalent to 3 mg of protein) was pre-treated as indicated in Table 3. After treatment the solution was mixed with 0.05 μmole of ^{14}C-maculosin (specific activity, 0.11 μCi/μmole) for 1 hour at room temperature. The mixture was passed through a Sephadex G-25 column and the radioactivity was determined.

Table 3. Effect of various treatments on ^{14}C-maculosin binding of leaf extracts. The leaf homogenate was prepared by centrifugation at 10,000 x g for 30 min. The supernatant was incubated with maculosin (0.05 μmole) in 25 mM Tris-HCl buffer (pH 7.5) as previous indicated in the text.

Treatment	Toxin Bound (dpm)	Relative binding activity % of control
None (Control)	2099	100
Heat (85°C / 3 min)	117	6
DNase (1 mg / 30 min / 30°C)	1864	89
RNase (1 mg / 30 min / 30°C)	2011	96
Protease (1 mg / 60 min / 30°C)	839	40

The maculosin binding substance was heat-labile and pretreatment with protease (Bacillus subtilis) largely abolished its binding activity. Incubation with deoxyribonuclease (from beef pancreas), and ribonuclease (from bovine pancreas) had little effect on binding activity (Table 3).

Fractionation of Binding Site

A toxin receptor site may be one or it may be a series of proteins that are widely distributed in the cell such as on the cell surface and/or in the cells (17,23). They are often found in the plasma membrane, cytosol, mitochondria, chloroplasts, nuclear envelope and in the other organelles.

Therefore, the leaf homogenate was mainly divided into two fractions; the membrane and the cytosol. Three to four month old leaves were homogenized in 25 mM Tris buffer (pH 7.5). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 3,000 x g for 20 min. The supernatant was further centrifuged at 100,000 x g for 1 hour. The pellet was taken up in the same buffer and the supernatant was saved for the determination of binding activity according to the standard equilibrium dialysis method. The results in Table 4 show that most of toxin binding activity was present in the cytosolic fraction and a relatively small amount of binding activity was detected in the native membrane fraction. However, this result did not rule out the possibility of the presence of some binding

Table 4. Distribution of ^{14}C-maculosin binding activity from leaves of spotted knapweed. The leaf homogenate was centrifuged at 3,000 x g for 20 min and 100,000 x g for 1 hr. The binding assay was performed via an equilibrium dialysis (30).

100,000 x g Fraction	Specific binding activity (dpm/mg protein)
Supernatant	698
Precipitant	212

activity being present in the membrane since they were not solubilized by detergents. In fact, more binding activity may be present in the membrane fraction since most of integral proteins are embedded in the lipid bilayer impeding toxin access.

Binding Activity of Various Detergent Treated Membrane Protein

There are many detergents available for use in solubilizing membrane proteins. Furthermore, there are several types of strategies which can be used to purify integral membrane proteins (24). Thus, it is important to choose the appropriate detergent to release the membrane proteins in the cells of spotted knapweed.

The various detergent solubilized proteins possessed binding activities (Table 5). The membrane fraction, when treated with various detergents, showed increased binding

Table 5. ^{14}C-maculosin binding activity of various detergent treated membrane fractions. The crude membrane was prepared by a sequential centrifugation at 3,000 x g for 20 min and 100,000 x g for 1 hour. The pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.5) and made up to 0.5% concentration of each detergent. After mild extraction and incubation for 2 hr at 4°C, the membrane fraction treated with each detergent was centrifuged again at 100,000 x g for 1 hour. The supernatant was collected and detergent removed. The binding activity was measured by an equilibrium dialysis method.

Detergent	Specific binding (dpm/mg protein)
Digitonin	58
Trichloroacetate (sodium salt)	538
Triton X-100	440
Deoxycholate	525
Cholate	476

activity as compared to the non-treated membrane fraction (Table 4) except in the case of the digitonin treatment.

Purification of Cytosolic Maculosin Binding Protein

Because the major binding activity was located in the cytosolic fraction, purification of the activity was started from the 12,000 x g supernatant of homogenized leaves in 25 mM Tris buffer (pH 7.5). The purification scheme is illustrated in Figure 3. After centrifugation, the binding activity was found in the 20 - 60% ammonium sulfate fraction. The elution pattern of the maculosin-bound protein from Sephadex G-200 showed one peak of binding activity

(Figure 5). Elution of the affinity column (maculosin bound onto epoxy-activated Sepharose 6B) reproducibly yielded proteins obtained from the Sephadex G-200 step. After extensively washing the column with elution buffer, the maculosin-binding protein was eluted at 0.3 M NaCl solution, which was sufficient for resolving the maculosin-binding proteins (Figure 6), whereas the other trace proteins could not be detected up to 0.9 NaCl solution.

The protein eluted from Sephadex G-200 column was applied to the tyrosine affinity column. After washing extensively with the same buffer, the column was eluted with a stepwise salt gradient up to 0.9 M NaCl, and a pH gradient (pH 3 - 9). But with these systems, it was found that the tyrosine affinity matrix did not retain any proteins eluted by Sephadex G-200 chromatography. The purification procedure resulted in a 16-fold purification of the maculosin-binding activity on a protein basis (per mg) with an overall yield of 3%. The specific maculosin-binding activity of the final preparation was 43.2 nmole maculosin bound/mg of protein (Table 6).

Characterization of Binding Protein

The purified binding protein from the affinity column was subjected to disc gel electrophoresis using a 10% acrylamide gel in the presence of 0.1% sodium dodecyl sulfate. After electrophoresis, the gel was stained with

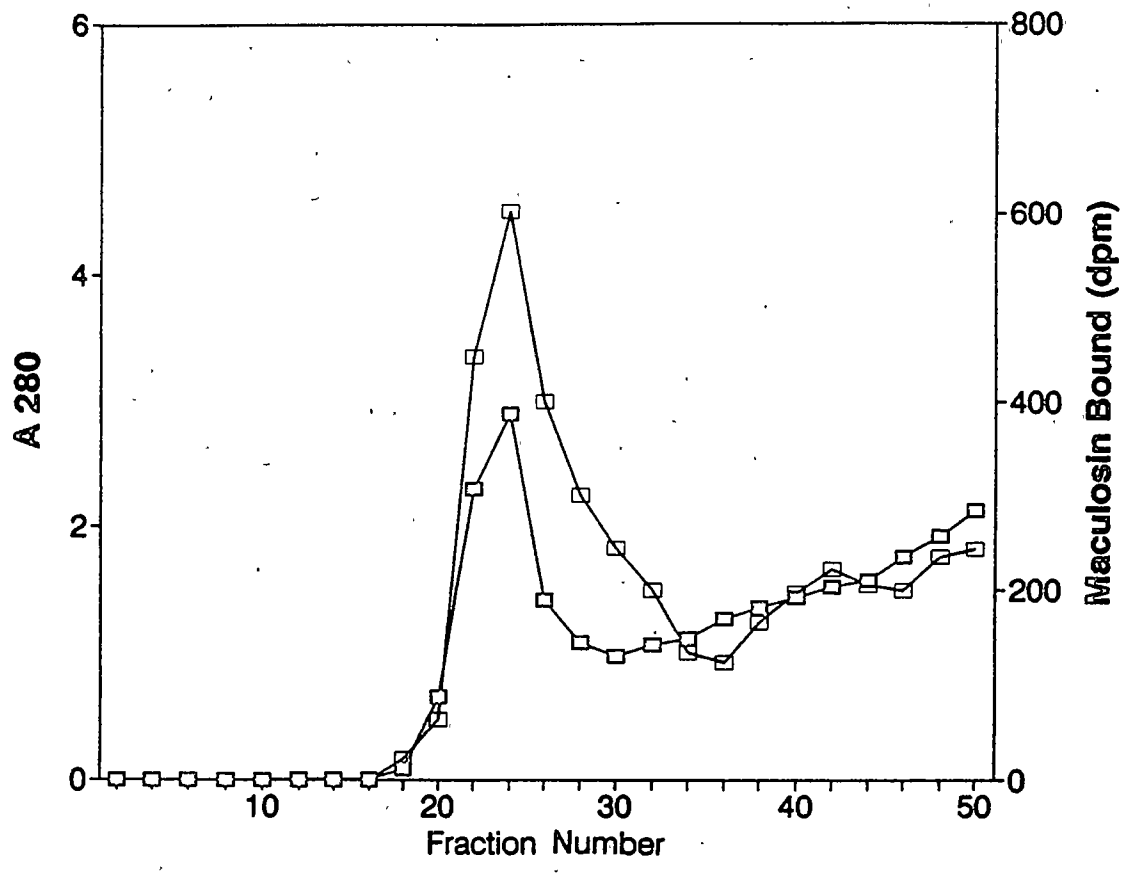


Figure 5. Elution profile of 20-60% ammonium sulfate fraction of cytosolic maculosin binding protein on Sephadex G-200 column. The protein was detected at 280 nm (□) and the radioactivity was counted as dpm (□).

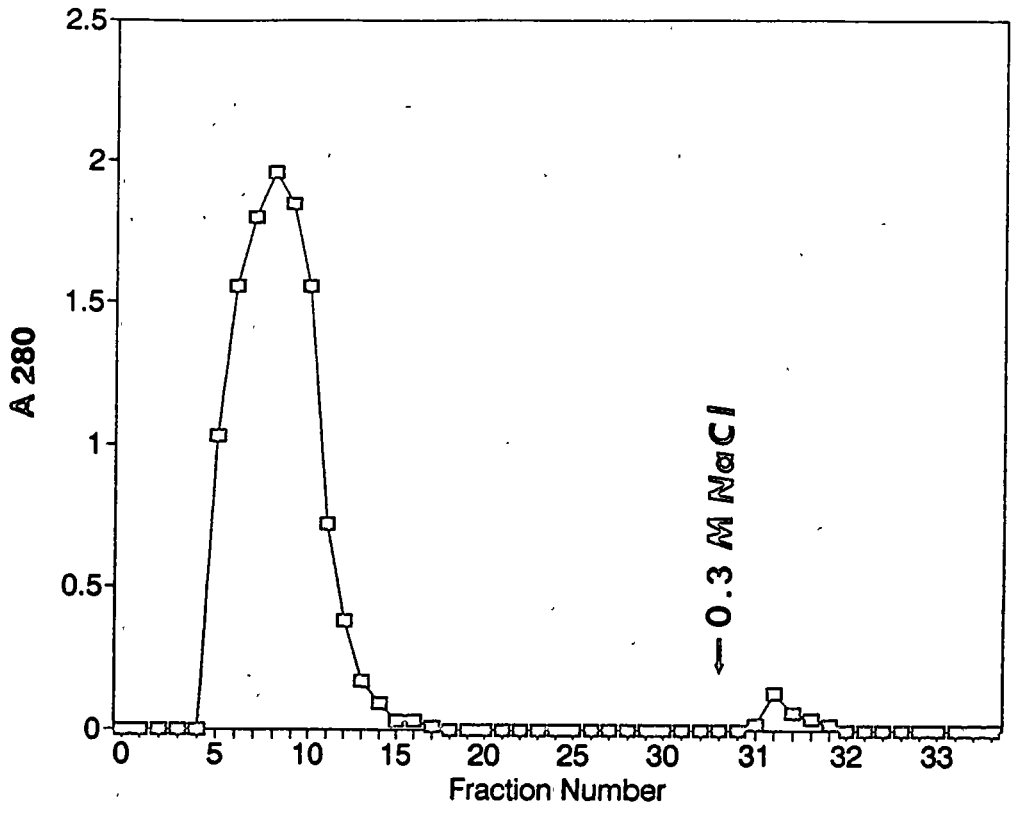


Figure 6. Elution profile of binding protein on maculosin-affinity chromatography. The protein eluted from Sephadex G-200 was applied to the affinity column and eluted with 0.3 M NaCl in 10 mM Tris-buffer (pH 7.5). The protein was detected at 280 nm.

Table 6. Purification of cytosolic maculosin binding protein from the leaves of spotted knapweed

Step	Total activity (nmole bound)	Total protein (mg)	Specific activity (nmole/mg protein)	Yield (%)
12,000 x g supernatant	518	192	2.7	100
100,000 x g supernatant	469	161	2.9	91
20-60% ammonium sulfate	415	67	6.2	80
Sephadex G-200	380	21	19.0	70
Maculosin-affinity column	18	0.41	43.2	3

Figure 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified maculosin-binding protein according to Laemmli (33).

Coomassie blue and it showed three closely migrating bands around 60 kdaltons and a weak band near 14 kdaltons (Figures 7, 8).

The molecular weight of the maculosin-binding proteins was estimated by chromatography on a Sepharose 6B column with several proteins of known molecular weight for reference purposes. The binding protein eluted as a aggregated molecular mass of 604 kdaltons (Figure 9). However, non-dissociating 7.5% polyacrylamide gel electrophoresis of the maculosin-binding protein yielded three bands which were of high molecular weight as estimated by their migration position at the top of the gel, and are presumably in a highly aggregated state (Figure 10). Accordingly, there were several proteins adhering to the affinity gel. By its molecular weight, abundance, and the electrophoretic mobility of its subunits, one of the binding proteins was surmised to be ribulose-1,5-biphosphate carboxylase (RuBPCase) which is the most abundant enzyme located in chloroplasts (21,41). Therefore, the affinity purified protein was applied to a double immunodiffusion test employing antibody to RuBPCase from tobacco. Cross reactivity between the purified protein and antiserum of RuBPCase was observed (Figure 11), but when the sample was pre-incubated with 1% SDS in 50 mM Phosphate buffer pH 7.4 at 4°C for 1 hour, the cross reactivity was more intense than the non-treated sample, indicating that this protein was an aggregate.

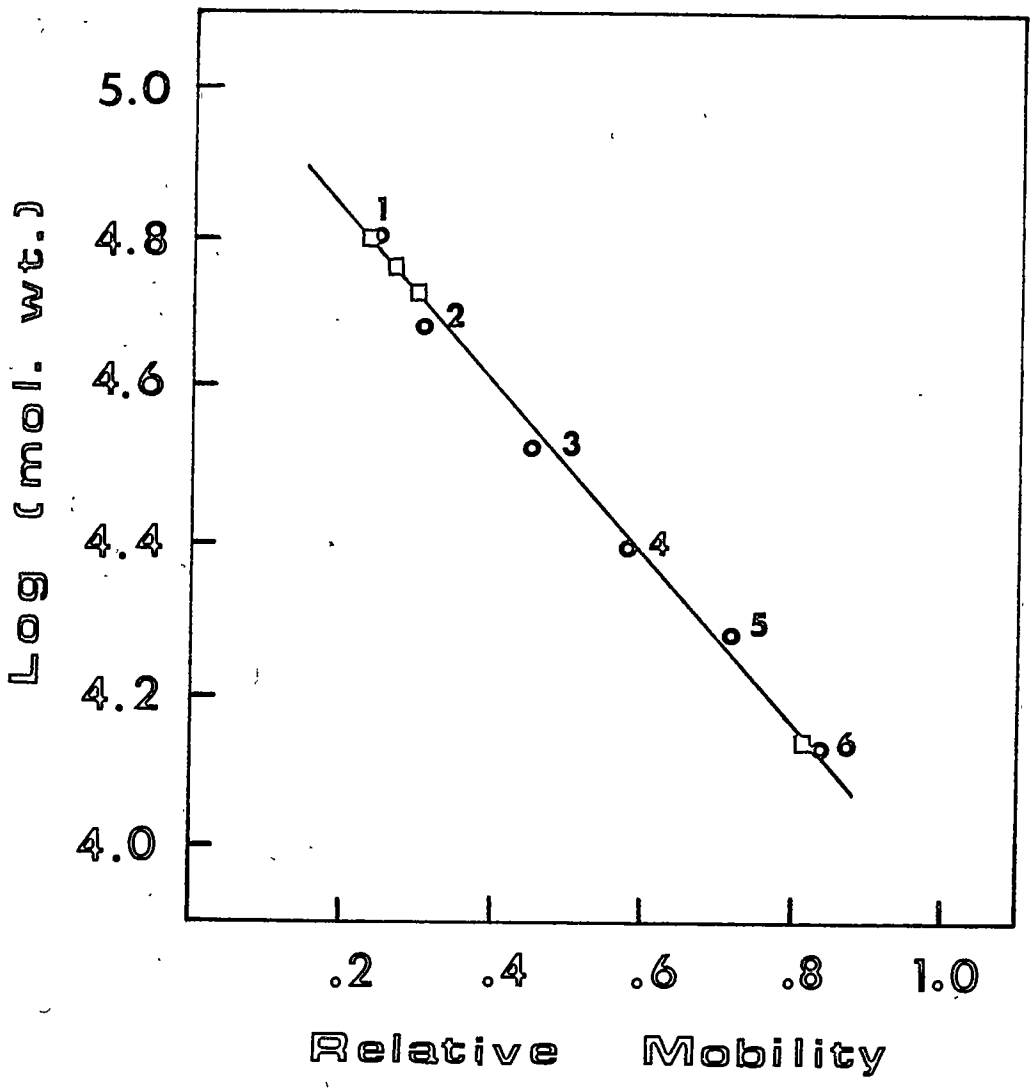


Figure 8. A plot of molecular weights of the purified maculosin binding protein and its subunits (□) by sodium dodecyl sulfate gel electrophoresis. Standard proteins used were: 1. albumin, bovine (66,000), 2. albumin, egg (45,000), 3. pepsin (34,700), 4. trypsinogen (24,000), 5. beta-lactoglobulin (18,400), 6. lysozyme (14,300).

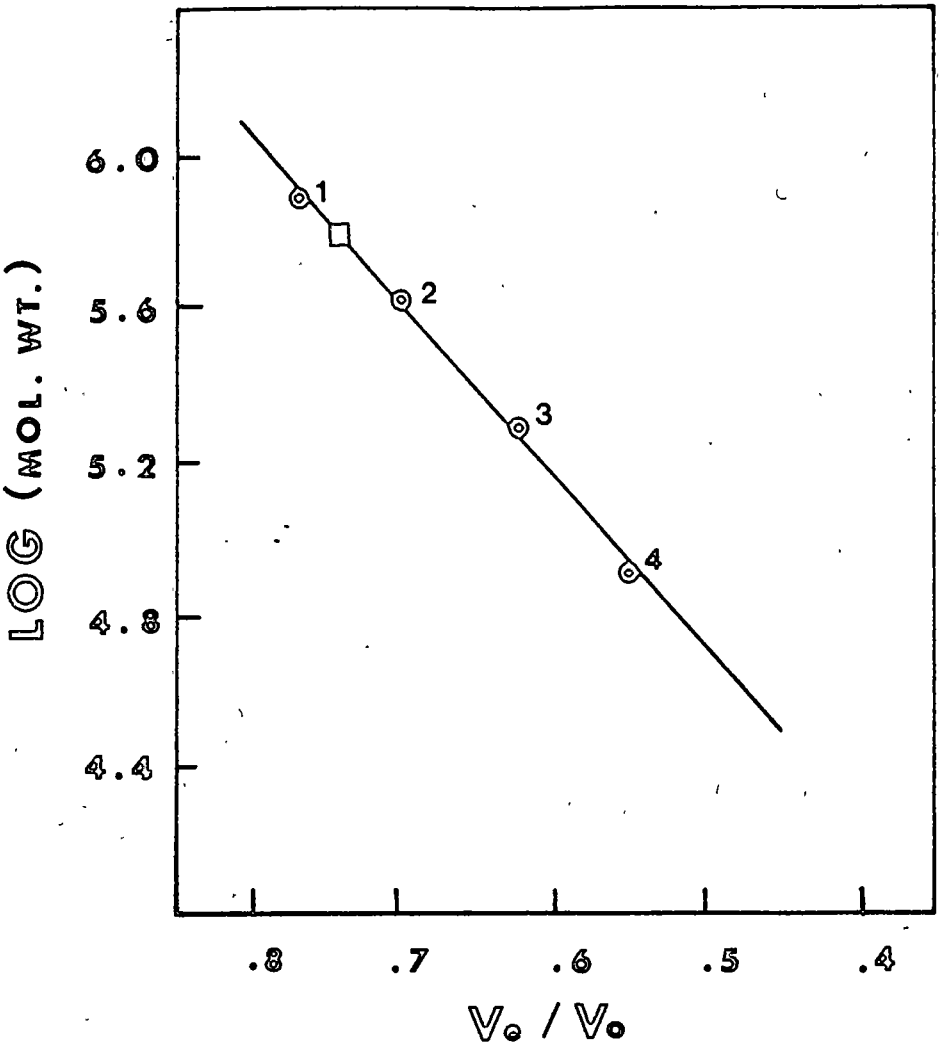


Figure 9. Molecular weight estimation of the purified maculosin-binding protein (□) by chromatography on Sepharose 6B. Standard proteins used were: 1. thyroglobulin (669,000), 2. apoferritin (443,000), 3. alpha-amylase (200,000), 4. bovine serum albumin (66,000).