

**INDUCTION, PURIFICATION AND CHARACTERIZATION  
OF CHITINASES IN CUCUMBER (*Cucumis sativus* L.) AND  
CARROT (*Daucus carota* L.)**

by

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Induction, purification and characterization of chitinases

in cucumber (Cucumis sativus) and carrot (Daucus carota)

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## Abstract

The profiles of chitinases (EC 3.2.1.14) in cucumber (*Cucumis sativus* L.) and carrot (*Daucus carota* L.) were studied. Chitinase isoform banding patterns were visualized using a polyacrylamide gel electrophoresis/overlay gel technique. In cucumber cotyledons, chitinase isoforms were induced by treatment with chitosan, salicylic acid, wounding, fungal pathogen or non-pathogen inoculation. The induction of chitinase isoforms was not specific to the inducing agents. Chitinase isoform patterns in true leaves and roots were similar to those in cotyledons, and no tissue-specific isoforms were observed. The uniform induction pattern in cotyledons, true leaves and roots suggested that the induction was systemic. Similar isoform patterns were observed in four cucumber cultivars and were not correlated with their resistance to powdery mildew (*Sphaerotheca fuliginea*). A time-course study showed that the overall increase in activity after induction could be attributed to the enhanced expression of four constitutive isoforms and the induction of three additional isoforms. The expression of the different isoforms was classified into three groups (low constitutive, enhanced constitutive and newly induced). The pIs for these isoforms ranged from pH 4 to 6, and the molecular weight was estimated to be around 25,600. Chitinase-containing extracts from cucumber tissues were shown to have antifungal activity *in vitro* against *Trichoderma* and *Thielaviopsis*.

In mature carrot roots (cv. Eagle), multiple chitinase isoforms (8-10) were produced. Some of these isoforms were shown to have differential cross-reactivity to antisera raised against chitinases from classes I, II, and III. The molecular weight of carrot chitinases was estimated to range from 20,000 to

40,000. One major chitinase, which did not react with any of the antisera tested, was purified and found to be an acidic protein with pI at 4.3 and a molecular weight of 39,500. The optimum pH for enzymatic activity was around 5 and the optimum temperature was 25 °C. The enzyme was stable at pH values below 8 and temperatures below 60 °C. The protein did not have a chitin-binding domain, but showed similarity to tobacco class I chitinase in its amino acid composition. The N-terminal amino acid sequence did not resemble any of the described classes of chitinases. The chitinase did not possess lysozyme activity and showed antifungal activity when tested against *Trichoderma* sp.

## Dedication

To my parents

谨献给家乡的父老乡亲

## **Acknowledgments**

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A special tribute to my wife, Lixing Liu, for her enormous help in every sphere of my life and for her comprehension and continuous encouragement.

## Abbreviations

<b>BA</b>	benzylaminopurine
<b>CAPS</b>	3-(cyclohexylamino)-1-propanesulfonic acid
<b>CM</b>	carboxymethyl
<b>Da</b>	dalton
<b>DEAE</b>	diethyl amino ethyl
<b>2,4-D</b>	2,4-dichlorophenoxyacetic acid
<b>GlcNAc</b>	N-acetyl-D-glucosamine
<b>HPLC</b>	high performance liquid chromatography
<b>IEF</b>	isoelectric focusing
<b>M<sub>r</sub></b>	molecular mass
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PVDF</b>	polyvinylidene difluoride
<b>R<sub>f</sub></b>	relative mobility
<b>SAR</b>	systemic acquired resistance
<b>SDS</b>	sodium dodecyl sulfate



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**photographed. ....101**

# Chapter I

## Introduction: An Overview of Plant Chitinases

### *1. Nomenclature*

Chitinases are enzymes that catalyze the hydrolysis of chitin, a linear homopolymer of  $\beta$ -1,4-linked N-acetyl-D-glucosamine (GlcNAc) (Fig. 1.1). Chitinases can be divided into two groups: exochitinases (EC 3.2.1.30) and endochitinases (EC 3.2.1.14) according to the compounds which are released after hydrolytic reactions (McCreath and Gooday 1992; Robbins et al. 1988). Exochitinases act on the non-reducing end of the chitin chain and release only GlcNAc monomers. Different names, such as  $\beta$ -N-acetylglucosaminidases, chitobiases, are also used in the literature to describe exochitinases (Flach et al. 1992; Tronsmo and Harman 1993). Endochitinases randomly hydrolyze internal  $\beta$ -1,4-linkages of GlcNAc and split the chitin polymer, releasing oligosaccharides of GlcNAc (Boller et al. 1983; Molano et al. 1979).

### *2. Plant chitinases - General characteristics*

In higher plants, chitinase was discovered first in bean seeds (Powning and Irzykiewicz 1965). When Ables et al. (1970) first proposed that chitinases may have a role in plant defense against fungal pathogens, it attracted little attention. The studies on chitinases conducted in the late

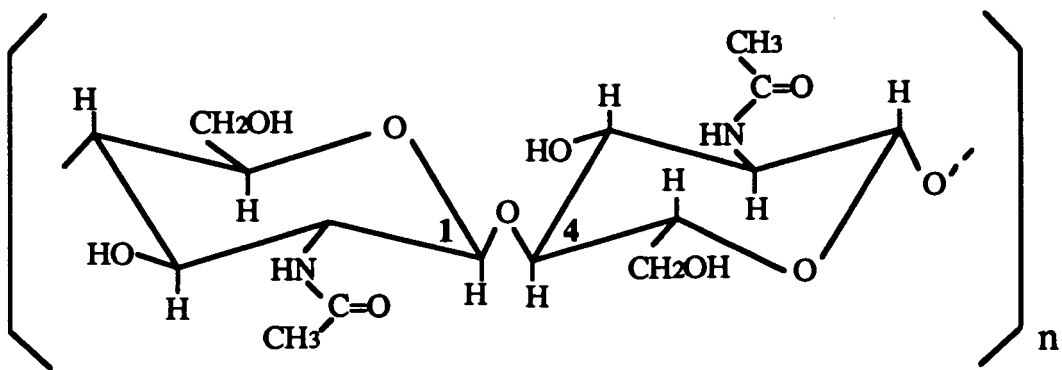


Fig. 1.1.  $\beta$ -1,4-linked N-acetylglucosamine.

1970's and early 1980's have provided evidence for the association between chitinase production and host infection, and has since reinforced the postulated role of chitinases in plant defense. Many previously unidentified pathogenesis-related (PR) proteins were later found to be chitinases (Legrand et al. 1987).

Plant chitinases in general have a low molecular mass (from 20 to 40 kDa) and may be either acidic or basic proteins. Like many other PR proteins (Bowles 1990), plant chitinases are acid extractable, resistant to proteolytic degradation, and relatively thermostable (Bol et al. 1990; Garcia Breijo et al. 1990; Linthorst 1991). The proteins are normally produced as monomers, although a dimer has also been reported (Ary et al. 1989). The optimal pH for chitinolytic activity varies among plant chitinases according to their source, but is usually acidic (from pH 3.5 to 6.0).

Most reported plant chitinases are endochitinases (Boller 1988; Punja and Zhang 1993), although exochitinases (Kirsch et al. 1993; McLeod and Poole 1994; Roby and Esquerré-Tugayé 1987) and endochitinases with exochitinase activity (Kragh et al. 1993; Melchers et al. 1994; Nielsen et al. 1993) have also been reported from plants. Many purified plant chitinases also have lysozyme (EC 3.2.1.17) activity and are capable of breaking down  $\beta$ -1,4-linkages between N-acetylmuramic acid and GlcNAc residues found in peptidoglycans of bacterial cell walls (Boller 1988; Majeau et al. 1990; Roberts and Selitrennikoff 1988; Trudel et al. 1989).

Chitinases have been reported from a wide range of higher plant species, including both monocotyledonous and dicotyledonous species (Punja and Zhang 1993). Tobacco, bean, tomato, *Arabidopsis*, and barley are among

the most studied plant species (for review papers, see Collinge et al. 1993; Flach et al. 1992; Graham and Sticklen 1994; Pospeshny 1993; Punja and Zhang 1993).

### ***3. Detection of chitinases***

Chitin occurs as a structural and defensive material in animals, insects, and fungi, and is relatively intractable (Roberts 1992). There are a limited number of solvent systems available to make a chitin preparation, such as acid solvents, organic solvents, and a few neutral salts; however, none of them are suitable for biological analysis. Bioassays for chitinase activity either employ a shaking device to keep the chitin powder suspended in the reaction mixture or utilize a soluble substrate, such as regenerated chitin (Molano et al. 1977), colloidal chitin (Lingappa and Lockwood 1962), or glycol chitin (Koga and Kramer 1983).

Many methods have been developed to detect chitinolytic activity (Wood and Kellogg 1988). These methods can be grouped into the following categories according to the manner in which the chitinase activity is measured.

#### **3.1. Viscometric assay**

Viscometric assays measure the turgidity changes during the enzymatic reaction of chitinase on chitin (Ohtakara 1988). These assays use a soluble substrate (glycol chitin) and are very sensitive to endochitinases. However, the assay procedure is somewhat cumbersome and too time-consuming to

determine the chitinase activity of large numbers of samples. As a result, this method is not widely used.

### **3.2. Radiochemical assay**

The radiochemical assay is based on the release of soluble oligosaccharides from tritium-labeled chitin after the hydrolytic reaction, which can be monitored radiochemically (Molano et al. 1977). This method is very sensitive, rapid, simple to carry out, and suitable for both exochitinases and endochitinases. However, it requires a radioactively-labeled substrate and specific equipment. It is the method recommended for routine work if tritiated chitin and scintillation equipment are available.

### **3.3. Colorimetric assay**

Monreal and Reese (1969) described a method to determine the reducing sugars released by chitinases with ferri-ferrocyanide reagent. Although this assay is frequently used in microbiological studies, it is unsuitable for crude plant enzyme preparations, which usually contain large amounts of reducing sugars. However, the simplicity of this assay makes it a good alternative to be used in plant chitinase purification (Tsukamoto et al. 1984; Yamagami and Funatsu 1993).

Ables et al. (1970) developed a method to measure chitinolytic activity by mixing the enzyme with a reaction mixture containing colloidal chitin. The hydrolytic product was detected with a color reaction with p-dimethylaminobenzaldehyde (Reissig et al. 1955). However, this assay was later found to be suitable only for exochitinase activity because only the monosaccharides were accounted for in the color reaction (Boller et al.



1983). Since plant chitinases are generally endochitinases and the principal products are oligomers, Ables' method then had to be modified and a second enzyme, snail gut enzyme (cytohellicase) (Cabib and Bowers 1971), was added to help convert the chitin oligosaccharides into GlcNAc monomers. The method employed by Boller et al. (1983) describes a non-linear relationship between the amount of enzyme and the amount of reaction product produced. Therefore, a series of controls, such as enzyme blank, substrate blank, reagent blank, internal standards, serial dilution, and duplications have to be included in the assay to ensure the accuracy of the test. This method is equivalent in sensitivity to the radiochemical assay and is applicable to crude plant extracts as well as to other sources of chitinases. It can also detect both exochitinase and endochitinase activity. However, it was reported that the color reaction (Reissig et al. 1955) also recognized all soluble N-acetyl-D-glucosamine oligomers (Domard and Vasseur 1991). Thus, the estimation of exochitinase activity using this method should be viewed with caution.

### **3.4. Electrophoresis assay**

Chitinase activity can be detected after native or denaturing polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing (IEF) (McBride et al. 1993; Pan et al. 1991; Tronsmo and Harman 1993; Trudel and Asselin 1989). A gel containing the substrate (glycol chitin) is overlaid onto a native PAGE gel or an IEF gel and the bands with chitinolytic activity are allowed to react with the substrate. In a denatured sodium dodecyl sulfate (SDS) PAGE system, however, proteins can be renatured and the enzymatic activity is recovered (Trudel and Asselin 1989). A

staining procedure is applied to visualize the bands on the overlay or SDS-PAGE gel. Fluorescent-labeled substrates can also be used to detect chitinase activity after gel electrophoresis (Kim et al. 1991; McBride et al. 1993). The use of the PhastSystem (Pharmacia, Sweden) has the advantage of saving time (McBride et al. 1993). These electrophoretic methods are very sensitive and are especially suitable for detecting chitinase isozymes. However, quantitative measurements of chitinase activity using these methods are not very accurate and are therefore not recommended.

### **3.5. Other methods**

Other methods have been recently developed for chitinase detection to suit various special needs. Micro-scale assays for detecting chitinolytic activity using 4-methylumbelliferyl or 4-ethylumbelliferyl derivatives and dye-labeled substrates are designed to be used in a microtiter plate reader so that many samples can be analyzed over a short time with ease (McCreath and Gooday 1992; O'Brien and Colwell 1987; Wirth and Wolf 1990, 1992). Recently, near-infrared reflectance spectroscopy (NIRS) technology has been used to determine chitinase activity in plant breeding studies, which offered the advantage of analysis of over 1000 samples within a week with minimum sample preparation (Roberts et al. 1994). Methods using substrates such as p-nitrophenyl-labeled derivatives (Nagal et al. 1990; Tronsmo and Harman 1993) and chitin-azure (Evrall et al. 1990) have been described to detect enzymatic activity. Colloidal gold labeling techniques, using lectin-gold complex and chitinase-gold complex, have been developed for ultrastructural studies (Benhamou et al. 1993; Benhamou and Chet 1993; Chamberland et al. 1985; Mauch et al. 1992;

Wubben et al. 1992). Chromatography techniques, such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), are used in the detection of soluble GlcNAc oligomers after chitin hydrolysis.

#### ***4. Classification of Chitinases***

##### **4.1. General**

The first classification scheme for plant chitinases was proposed by Shinshi et al. (1990) based on work on chitinases from tobacco (*Nicotiana tabacum*). These chitinases were grouped into three classes (I-III): vacuolar basic chitinases, extracellular acidic chitinases, and bifunctional lysozyme/chitinases, respectively. Since then, many chitinases have been purified from various plant species and the polypeptides have been sequenced, and some of the genes encoding the enzymes have been isolated and sequenced. Additional classes were therefore needed to categorize chitinases that did not fit into the original three classes (Collinge et al. 1993; Lerner and Raikhel 1992; Margis-Pinheiro et al. 1991; Melchers et al. 1994; Mikkelsen et al. 1992). A working group on chitinases organized by the International Society of Plant Molecular Biology has proposed a new scheme for the classification of chitinases (Meins et al. 1994) which now groups chitinases into six families (Table 1.1).

The new system uses a simple criterion for classification of chitinases based on amino acid sequence similarity of more than 50% (Meins et al. 1994) and is flexible enough to accommodate any possible new findings of

Table 1.1. System for classification of chitinases in higher plants.

Class	Criteria	Amino acid sequence and structure	Reference
I	more than 50% identical to tobacco class I chitinase	conserved cysteine-rich domain, catalytic domain	Meins et al. 1992 Shinshi et al. 1990
II	more than 50% identical to tobacco class II chitinase	amino acid sequence similar to class I, without cysteine-rich domain	Meins et al. 1992 Shinshi et al. 1990
III	more than 30% identical to tobacco class III chitinase	no sequence similarity to class I and II, significant similarity within the class	Lawton et al. 1992
IV	more than 50% identical to <i>Phaseolus vulgaris</i> PR4 chitinase	similar to class I, deletions in the cysteine-rich domain, a truncated C-terminal	Collinge et al. 1993 Mikkelsen et al. 1992
V	more than 50% identical to stinging nettle ( <i>Urtica dioica</i> ) lectin precursor	similar to class I, a duplicated N-terminal cysteine-rich lectin domain	Lerner and Raikhel 1992
VI	more than 50% identical to tobacco class V endochitinase	sequence similarity to bacterial exochitinases, no similarity to the class I-V proteins	Melchers et al. 1994

chitinase classes. Classes I-III correspond to chitinase classes I-III proposed earlier (Meins et al. 1992; Shinshi et al. 1990). Class IV corresponds to class IV chitinases proposed by Mikkelsen et al. (1992) and Collinge et al. (1993). Class VI corresponds to the new class proposed by Melchers et al. (1994).

#### **4.2. Protein structure**

The proposed new classification system has adopted the grouping scheme used earlier and integrated new findings on chitinases into an expanded system. The original class I chitinases were defined as basic vacuolar chitinases (Shinshi et al. 1990), but were later divided into two subclasses, Ia and Ib, due to the discovery of acidic extracellular class I chitinases which lacked the C-terminal extension (Collinge et al. 1993; Esaka et al. 1990; Stintzi, et al. 1993; Wu et al. 1994). Class Ia is for basic vacuolar chitinases and class Ib is for acidic extracellular chitinases. The structural features of a typical class I chitinase include a hydrophobic signal peptide at the N-terminal, a cysteine-rich domain, a proline- and glycine-rich linker region which may vary among chitinases, a catalytic domain, and a C-terminal extension (Ib) (Collinge et al. 1993; Beerhues and Kombrink 1994). The cysteine-rich domain, found in class I and class IV chitinases, is a chitin binding domain, but is not essential for chitinolytic activity (Payne et al. 1990), or antifungal activity (Iseli et al. 1993). The class I and class II chitinases are serologically related, while class I and IV chitinases are serologically distinguishable. Figure 1.2 illustrates the protein structures of six classes of chitinases adapted from Collinge et al. (1993).

### **4.3. Biological properties**

Table 1.2 lists all of the chitinases belonging to the different classes reported to date. There are few members in class V and class VI since these are newly defined classes (Meins et al. 1994). It is not uncommon for one plant species to produce chitinases which belong to more than one class. Tobacco plants, for example, contain four classes of chitinases (class I, II, III, and VI); bean chitinases may belong to three classes (I, III, and IV). Chitinases appear to be part of a multienzymatic chitinolytic pathway in many plants (Danhash et al. 1993; Legrand et al. 1987; Ride and Barber 1990; Rousseau-Limouzin and Fritig 1991).

Although the amino acid sequence information is a very important parameter to classify chitinases, other criteria, especially those of biological significance, such as antifungal activity and substrate specificity, should also be considered. In tobacco, chitinases A and B share 87% homology in their amino acid sequence, and yet they are significantly different in their antifungal activity *in vitro* and in substrate specificity (Huynh et al. 1992). Class I chitinases are the most active against fungi, while class II chitinases have almost no antifungal activity (Sela-Burlage et al. 1993). In tobacco, class I chitinase was very active against *Fusarium solani*, while class II chitinase showed no inhibitory activity (Sela-Buurlage et al. 1993). In addition, genes encoding acidic and basic isoforms of chitinases may be differentially regulated in plants. In healthy tobacco plants, mRNAs encoding basic chitinases were found to be expressed at higher levels, while mRNAs encoding acidic chitinases were present at low levels (Memelink et al. 1990). Other characteristics, such as the mode of action of chitinases

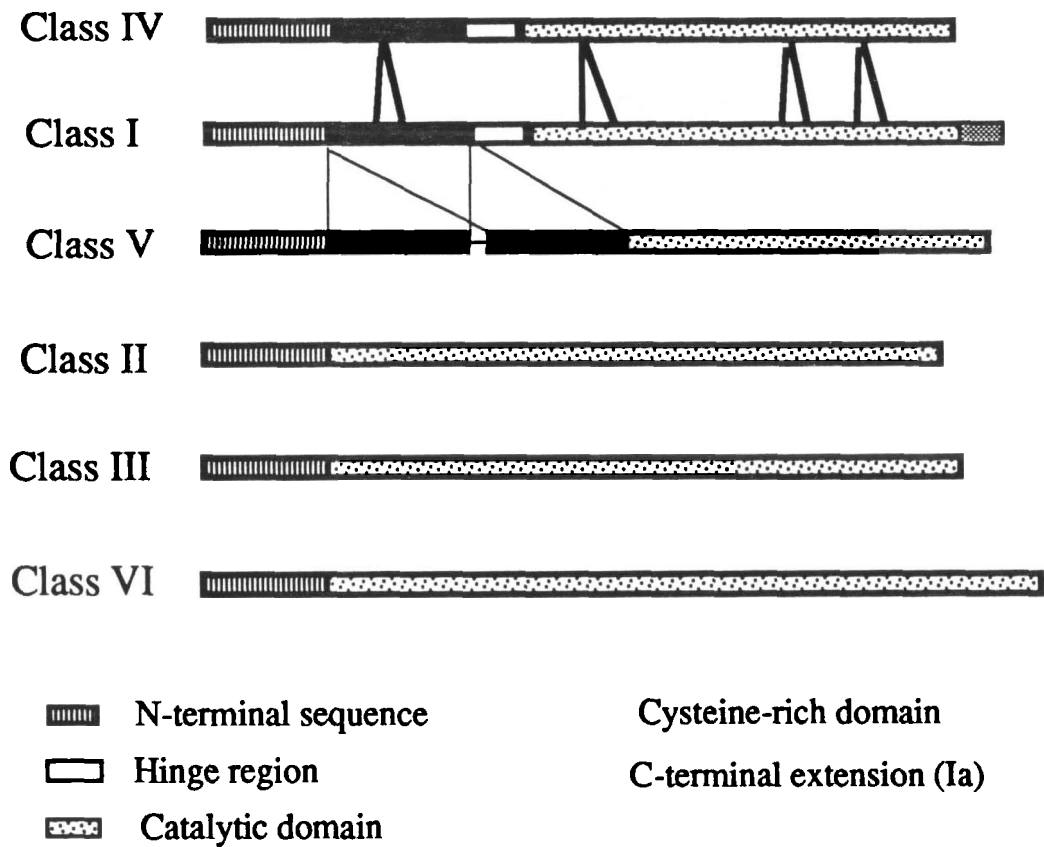


Fig. 1.2. The protein structure (schematic) of different classes of chitinases (adapted from Collinge et al. 1993)

Table 1.2. Chitinases in plant species grouped according to classes.

Class	Plant species	Reference
<b>Class I</b>		
	<i>Arabidopsis thaliana</i>	Samac et al. 1990 Verburg and Huynh 1991
	Barley ( <i>Hordeum vulgare</i> )	Jacobsen et al. 1990; Kragh et al. 1991 Swegle et al. 1989
	Bean ( <i>Phaseolus vulgaris</i> )	Brogliè et al. 1986; Hedrick et al. 1988; Lucas et al. 1985
	Chickpea ( <i>Cicer arietinum</i> )	Vogelsang and Barz 1993
	Chinese elm ( <i>Ulmus parvifolia</i> )	Graham and Sticklen 1994
	Dutch elm ( <i>Ulmus americana</i> )	Hajela et al. 1992
	Garlic ( <i>Allium sativum</i> )	Van Damme et al. 1993
	Job's tears ( <i>Croix lachrymosa-lobi</i> )	Ary et al. 1991
	Maize ( <i>Zea mays</i> )	Huynk et al. 1992; Wu et al. 1994
	Pea ( <i>Pisum sativum</i> )	Vad et al. 1991, 1993
	Peanut ( <i>Arachis hypogaea</i> )	Hergert et al. 1990
	Poplar ( <i>Populus hybrid</i> )	Parsons et al. 1989; Davis et al. 1991; Clarke et al. 1994
	Potato ( <i>Solanum tuberosum</i> )	Gaynor 1988, 1989 Laflamme and Roxby 1989
	Rice ( <i>Oryza sativa</i> )	Huang et al. 1991; Zhu and Lamb 1991 Nishizawa and Hibi 1991 Nishizawa et al. 1993
	Rye ( <i>Secale cereale</i> )	Yamagami and Funatsu 1993, 1994
	Tobacco ( <i>Nicotiana tabacum</i> )	Fukuda et al. 1991; Neale et al. 1990 Hooft van Huidsdijnen et al. 1987 Ponstein et al. 1994; Shinshi et al. 1987 Van Buuren et al. 1992
	Tomato ( <i>Lycopersicon esculentum</i> )	Danhash et al. 1993
	Yam ( <i>Dioscorea japonica</i> )	Araki et al. 1992a, 1992b

– Continued –



Class	Plant species	Reference
<b>Class II</b>		
	<i>Arabidopsis thaliana</i>	Samac et al. 1990
	Barley ( <i>Hordeum vulgare</i> )	Leah et al. 1987, 1991
	Chestnut ( <i>Castanea sativa</i> )	Collada et al. 1992
	Chestnut ( <i>Castanea crenata</i> )	Collada et al. 1993
	Orange ( <i>Citrus sinensis</i> )	Osswald et al. 1994
	Petunia ( <i>Petunia hybrida</i> )	Linthorst et al. 1990
	Potato ( <i>Solanum tuberosum</i> )	Pierpoint et al. 1990
	Pumpkin ( <i>Cucurbita</i> spp.)	Esaka et al. 1990
	Rye ( <i>Secale cereale</i> )	Yamagami and Funatsu 1993
	Tobacco ( <i>Nicotiana tabacum</i> )	Linthorst et al. 1990; Payne et al. 1990 Hooft van Huidsduijnen et al. 1987

### Class III

	<i>Arabidopsis thaliana</i>	Samac et al. 1990
	Azuki bean ( <i>Vigna angularis</i> )	Ishige et al. 1991, 1993
	Barley ( <i>Hordeum vulgare</i> )	Kragh et al. 1993
	Bean ( <i>Phaseolus vulgaris</i> )	Margis-Pinheiro et al. 1993
	Chickpea ( <i>Cicer arietinum</i> )	Vogelsang and Barz 1993, 1993
	Cucumber ( <i>Cucumis sativus</i> )	Métraux et al. 1989; Lawton et al. 1994
	Rubber tree ( <i>Hevea brasiliensis</i> )	Jekel et al. 1991
	Papaya ( <i>Carica papaya</i> )	Howard and Glazer 1969
	American ivy ( <i>Parthenocissus quinquefolia</i> )	Bernasconi et al. 1987
	Orange ( <i>Citrus sinensis</i> )	Osswald et al. 1994
	Blackberry ( <i>Rubus hispidus</i> )	Bernasconi et al. 1986
	Sugar beet ( <i>Beta vulgaris</i> )	Fleming et al. 1991; Nielsen et al. 1993
	Tobacco ( <i>Nicotiana tabacum</i> )	Lawton et al. 1992, 1994
	Yam ( <i>Dioscorea japonica</i> )	Nomura et al. 1993

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<b>Class</b>	<b>Plant species</b>	<b>Reference</b>
<b>Class IV</b>		
	<i>Arabidopsis thaliana</i>	
	Bean ( <i>Phaseolus vulgaris</i> )	Margis-Pinheiro et al. 1991, 1994
	Carrot ( <i>Daucus carota</i> )	Kragh et al, 1994
	Maize ( <i>Zea mays</i> )	Huynk et al. 1992; Wu et al. 1994
	Rape ( <i>Brassica napus</i> )	Rasmussen et al. 1992
	Sugar beet ( <i>Beta vulgaris</i> )	Mikkelsen et al. 1992
<b>Class V</b>		
	Stinging nettle ( <i>Urtica dioica</i> )	Lerner and Raikhel 1992
<b>Class VI</b>		
	Tobacco ( <i>Nicotiana tabacum</i> )	Melchers et al. 1994

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(some release elicitors, others degrade chitin in the cell walls - with major antifungal activity), should also be taken into consideration for classification. Some chitinases, even though they belong to the same class, may respond differently to pathogen infection. Genes encoding tobacco class I chitinases, CHN14, CHN14', CHN48, and CHN50, although regulated in the same way by hormones such as auxin and cytokinin, responded differently to pathogen infection; transcripts encoded by CHN48 and CHN50 were induced by *Cercospora nicotianae*, while transcripts of CHN14 and CHN14' were not detected in leaves infected with *C. nicotianae* (Van Buuren et al. 1992).

### ***5. Induction of chitinases in plants***

Chitinases may be produced constitutively at low or undetectable levels in plants, and are induced significantly in response to a number of abiotic and biotic stresses, such as mechanical wounding or insect/nematode feeding, ethylene, chitosan, salicylic acid, fungal cell wall fragments, and viral, bacterial, and fungal inoculation (Table 1.3). Generally, the induction of chitinases does not seem to be specific to the inducing agents (Meins and Ahl 1989; Zhang and Punja 1994), although some isoforms may be associated with certain biotic factors (Cordero et al. 1994; Herget et al. 1990; Koga et al. 1992; Furosaki et al. 1990). For example, in cultured carrot cells, fungal culture induced more chitinase isoforms than fungus-free culture filtrate (Kurosaki et al. 1990).

Table 1.3. Abiotic and biotic factors reported to induce chitinases in plants.

Inducing agents	Plant species	References
<b>Abiotic</b>		
<u>chitosan</u>	celery	Krebs and Grumet 1993
	cucumber	El Ghaouth et al. 1994; Zhang and Punja 1994
	rice	Masuta et al. 1991; Notsu et al. 1994
<u>ethylene</u>	azuki bean	Ishige et al. 1991, 1993
	bean	Gomez et al. 1989; Ishige et al. 1991, 1993
	tobacco	Keefe et al. 1990; Broglie et al, 1989
<u>ozone</u>	tobacco	Ernst et al. 1992; Schraudner et al. 1992
<u>wounding</u>	bean	Hedrick et al. 1988
	cucumber	Zhang and Punja 1994
	poplar trees	Parsons et al. 1989; Bradshaw et al. 1991
	pumpkin	Esaka et al. 1993
<u>polysaccharides</u>	clover	Staehelin et al. 1994b
	Indian mulberry	Doernenburg and Knorr 1994
	rice	Park and Kim 1993; Um et al. 1994
	tobacco	Broekaert et al. 1988
<u>salicylic acid</u>	carrot	Schneider-Müller et al. 1994
	onion	Williams et al. 1993
	potato	Pierpoint et al. 1990
	sunflower	Lung et al. 1993; Siefert et al. 1994
	barley	Jacobsen et al. 1992
<u>salt solutions</u>	cucumber	Cherif et al. 1994; Raz and Fluhr 1992
	rape	Jacobsen et al. 1992
	soybean	Ham et al. 1991
	tomato	Cohen et al. 1994
	bean	Margis-Pinheiro et al. 1993
<u>UV light</u>	bean	Margis-Pinheiro et al. 1993
	tobacco	Brederode et al. 1991

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Inducing agents	Plant species	References
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### Biotic

<u>bacteria</u>	bean	Voisey and Slusarenko 1989
	rutabaga	Conrads-Strauch et al. 1990
	tobacco	Meins and Ahl 1989
<u>feeding</u>	nematode	tall fescue Bowles et al. 1991; Roberts et al. 1992
	insect	nightshade Bronner et al. 1991
<u>fungal cell wall fragments</u>	carrot	Kurosaki et al. 1987, 1988, 1990
	American ivy	Flach et al. 1993
<u>fungi</u>	pea	Kendra et al. 1989
	rice	Ren and West 1992
	bean	Daugrois et al. 1990
	cucumber	Zhang and Punja 1994
	melon	Roby and Esquerré-Tugayé 1987
	pea	Dumas-Gaudot 1994; Rasumussen et al. 1992
	peanut	Sathiya Bama and Balasubramanian 1991
	potato	Schröder et al. 1992
	rice	Du and Wang 1992
	spruce	Sharma et al. 1993
	sugar beet	Rousseau-Limouzin and Fritig 1991
	tomato	Wubben et al. 1994
	tobacco	Meins and Ahl 1989; Van Buuren et al. 1992
	tomato	Joosten and de Wit 1989
<u>viroid</u>	tomato	Garcia Breijjo et al. 1990
		Bol et al. 1990
<u>virus</u>	lima bean	Sehgal 1992
	tobacco	Hoof et al. 1987; Volegi-Lange et al. 1988 Trudel et al. 1989; Lawton et al. 1992

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## **6. Localization of chitinases in plants**

### **6.1. Tissues**

Chitinases can be found in different tissues of many higher plant species and they can be present at different levels in various tissues (Gomez Lim et al. 1987; Sweigle et al. 1992). Table 1.4 lists examples of tissues from which chitinases have been isolated (1990-1994). Different chitinase isozymes may be associated with certain plant tissues or organs in some plants (El Ghaouth et al. 1991; Esaka et al. 1993; Zhu and Lamb 1991), while some are developmentally regulated (Leah et al. 1991; Lotan et al. 1989; Neale et al. 1990; Zhu et al. 1993).

### **6.2. Subcellular targeting**

Chitinases are either retained in the central vacuole of the cells (Boller and Vögeli 1984; Mackenbrock et al. 1992; Mauch and Staehelin 1989) or are secreted extracellularly (Boller and Métraux 1988). The vacuolar targeting information is reported to reside in a short C-terminal propeptide found in class I chitinases, which is removed during or after transport to the plant vacuoles (Bednarek and Raikhel 1991; Melchers et al. 1993; Neuhaus et al. 1991, 1994; Rasmussen et al. 1992; Sticher et al. 1993). Chitinases in oat (Fink et al. 1988) and tomato (Joosten and De Wit 1989) are reported to be located in the apoplastic compartment. It is generally believed that basic isoforms are intracellular whereas the acidic isoforms are secreted into the extracellular space (Dore et al. 1991; Keefe et al. 1990; Nasser et al. 1990). The localization of chitinases might be

Table 1.4. A selected list of plant tissues in which chitinases have been reported (1990-1994).

Plant tissue	Plant species	Reference
embryos	barley	Swegle et al. 1992
	cucumber	Majeau et al. 1990
	tobacco	Broekaert et al. 1988
seeds	barley	Leah et al. 1991; Swegle et al. 1992
	corn	Neucere et al. 1991
	cucumber	Majeau et al. 1990
	maize	Huynh et al. 1992; Cordero et al. 1994
	rye	Yamagami and Funatsu 1993
	sorghum	Darnetty et al. 1993
	soybean	Wadsworth and Zikakis 1984
	wheat	Darnetty et al. 1993
tubers	potato	Gozia et al. 1993
	yam	Araki et al. 1992a; Nomura et al. 1993
roots	celery	Krebs and Grumet 1991, 1993
	Chinese cabbage	Ludwig et al. 1994
	cucumber	Cherif et al. 1994
	leek	Dumas-Gaudot et al. 1992
	onion	Dumas-Gaudot et al. 1992; Kim et al. 1992
	spruce	Sauter and Hager 1989; Asiegbu et al. 1993
	tobacco	Tahiri-Alaoui et al. 1990
	cotyledons	chestnut
cucumber		Zhang and Punja 1994
rape		Rasmussen et al. 1992

– *Continued* –

<b>Plant tissue</b>	<b>Plant species</b>	<b>Reference</b>
leaves	barley	Jacobsen et al. 1990; Kragh et al.1990, 1993
	bean	Mauch et al. 1992
	cucumber	Métraux et al. 1990
	garlic	Van Damme et al. 1993
	tobacco	Boller et al. 1983
	wheat	Ride and Barber 1990
stems	bitter melon	Pei et al. 1993
	tomato	Pegg and Young 1982
	tobacco	Meins and Ahl 1989
flowers	petunia	Leung 1992; Lotan et al. 1990
	strawberry	El Ghaouth et al. 1991
	tobacco	Neale et al. 1990
fruits	kiwifruit	McLeod and Poole 1994
	tomato	Mehta et al. 1991
Pods	chickpea	Nehra et al. 1994



important with regard to their role in plant defense against pathogenic invasion (see section 8.5).

## **7. Substrate specificity of plant chitinases**

### **7.1. Chitinolytic activity**

Plant chitinases appear to be relatively specific regarding the polysaccharides that are utilized as substrate (Krebs and Grumet 1991). The enzymes hydrolyze chitin and the soluble chitin derivative, glycol chitin (Boller et al. 1993), and partially hydrolyze colloidal chitin (Boller 1988). Wheat germ chitinase is shown to degrade chitin and glycol chitin but not cellulose ( $\beta$ -1,4-glucan),  $\beta$ -1,3-glucan or  $\beta$ -1,6-glucan (Molano et al. 1979). The only natural substrate for bean chitinase other than chitin is bacterial cell wall peptidoglycan (Boller et al. 1983). Chitinases from yam can hydrolyze glycol chitin, but not p-nitrophenyl derivatives or *Micrococcus lysodeikticus* cell walls (Tsukamoto et al. 1984). Chitin is the preferred substrate for many plant chitinases (Bernier et al. 1971; Tata et al. 1983).

### **7.2. Lysozyme activity**

Many purified plant chitinases are reported also to have lysozyme activity and can hydrolyze the bacterial cell wall peptidoglycan (Düring 1993; Jekel et al. 1991; Martin 1991; Uhm and Kim 1993). The enzymes are primarily class III chitinases with bifunctional enzymatic activity (chitinase/lysozyme), such as chitinases from cucumber (Métraux et al. 1989), bean (Boller et al. 1983), and *Hevea* (Martin 1991). Lysozymes are

also reported to act as endochitinases (Bernier et al. 1971; Bernasconi et al. 1985). However, these plant lysozymes act primarily as chitinases because they hydrolyze chitin much more rapidly than peptidoglycan (Boller 1985). Recently, an evolutionary connection between plant endochitinases and lysozymes from animals and phage was made after a search of the database of known three-dimensional protein structures (Holm and Sander 1994). Plant chitinases and lysozymes from animals and phage showed unambiguous similarities in overall topology of folding, overlapping substrate specificity and remarkable conservation of some sequence and architectural detail around the active site (Holm and Sander 1994).

### **7.3. Chitosanase and other activities**

Other enzymatic activities are also reported for some purified chitinases. Some citrus chitinases possess chitosanase (EC 3.2.1.99) activities (Osswald et al. 1993, 1994). However, it has been suggested that since chitosan is usually incompletely deacetylated, the apparent chitosanase activity of chitinases may be due to hydrolysis of stretches of GlcNAc units in chitosan (Boller 1988).

An insect  $\alpha$ -amylase inhibitor purified from seeds of Job's Tears (*Coix lachryma-jobi*) also has endochitinase activity (Ary et al. 1989), and the combination of functions may be relevant to protection of the grain from insect feeding and fungal infection (Ary et al. 1989).

More recently, a basic inducible protein containing chitinase and beta-1,3-glucanase (EC 3.2.1.39) activity concomitantly was purified from cell suspension culture media of rice after treatment with chitooligosaccharides (Um and Kim 1994). An acidic protein purified from rice leaves was also

found to have chitinase, beta-1,3-glucanase and lysozyme activities (Um and Kim 1994).

## ***8. Role of chitinases in plant defense***

### **8.1. Constitutive expression**

Chitinases are commonly produced in higher plants, but the substrate for these enzymes has never been reported to occur in higher plants (Molano et al. 1970; Boller et al. 1983). Apparently, chitinases are not produced for the plant's own metabolism, although a possible role during development, such as during carrot somatic embryo development, has been proposed (De Jong et al. 1992).

The constitutive activity of chitinases is normally low or undetectable in many plants (Somssich et al. 1986; Kombrink et al. 1988). In some plants, resistance to disease is often associated with high constitutive levels of chitinases (Gentile et al. 1993; Ahl Goy et al. 1992). A tobacco hybrid was found to be much more resistant than either parental species to viral, bacterial and fungal pathogens, and the enhanced resistance was attributed to the high levels of chitinases and other hydrolases in the hybrid (Ahl Goy et al. 1992). Increases in the levels of these enzymes in the parental species with preinduction to the levels comparable with those found in the hybrid was able to enhance resistance to subsequent infection by several fungal and bacterial pathogens (Ahl Goy et al. 1992).

The constitutive expression of chitinases may be a response to chronic levels of stress, a preemptive measure anticipating possible pathogen

infection, or a result of leakiness of the mechanism by which gene expression is repressed (Garcia-Garcia et al. 1994).

## **8.2. Induction of plant chitinases**

Higher plants synthesize a number of antimicrobial proteins in response to pathogen invasion and environmental stresses (Asselin 1993). Chitinases are found to be associated with plant-microbe interactions, and are a group of major pathogenesis-related (PR) proteins produced in plants (Awade et al. 1989; Bowles 1990; Kombrink et al. 1988; Nasser et al. 1988).

Chitinase activity is reported to increase after treatment of plants with fungal cell wall component, fungal filtrate, or fungal inoculation (Punja and Zhang 1994). Chitinase mRNA became detectable 1-1.5 h after treatment in bean leaves (Margis-Pinheiro et al. 1993), and the increase in activity was as high as up to 600-fold in cucumber leaves (Métraux and Boller 1986). The activation of chitinase transcription in bean is very rapid, with a 10-fold stimulation after 5 minutes and a 30-fold increase within 20 minutes (Hedrick et al. 1988). Tobacco plants infected with *Phytophthora parasitica* showed a 230-fold increase in chitinase activity within 7 days (Meins and Ahl 1989).

## **8.3. Role of chitinases in host-pathogen interactions**

Chitinases are induced to a higher level in some incompatible host-pathogen interactions than in compatible interactions (Boyd et al. 1994; Vogelsang and Barz 1990). In chickpea, resistant cell lines contained a 5-fold higher level of chitinase activity in comparison to the susceptible cell lines (Vogelsang and Barz 1990). Chitinases also accumulate earlier and

more rapidly in some incompatible interactions than in compatible interactions (Benhamou et al. 1990; Daugrois et al. 1990; Joosten and De Wit 1989; Mahe et al. 1993; Nielsen et al. 1994; Vöisey and Slusarenko 1989). In the interactions of French bean and *Pseudomonas syringae* pv. *phaseolicola*, the avirulent pathogen produced an incompatible reaction in which increases in chitinase activity in leaf extracts began 6-9 h after infection and rose to 19-fold over uninfected leaves by 48 h; however, chitinase activity did not increase until 24 h post-infection by the virulent race (Vöisey and Slusarenko 1989). In compatible interactions, chitinase activity was unchanged (Fink et al. 1990) or the increase in activity was delayed (Jakobek et al. 1993; Rasmussen et al. 1992; Voisey and Slusarenko 1989). *Puccinia* infections induced a rapid increase in chitinase activity in incompatible oat systems while no activity changes were observed in compatible interactions (Fink et al. 1990). The enzymatic activity in coffee leaves increased on the first day after inoculation in an incompatible combination, whereas in the compatible interaction, the increase was delayed until the 21st day and was at lower level (Maxemiuc et al. 1992).

The timing and levels of chitinase accumulation seem to be a factor in determining the type of host-pathogen interactions: incompatible (resistant) or compatible (susceptible). Chitinase activity in compatible interactions may eventually increase to levels comparable to, or even higher, than that of incompatible interactions. However, the time course of the induction in susceptible plants is usually delayed compared with that in resistant plants, and may not be as effective in inhibiting pathogen invasion since the initial infection by pathogens is already established. A differential induction was observed between resistant and susceptible cultivars of rape, where

chitinase transcript levels in resistant cultivars was 3-fold higher one day after inoculation compared with a susceptible cultivar; however, this difference diminished 8 days after inoculation (Rasmussen et al. 1992). The synthesis of two melon chitinases peaked at 5.5 days postinfection and then dropped to near control levels by 6.5 days (Roby and Esquerré-Tugayé 1987). A comparison of overall chitinase activity at later stages of induction between compatible and incompatible interactions may lead to a misleading conclusion as to the role of chitinase.

#### **8.4. Effect of chitinase localization**

The localization of chitinases may have functional significance and be attributed to a balanced plant defense system. Chitinases are found to accumulate in areas where host walls are in close contact with fungal hyphae (Benhamou et al. 1990). Acidic chitinases are located extracellularly, and they may be involved in the recognition process during pathogen infection upon making contact with the pathogen, recognize the cell wall component using chitinolytic or lysozyme activity, and release oligosaccharides as elicitors, and thus trigger the plant defense system (Graham and Sticklen 1994). The extracellular matrix is believed to be the site where signals originate to elicit defense responses in plants (Bowles 1990). In addition, acidic chitinase may also act as the first line of defense by interfering with the pathogen invading structure and therefore limiting its spread. Basic chitinases are generally vacuolar-localized in large quantities. When plant cells are sensitized or upon pathogen invasion, a large concentration of enzyme with strong antifungal activity may be released upon cell death and make contact with the invading structure. The

sudden release of enzymes can have a dramatic effect on the pathogen, and overwhelm the natural balance of cell wall construction in the growing tip, and cause a fungicidal effect, digest cell walls, lyse hyphal tip, inhibit spore germination, and kill the pathogen (Mauch and Stahelin 1989). The synergistic effect of basic chitinases with other hydrolytic enzymes such as  $\beta$ -1,3-glucanases would greatly increase their ability to successfully attack the fungal cell wall.

### **8.5. Elicitation of chitinases**

Fungal cell wall fragments and chitin oligosaccharides released by the action of plant chitinases on fungal cell walls can have an elicitor effect and trigger the defense system in plants to lead to other defense responses, such as the accumulation of phenolic compounds and lignin (Boller 1987; Flach et al. 1993; Fritig et al. 1989; Furosaki et al. 1988; Pearce and Ride 1982; Ride and Barber 1990; Roby et al. 1987; Ryan 1987).

The effect on a pathogen may depend on its ability to avoid the elicitation of chitinase and other antimicrobial proteins or to suppress the production of these proteins (Stahelin et al. 1994b; Yoshioka et al. 1992). A pea pathogen, *Mycosphaerella pinodes*, secretes both an elicitor and a suppressor in its pycnospore germination fluid. The elicitor induced the activation of endochitinase and  $\beta$ -1,3-glucanase. The suppressor, however, suppressed the activation in compatible host plants but not in nonhost plants (Yoshioka et al. 1992).

Chitinases are also involved in the specificity of the symbiotic microorganism-host plant interactions by differentially inactivating chitinase resistant and susceptible nodulation-inducing (Nod) factors

(Stahelin et al. 1994a). In the interactions of *Rhizobium*-plant symbiosis, plant chitinases were involved in controlling the biological activity of Nod factors by cleaving and inactivating susceptible ones (Stahelin et al. 1994a). Successful colonization by mycorrhizal fungi also depends upon suppression of chitinase expression and production (Lambais and Mehdy 1993). Colonization of soybean roots by mycorrhizal fungi did not induce chitinase activity in the central region of effective nodules (Stahelin et al. 1992). Chitinase levels fell to below that found in the control during mycorrhizal colonization of leek (*Allium porrum*) roots (Spanu et al. 1989).

#### **8.6. Antifungal activities**

Many fungi, more specifically, pathogenic fungi, have chitin as an important component of their cell walls (Ruiz-Herrera 1992; Sivan and Chet 1989; Van Pelt-Heerschap and Sietsma 1990). Chitinases isolated from plants are capable of digesting fungal cell walls, lysing growing tips, inhibiting mycelium growth, and have antifungal activity in vitro (Schlumbaum et al. 1986). Chitinase affects the extreme tip of hyphae, causing cell wall thinning, the swelling and a rupture of the plasma membrane, and arrests hyphal growth (Arlorio et al. 1992; Benhamou et al. 1993a, 1993b). These enzymes also cause rapid and extensive bursting of the hyphal tips (Shapira et al. 1989; Woloshuk et al. 1991), inhibit spore germination (Broekaert et al. 1988), and change the usual fungal infection structure (Toyoda et al. 1991). Basic chitinases are generally more antifungal, whereas some acidic chitinases have less or no inhibitory activity (Vogelsang and Barz 1993). Different plant chitinases may act



synergistically against fungi (Ponstein et al. 1994). Plant chitinases are more effective against fungi than enzymes such as microbial chitinases, egg white lysozyme, and papaya protease (Park et al. 1992; Roberts and Selitrennikoff 1988; Schlumbaum et al. 1986)

### **8.7. Synergism with $\beta$ -1,3-glucanases**

Plant chitinases often act synergistically with other proteins, especially  $\beta$ -1,3-glucanases (EC 3.2.1.39) which hydrolyze  $\beta$ -1,3-glucan, another major component of fungal cell walls (Mauch and Staehelin 1989). The combined action of chitinase and  $\beta$ -1,3-glucanase would synergistically enhance the substrate accessibility and thus accelerate cell wall disruption, leading to efficient cellular lysis. When chitinase and  $\beta$ -1,3-glucanase were combined, more GlcNAc-containing oligosaccharides were released than by chitinase alone (Young and Pegg 1982; Mauch et al. 1988). The combination of chitinase and  $\beta$ -1,3-glucanase isolated from pea pods strongly inhibited growth of all fungi tested with chitin-glucan cell walls, such as *Fusarium solani* and *Trichoderma viride*, even at concentrations at which each enzyme alone showed no inhibitory effect (Mauch et al. 1988). Besides the direct enzymatic activity on the cell walls, chitin and glucan fragments released by the enzymes are active elicitors of plant defense system and synergistic effects between different elicitors appear to be widespread (Dixon and Lamb 1990). However, the lytic activity of chitinase on the fungal cell walls is not enhanced by addition of  $\beta$ -1,3-glucanase, even though hydrolytic activity of  $\beta$ -1,3-glucanase on the cell walls is substantially increased by adding chitinase (Krebs and Grumet 1993).

## **8.8. Role in plant defense**

Although it is hard to imagine that a large amount of chitinase rapidly induced by pathogen infection has no function in plant defense, it has still not been shown conclusively that chitinase plays a decisive role in plant disease resistance. Because chitinases are induced simultaneously with other defense reactions and PR proteins in the same plant tissues, elucidation of the specific roles of these enzymes in resistance is difficult. The multienzyme system and different localization of plant chitinases further attribute to the complexity of the situation, since different isozymes displayed in the same tissue may have different roles (Wu et al. 1994).

In summary, the following facts lead to the proposal that chitinases play a major role in plant defense against fungal and other diseases:

- No substrate is found in plants.
- Chitin is a major component of fungal cell walls.
- Chitinase activity increases dramatically upon fungal attack.
- The activation of genes encoding chitinases is associated with fungal infection.
- Plant chitinases hydrolyze fungal cell walls and have antifungal activity *in vitro*.
- The oligosaccharide and chitin fragments released by plant chitinases from fungal cell walls have elicitor effects that can trigger the plant defense system.
- Fast and higher induction of chitinases upon pathogen infection result in disease resistance.
- Suppression of chitinase production causes susceptibility to diseases.

- Increased constitutive expression of chitinase through gene transformation can enhance disease resistance.

It is generally believed that hydrolytic enzymes, especially chitinases, are a part of defense responses in plants against diseases. However, the role chitinase is playing in plant defense system, the extent to which it is involved, and how it is working, are still not clear.

### ***9. General defense mechanisms***

Higher plants have evolved a broad array of defense mechanisms believed to be involved in disease resistance (Cramer et al. 1993; Kuc 1990). Chemical changes include releasing compounds such as signals and elicitors, synthesis of phytoalexins and proteinase inhibitors (Geoffroy et al. 1990), deposition of cell wall materials such as callose (Kauss 1992), accumulation of hydrolytic enzymes (Boller 1987) and other proteins (Fig. 1.3). The multifactorial mechanism of disease resistance appears to depend on the ability of the host to recognize the pathogen and induce these defense responses rapidly to kill the pathogen or limit its spread (Kuc 1990). Chitinases may be involved in the recognition of the pathogen, releasing the elicitors, triggering other defense responses, or affecting the pathogen directly due to its hydrolytic or lytic activity on the pathogen cell wall. Plant defense is a complicated system and chitinase is one component of the arsenal the plant may use to protect itself. In many cases, chitinases alone may not be enough to cope with a wide range of potential pathogens, and coordinated responses with other defense mechanisms are needed to successfully protect the host (Ward et al. 1991). Because of the

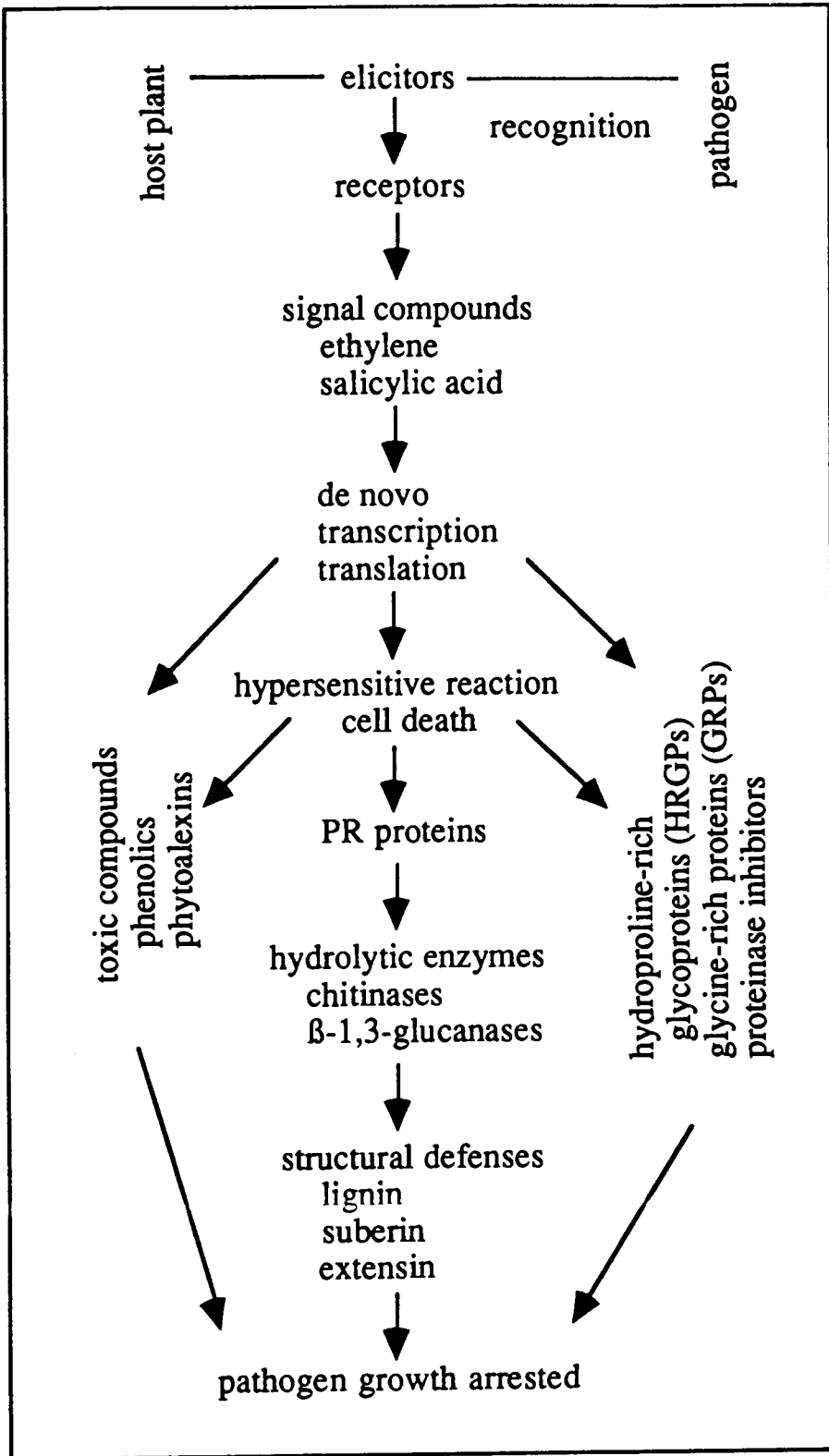


Fig. 1.3. Host defense responses that lead to disease resistance in plants (general scheme).

coordinated induction of all the defense responses, it has been difficult to determine whether they are functional defense responses, and if they are, how they specifically contribute to disease resistance (Lindgren et al. 1992). In order to study the role of chitinases in plant defense system, it is important to have a good control system so that the effect can be shown.

### **9.1. Chitinases and the hypersensitive reaction**

The role of chitinases in plant defense has been studied in a very special case of host-pathogen interactions, the hypersensitive reaction (Pierpoint 1983). The hypersensitive reaction describes the rapid cell death in the area of contact with pathogens which results in limited pathogen development and restricted spread of the pathogen (De Wit 1992).

Hypersensitive reactions are marked by a dramatic and rapid enhancement of enzyme levels in the area of infection (Meier et al. 1993). Much attention on chitinase has been focused on whether chitinase induction is a specific disease resistance response, as opposed to a non-specific stress response. Some believe that the induction of chitinases is part of the hypersensitive reaction (Vögeli-Lange et al. 1988; Meier et al. 1993), while others propose that induction of chitinases is more of a general, non-specific mechanism which is distinct from the more specific mechanism associated with the induction of the hypersensitive reaction (Jakobek and Lindgren 1993). In the case of the hypersensitive reaction developed during non-host and virus interactions, chitinases are likely part of a general hypersensitive response since there is no evidence for an antiviral function for these enzymes (Vögeli-Lange et al. 1988).

### **9.2. Chitinases and systemic acquired resistance (SAR)**

Chitinases are often associated with systemic acquired resistance (Enkerli et al. 1993; Métraux et al. 1990). The induction of chitinase activity coincides with the onset of immunization in plants (Brown and Davis 1992). Preinoculation on the first leaves of cucumber with tobacco necrosis virus (TNV) enhanced resistance to *Colletotrichum lagenarium* infection of the second leaves 48 hours later, and the resistance was correlated with an increase in chitinase activity (Métraux and Boller 1986). The accumulation of chitinase was also correlated with the acquisition of SAR in tobacco (Tuzun et al. 1989; Ye et al. 1990). The increase in chitinase activity was much earlier in *Peronospora tabacina* immunized tobacco plants than in normal plants (Tuzun et al. 1989; Ward et al. 1991), and the induction of chitinases systemically protected plants against blue mold diseases caused by *Peronospora tabacina* (Pan et al. 1992). The accumulation of chitinases and other PR proteins correlated with induction of resistance in tobacco against *Peronospora tabacina*, an oomycete which does not contain chitin as a cell wall component (Tuzun et al. 1989). Therefore, the induction of chitinase does not require a pathogen with chitin in its cell wall and the enzyme is unlikely to act directly against such pathogens.

However, chitinase is not always associated with SAR. Salicylic acid treated *Arabidopsis* plants that acquired systemic resistance did not show an increase in chitinase activity (Uknes et al. 1992). *Pseudomonas syringae* pv. *syringae*-infected rice plants developed SAR to *Pyricularia oryzae*, but this SAR was not correlated with a systemic increase in chitinase activity (Smith and Métraux 1991).

### 9.3. Role of chitinases in biocontrol

Chitinases are also considered to be involved in hyperparasitism and biocontrol (Benhamou and Chet 1993; Di Pietro et al. 1993; Ordentlich et al. 1988). Chitinases produced by *Trichoderma* inhibited the growth of a pathogenic fungus, *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Chérif and Benhamou, 1990). Chitinases from *Serratia marcescens* were reportedly involved in the biocontrol of *Sclerotium rolfsii* (Ordentlich et al. 1988). A chitinolytic bacterium isolate, *Aeromonas caviae*, controlled *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *S. rolfsii* and resulted in 78, 57, and 60% disease reduction, respectively (Inbar and Chet 1991). The production of chitinase appears to be an important characteristic of some bacteria that suppress root and seedling diseases (Weller et al. 1988). The spraying of viable cells, or their chitinase preparation, of *Escherichia coli* containing a plasmid carrying the ChiA gene from *Serratia marcescens* was effective in disease reduction (62%) caused by *S. rolfsii* in bean and *R. solani* in cotton (Shapira et al. 1989). Meanwhile, reduced chitinase production resulted in reduced inhibition of fungal spore germination and reduced biological control of fungal pathogens (Jones et al. 1986).

Chitinases are also believed to have roles in defence against insects (Bronner et al. 1991; El Sayed et al. 1989) and nematodes (Mercer et al. 1992; Opperman and Conkling 1994). Various pesticides and drugs have been developed using chitin as a selective target to destabilize, perturb, or inhibit crucial biochemical and physiological targets related to metabolism, growth, and development of fungi and insects (Cohen 1993; Kendra et al. 1989).

## ***10. Regulation of chitinase expression***

### **10.1. Signaling**

Although there are many abiotic and biotic factors which can enhance the levels of chitinases in plants, the pathway for regulation of chitinase is not well understood. Physical injury leads to a wound response that involves the re-establishment of a diffusion barrier to keep the plant surface intact, repair the damage, and stimulate new growth and differentiation. Injured plants are vulnerable to microbial infection and the wound sites are important potential entry points for many pathogens. The wound stimulus seems to release a stress signal which induces defense responses in the whole plant (Clarke et al. 1994).

Chemical treatments, such as fungal cell wall fragments, culture filtrates, chitin oligosaccharides, or salicylic acid, can act as elicitors and trigger general plant defense responses. A cis-acting element that is responsive to fungal elicitors was identified in the promoter of a tobacco class I chitinase gene (Fukuda and Shinshi 1994). Some chemicals, such as salicylic acid, may involve or interfere with the signal transduction pathways leading to defense responses (Bowles 1990). The endogenous salicylate increases upon pathogen infection (Malamy et al. 1990; Métraux et al. 1990; Yalpani et al. 1991). The involvement of salicylic acid in systemic acquired resistance has lead many to believe that salicylic acid is the putative signal compound (Gaffney et al. 1993; Malamy et al. 1990; Métraux et al. 1990; Yalpani et al. 1991; Ward et al. 1991) which, upon its release, would rapidly be transported throughout the host plant and trigger



the defense system. Transgenic plants, expressing a bacterial gene for salicylate hydroxylase, did not accumulate salicylic acid and were defective in the development of systemic acquired resistance (Gaffney et al. 1993). However, salicylic acid does not induce basic class I chitinases, and cannot be solely responsible for a multienzymatic chitinase induction.

Furthermore, a recent report has showed that transgenic tobacco rootstocks, although unable to accumulate salicylic acid, were fully capable of delivering a translocating signal that renders nontransgenic scions resistant to further pathogen infection (Vernooij et al. 1994). This result indicates that salicylic acid is not the translocating signal. However, the translocating signal requires the presence of salicylic acid in the reciprocal graft tissue distant from the infection site to induce systemic resistance (Vernooij et al. 1994).

Ethylene production is stimulated by wounding, elicitors, or fungal, bacterial and viral infection (Boller 1988). Transcription of chitinase genes was strongly induced by exogenously applied ethylene. Chitinase mRNA was induced 30 hours after ethylene treatment in bean leaves, and continued to increase until 48 hours (Boller 1988; Boller et al. 1983). The level of increase was as much as 36-fold (Boller et al. 1983). Withdrawal of ethylene resulted in a decrease of translatable chitinase mRNA (Boller 1988). A promoter region specific for ethylene induction was identified in the bean chitinase gene CH5B (Broglie et al. 1989). Although ethylene may be involved in chitinase induction, it may be one of several signal compounds responsible for the induction. *Rubus hispidus* cells produced little ethylene and exogenously applied ethylene did not induce chitinase activity while elicitors did (Bernasconi et al. 1986). Plant growth

regulators, such as auxin and cytokinin, generally have a suppressive effect on chitinase induction which is ethylene-dependent (Hughes and Dickerson 1991; Shinshi et al. 1987). However, the actual signals, their receptors, and the transduction pathways are yet to be found, and the relevance to a defense response in vivo is still unknown.

## **10.2. Chitinase encoding genes**

Chitinases exist as isoenzymes, and are probably encoded by multigene families (Fukuda et al. 1991; Rasmussen et al. 1992). Chitinase in rice is encoded by a family of four to six genes (Nishizawa and Hibi 1991). Acidic and basic chitinases in tobacco are each encoded by two to four genes (Hooft van Huijsduijnen et al. 1987). There are six to eight closely related chitinase genes contained in the barley genome (Leah et al. 1994). In poplar trees, there are at least three different chitinase gene families (*win6*, *win8* and *chiX*) which comprise multiple members (Clarke et al. 1994). However, some chitinases are encoded by one or two genes (Danhash et al. 1993). Chitinases in *Arabidopsis* are encoded by single copy genes (Samac et al. 1990).

## **10.3. Gene expression**

The expression of chitinases is regulated by complex tissue, developmental and environmental determinants (Lawton et al. 1992). Class I chitinase mRNA is regulated by ethylene, a phytohormone that has been associated with stress and wound responses in plants (Boller and Kende 1980; Yang and Hoffmann 1984), whereas class II and class III chitinases are induced by salicylic acid (Ward et al. 1991). A class IV chitinase from

bean is also reported to be induced by salicylic acid (Margis-Pinheiro et al. 1994). Chitinase production is inhibited by some plant growth regulators, auxin and cytokinin (Shinshi et al. 1987; Siefert et al. 1994).

#### **10.4. Organ-specific expression**

Some plant organs, such as roots and flowers, consistently display higher chitinase activity than others, and some isoforms are present only in these organs. Chitinase expression is developmentally regulated in an organ-specific manner (Broglie and Broglie 1993). Different genes may be involved in the tissue-specific expression of chitinases (Leah et al. 1994). This biased distribution of chitinases may indicate a differential need for protection of critical (flower) or readily infected (root) tissues (Graham and Sticklen 1994). Higher chitinase levels are displayed in roots of *Arabidopsis* (Samac et al. 1990), cucumber (Trudel et al. 1989), and tobacco (Neale et al. 1990; Shinshi et al. 1987). Chitinase activity is more than 10 times higher in tobacco roots than leaves (Broglie et al. 1991). This may reflect the fact that, roots are under constant challenge of infection or colonization from soil microorganism, so high chitinase activity may provide better protection. On the other hand, roots are sensitive to the environmental conditions such as soil conditions and water saturation, changes that may stress the plant and thus induce chitinase in the roots as a general stress response.

#### **10.5. Developmental regulation**

It seems that there are developmentally programmed defenses in higher plants (Leah et al. 1994; Zhu et al. 1993). High levels of chitinase in

flowers (Lawton et al. 1994; Lotan et al. 1989; Neale et al. 1990; Shinshi et al. 1987; Trudel et al. 1989) may be explained by the need for better protection from opportunistic pathogens during the reproductive stage. Consistently high levels of chitinase expression in sepals and petals, the outermost tissues in the developing flower, could be the place encountered first by pest and pathogens (Clarke et al. 1994). The accumulation is at the highest levels during open-flower stage just prior to senescence (Lawton et al. 1994). A basic class I chitinase, the most abundant soluble protein in potato pistils, may have a role in protecting the plant from fungal ingress during insect-mediated pollination (Wemmer et al. 1994). Other defense responses, such as the release of vacuolar enzymes and callose synthesis, are not suitable in this regard since they may also jeopardize the passage of pollen tubes. Developmentally regulated chitinases may be a factor in age-related resistance. The increase in chitinase activity correlates with the development of age-related resistance (Shinshi et al. 1987), and the tissues with higher increase in activity have shown more resistance (Lawton et al. 1994; Wyatt et al. 1991).

## ***11. Development of transgenic plants and evaluation of disease resistance***

### **11.1. Plant chitinases**

Introduction of 'foreign' chitinase genes into plants has been achieved by using *Agrobacterium*-mediated transformation (Table 1.5). The promoter

Table 1.5. Chitinase expression in transgenic plants

Plant species	Introduced gene	Chitinase class	Source	A	Pathogen tested	R/S	Reference
tobacco-1	CH5B	I	bean	≤44	<i>Rhizoctonia solani</i>	R	Brogie et al 1991
canola	CH5B	I	bean	≤33	<i>Pythium aphanidermatum</i>	U	Brogie et al 1991
tobacco-1	RCH10	I	rice	8	<i>Cercospora nicotianae</i>	R	Zhu et al 1994
tobacco-3	RCH10*	I	rice	nd	<i>C. nicotianae</i>	R	Zhu et al 1994
tobacco-3	SE2	III	sugar beet	nd	<i>C. nicotianae</i>	Ua	Nielsen et al 1993
tomato	Chi-I	I	tobacco-1		<i>Fusarium oxysporum</i>	U	Van den Elzen et al 1993
tomato*	Chi-I	I	tobacco-1	10	<i>F. oxysporum</i>	R	Melchers et al 1993, 1994
tobacco-1	Tab	I	tobacco-2	≤25	<i>C. nicotianae</i>	U	Beuhaus et al 1991
tobacco-2	Tab	I	tobacco-1	12.9	<i>R. solani</i>	R	Vierheilig et al 1993
	TabΔHb	I	tobacco-1	14.1	<i>R. solani</i>	R	Vierheilig et al 1993
	TabΔTC	I	tobacco-1	5.39	<i>R. solani</i>	U	Vierheilig et al 1993
tobacco-1	ChiA	exo	S.m.	1.4	<i>Alternaria longipes</i>	R	Suslow et al 1988
tobacco-1	ChiA	exo	S.m.	≤1.3			Jones et al 1988

tobacco-1	ChiA	exo	S.m.	<i>R. solani</i>	R	Howie et al 1994
tobacco-1	ChiA	exo	S.m.	<i>P. ultimum</i>	U	Howie et al 1994
tobacco-1	ChiA	exo	S.m.	<i>Botrytis cinerea</i>	R	Bedbrook 1993
carrot	CH5B	I	bean	<i>R. solani</i>	R	Jach et al 1992
cucumber	Chi-I	I	tobacco-1			Hernandez et al. 1995
cucumber		II	petunia	≤2.83 <i>Collectotrichum lagenarium</i>		Raharjo et al 1995
<i>Arabidopsis</i>	aRNA	I	<i>Arabidopsis</i>	≤0.15 <i>Botrytis cinerea</i>	S	Samac and Shah 1994

A: chitinase activity ratio (transgenic/non-transgenic)

R/S: more resistance (R), susceptible (S), or unaltered or not appreciable (U)

tobacco-1: *Nicotiana tabacum*; tobacco-2: *N. sylvestris*; tobacco-3: *N. benthamiana*.

S.m.: *Serratia marcescens*

\*: in combination with  $\beta$ -1,3-glucanase

a: preliminary trial with limited plant material

aRNA: antisense RNA

b: cysteine-rich lectin domain removed

c: C-terminal extension removed

d: Chitinase Total chitinase activity undetermined.

ChiA activity: transgenic plants  $\leq$  442 ng purified chiA; controls = 0.

nd: not determined or not tested

region of the cauliflower mosaic virus (CaMV) 35S transcript, a highly active promoter that is able to function in a wide variety of plants (Odell et al. 1988), has been used to ensure strong constitutive expression. There are several reports of the successful introduction of chimeric chitinase genes into plants (Benhamou et al. 1993b; Broglie et al. 1989, 1991; Van den Elzen et al. 1993; Vierheilig et al. 1993). Transgenic canola plants containing a bean chitinase have been shown to have enhanced resistance to infection by a soil-borne pathogen (Benhamou et al. 1993b). In infected transgenic plants, fungal colonization was restricted and severe hyphal alterations led to fungal lysis due to chitin degradation (Benhamou et al. 1993b). The introduction of an exochitinase gene from *Serratia marcescens* into tobacco resulted in enhanced exochitinase activity and reduction in disease severity (Nagel et al. 1990). When the 35S-chitinase gene construct from bean was introduced into tobacco, chitinase activity in the transgenic plants was increased by as much as 44 times in leaves, seedling mortality was reduced by 30 to 57% and fresh weight loss in root was reduced by 67 to 89% compared to plants without the chitinase gene (Broglie et al. 1991). The enhanced resistance appeared to be correlated with the level of bean chitinase expressed in tobacco since plants containing higher levels of the enzyme displayed a greater survival rate in fungal-infested soil (Broglie et al. 1993). In transgenic canola plants infected with *R. solani*, a reduction in fungal biomass was due to increased hyphal alterations, leading to fungal lysis and chitin breakdown (Benhamou et al. 1993b).

## 11.2. Chitinases and other hydrolytic enzymes

An effective strategy for engineering enhanced disease resistance is to take the advantage of the synergistic effect of some chitinases with other hydrolytic enzymes, such as  $\beta$ -1,3-glucanases, in antifungal activity (Mauch et al. 1988). The combined expression of antimicrobial genes in transgenic plants is an effective approach to engineering enhanced crop protection against diseases (Cornelissen and Melchers 1993; Lamb et al. 1992; Van den Elzen et al. 1993). High level resistance was observed in transgenic tomato plants expressing a combination of genes encoding hydrolytic enzymes, including chitinase, while transgenic tomato plants expressing individual hydrolytic enzyme alone at the same level did not substantially enhance resistance (Van den Elzen et al. 1993). Hybrid plants, expressing a basic class I chitinase gene from rice and an acidic  $\beta$ -1,3-glucanase gene from alfalfa, showed that the combination of the two transgenes gave substantially greater protection against the fungal pathogen *C. nicotianae* than either gene alone (Zhu et al. 1994). Resistance in transgenic plants was reflected by a delay in symptom development and limited symptom development, such as reduction in the number of lesions and the size of lesions (Zhu et al. 1994).

### **11.3. Bacterial chitinases**

Other encouraging results have shown the enhancement of disease resistance/tolerance through the expression of exochitinases from bacteria in plants (Bedbrook 1993; Howie et al. 1994). Transgenic tomato plants constitutively expressing bacterial chitinases resulted in increased resistance to *B. cinerea* infection of fruit (Bedbrook 1993). Transgenic tobacco plants expressing the *S. marcescens* ChiA gene provided tolerance to



infection by a fungal pathogen *R. solani*, and the highest level of ChiA expression showed the most dramatic disease reduction (Howie et al. 1994). The tolerance to the disease occurred only in progeny plants which inherited the ChiA gene (Howie et al. 1994). Greenhouse trials also showed significant tolerance among transgenic plants against *R. solani* infection (Howie et al. 1994).

#### **11.4. Antisense**

Instead of increasing chitinase activity in transgenic plants, an alternative to examining the role of chitinase in the plant defense system is to reduce the chitinase levels in plants and test their susceptibility to diseases.

*Arabidopsis* plants expressing a class I chitinase in the antisense orientation partially blocked induced expression of chitinase upon infection by a fungal pathogen, *B. cinerea*. The transgenic plants had less chitinase produced and were more susceptible to pathogens (Samac and Shah 1994).

#### **11.5. Negative reports and possible explanation**

Contrary to the successful protection conferred by chitinase gene introduction, preliminary infection experiments of transgenic tobacco plants expressing basic class I or acidic class III chitinase did not show increased resistance (Neuhaus et al. 1991; Nielsen et al. 1993). Transgenic *Nicotiana sylvestris* plants expressing high levels of tobacco class I chitinase showed no enhanced resistance to *C. nicotianae*, a leaf pathogen, despite the fact that *Cercospora* species are sensitive to chitinases in vitro. It is quite possible that the strongly expressed chitinase did not come into contact with the developing fungus, since it is located intracellularly, whereas

*Cercospora* species grow predominantly or even exclusively in the intercellular spaces (Cornelissen and Melchers 1993). A possible way to improve the effectiveness in resistance is to target the chitinase into the extracellular space by removing the C-terminal extension (Melchers et al. 1993). Targeting the protein into the right place seems to be critical to the effectiveness of chitinases in disease resistance, since the removal of the targeting sequence was shown to suppress resistance to *Rhizoctonia solani* in transgenic tobacco (Vierheilig et al. 1993). Accurate quantitative evaluation of the contribution of a transgene to disease resistance is hindered by the "position effect", i.e. the substantial variation in the transgene expression due to different insertion sites of transgene in the recipient genome (Zhu et al. 1994). One phenomenon that is often observed in transgenic plants is that some transformants exhibit chitinase levels lower than non-transformed plants (Hart et al. 1992; Neuhaus et al. 1991). This is the so-called "silencing effect", probably due to co-suppression, the inhibition of expression of both the homologous endogenous gene and the transgene in transgenic plants (Hart et al. 1992; Neuhaus et al. 1991).

Many microorganisms also have a complex chitinolytic system and different chitinases may have different functions (Rast et al. 1991). Not all of the chitinases have the same degree of effectiveness in antifungal activity (Roberts and Selitrennikoff 1988; Sela-Buurlage et al. 1993). Recent reports have suggested that the intracellular localization of basic chitinases may prevent its early involvement in defense against invading pathogens (Punja and Zhang 1993; Stintzi et al. 1993). It has also been suggested that basic chitinases have greater antifungal activity than acidic chitinases (Sela-

Buurlage et al. 1993; Vogelsang and Barz 1993). The targeting of chitinase to the right place is also crucial for the enzyme to be effective. Targeting basic chitinases into the extracellular spaces may improve the effectiveness of chitinases in plant defense (Melchers et al. 1993), whereas in transgenic tobacco expressing a chitinase without the C-terminal for vacuolar targeting showed no enhanced resistance (Vierheilig et al. 1993). Basic vacuolar class I chitinases, mainly contribute in protection with strongly hydrolytic and antifungal activity, may have a limited role against pathogens which lack a chitin-containing cell wall (Broglie et al. 1991). Furthermore, it is still not known whether an increase in the constitutive expression of chitinase alone is sufficient to confer protection against a wide range of pathogens often encountered by plants.

## Chapter II

### Induction and Characterization of Chitinase Isoforms in Cucumber (*Cucumis sativus* L.): Effect of elicitors, wounding and pathogen inoculation

#### 1. Introduction

In cucumber (*Cucumis sativus* L.), the appearance of chitinases was previously reported to be correlated with the manifestation of induced resistance to fungal, bacterial and viral infections (Métraux and Boller 1986). The induction of chitinases following pathogen infection is correlated with SAR (Métraux et al. 1989). Multiple isoforms (up to 13) of chitinase have also been described in seeds and germinating seeds of cucumber (Majeau et al. 1990). The chitinase was shown to be an acidic, 28 kDa extracellular protein which also has lysozyme activity (Boller and Métraux 1988; Métraux et al. 1988). A cDNA encoding an inducible, acidic chitinase has been isolated (Métraux et al. 1989). The deduced amino acid sequence of the encoding protein has no similarity to class I and class II chitinases, but has significant sequence homology to chitinases from *Parthenocissus* (Bernasconi et al. 1987), *Hevea* (Jekel et al. 1991), and *Arabidopsis* (Samac et al. 1990), and is classified as a class III chitinase. The chromosomal region encoding the acidic class III chitinase has been isolated and characterized (Lawton et al. 1994). Currently, there is little

information on the isoforms of chitinase present in cucumber seedlings or in different cucumber cultivars, and how expression of these isoforms may be enhanced by abiotic factors, treatments with elicitors, or pathogen infection. This information would be useful to elucidate whether stimulus- or pathogen-specific isoforms occur, and to help elucidate the potential role that chitinases may play in defense against pathogens.

The objectives of this study were to characterize the expression of chitinase isoforms in different cucumber cultivars following various inducing treatments, and to elucidate a role for the chitinases against fungal development in cucumber.

## ***2. Materials and methods***

### **2.1. Plant Materials**

Cucumber (*C. sativus*) seedlings were grown in plastic pots filled with Metro-Mix 290 (Grace Horticultural Products, Ajax, Ontario, Canada) in a Conviron growth chamber set at 26 °C/24 °C day/night temperature, 85 - 90% relative humidity, 14 h /10 h light/dark cycle (intensity of 100  $\mu\text{Em}^{-2}\text{sec}^{-1}$ ). Cucumber cultivars included in this study for chitinase induction following inoculation by the powdery mildew pathogen (*Sphaerotheca fuliginea* (Schlecht. ex Fr.) Pollacci) (provided by Dr. J. G. Menzies, Agriculture Canada) are listed in Table 2.1. The plants were assayed for chitinase activity at the cotyledonary stage, 2 weeks after seeding.

**Table 2.1. Cucumber cultivars and their susceptibility to powdery mildew (*Sphaerotheca fuliginea*).**

<b>Cultivar</b>	<b>Susceptibility</b>	<b>Source</b>
Calypso	susceptible	Campbell Seeds, Davis, CA, USA
Fidelio F1	tolerant	De Ruiters Seeds Inc., Columbus, OH, USA
Alaska	susceptible	Petoseed Company, Woodland, CA, USA
PSX 144388	resistant	Petoseed Company, Woodland, CA, USA

## 2.2. Induction of chitinases

**a) Abiotic.** Two-week-old seedlings of cucumber cv. Calypso were sprayed to runoff with salicylic acid (10 mM) or chitosan (1 mg/ml) (prepared by dissolving chitosan in 0.25 N HCl and adjusting the pH to 7 with 2 N NaOH). Physical wounding was achieved by puncturing the cotyledons with a needle at 10 evenly distributed locations. All treated plants were placed in the growth chamber under the conditions described above. Tissue samples were collected 3 days after treatment. Samples were also obtained from two-month-old calli grown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA) at 5  $\mu$ M each following established procedures (Punja et al. 1989).

**b) Biotic.** Inoculation with the powdery mildew pathogen (*S. fuliginea*) was conducted by dispersing fresh spores from previously infected plants onto the cotyledons of healthy seedlings and incubating the plants for 7 days in the growth chamber under the conditions described above (Section 2.1). Inoculation with *Colletotrichum lagenarium* (Pass) Ell. and Halst. race 1 (pathogenic to cucumber) and *C. lindemuthianum* (Saccardo et Magnus) Briosi et Cavara race B (pathogenic to bean and non-pathogenic to cucumber) (provided by Dr. J. Kuc, University of Kentucky, USA) was done by placing 10 drops of a conidial suspension ( $10^6$  conidia/ml) on each cotyledon leaf using a Pasteur pipet. Inoculated plants were placed within a plastic bag and incubated in the dark at 20 °C for 24 h and then returned to the growth chamber. All plant tissues were collected for assay 7 days after inoculation. In addition, samples were also collected

from true leaves and roots from powdery mildew-infected and control plants. All experiments were repeated a minimum of two times.

### **2.3. Enzyme extraction and preparation**

Plant tissues were frozen in liquid nitrogen immediately after being collected, and finely ground into a powder using a mortar and pestle. All extractions were performed at 4 °C unless indicated otherwise. The powder was extracted with 0.1 M sodium citrate buffer, pH 5.0 (2 ml/g fresh weight); the crude homogenate was filtered through four layers of Miracloth and centrifuged at 20,000 g for 30 min. at 4 °C. The supernatant fraction was put onto an ultrafiltration unit (Amicon YM10 filter) for protein concentration adjustment. Protein content was measured by the method of Bradford (Bradford 1976) using bovine serum albumin as a standard.

### **2.4. Native polyacrylamide gel electrophoresis (PAGE) and overlay gel**

Sample preparation and staining for chitinase activity after native-PAGE under conditions for acidic proteins (Davis buffer system) were performed as described by Pan et al. (1991) with the following modifications. Briefly, 15% PAGE gels (140 x 160 x 1.5 mm) were run under constant current (30 mA) at 6 °C. Following electrophoresis, the gels attached to supporting glass plates were incubated in sodium acetate buffer (0.1 M, pH 5.0) for 5 min. They were then covered with a 7.5% (0.75 mm thick) polyacrylamide overlay gel (attached to another supporting glass plate) containing 0.04% glycol chitin in sodium acetate



(0.1 M, pH 5.0). The gels were incubated at 40 °C for 2-4 h under moist conditions. The overlay gels were then incubated in freshly prepared 0.01% (w/v) fluorescent brightener 28 in 0.5 M Tris-HCl (pH 8.9) at room temperature for 5 min. The brightener solution was discarded and overlay gels were incubated in water at room temperature in the dark for 2-4 h. Chitinase isozymes were visualized as cleared zones by placing the overlay gels on a UV transilluminator with a UV transparent tray. Chitinase isozyme bands on the overlay gels were photographed with B & J 4x5 copy camera using Kodak Plus-X Pan professional film and a Hoya 80A blue filter. The exposed films were used as negatives to develop photographs in which the bands appeared as dark images instead of clear zones on the original overlay gel. R<sub>f</sub> values of isoforms were calculated by dividing the distances the bands traveled by the distance traveled by the tracking dye front.

## **2.5. SDS-PAGE and molecular mass determination**

Denaturing SDS-PAGE was performed as described by Trudel and Asselin (Trudel and Asselin 1989) with the following modifications. Briefly, samples were loaded onto an SDS-PAGE gel which contained glycol chitin as the substrate for chitinase. After running the gel, SDS was removed from the gel by shaking overnight at 38-40 °C in sodium acetate buffer (0.1M, pH 5.0) containing purified Triton X-100, and the proteins in the gel were renatured. The gels were then stained and destained as described above for native PAGE/overlay gels (Section 2.4), and lytic zones were photographed as described above for native gels. The

molecular mass of chitinase was determined by comparing standard markers on SDS-PAGE using a standard curve.

## **2.6. Western blot**

Proteins were separated by SDS-PAGE as described above. Proteins were transferred to 0.45  $\mu\text{m}$  nitrocellulose membranes at 5 mA/cm<sup>2</sup> for 30 min. in Bjerrum and Schafer-Nielsen transfer buffer (Bjerrum and Schafer-Nielsen, 1986) with a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell (Cat. No. 170-3940) as described in the instruction manual. Western blots were performed following the procedure described in the Bio-Rad Immun-Blot GAR-AP assay kit (Cat. No. 170-6509) instruction manual. The antibody raised against cucumber chitinase (provided by S. J. Stewart, Ciba-Geigy Corp., NC., USA) was used at 1:2000 dilution.

## **2.7. Time course of chitinase induction and activity assay**

One-week-old seedlings of cucumber cv. Calypso were inoculated with fresh powdery mildew spores; non-inoculated plants were used as controls. Cotyledons were harvested at 0, 1, 2, 3, 5 and 7 days after inoculation and ground into a fine powder in liquid nitrogen. The powder was extracted with sodium citrate buffer (0.05 M, pH 5.0) (1 ml/g), and then centrifuged at 20,000 g for 30 min. The collected samples were loaded onto native-PAGE gels without prior adjustment of the protein concentration. The banding patterns were visualized and photographed as described above. Samples were also used for a quantitative assay of chitinase activity using a modified colorimetric assay as described by Legrand et al. (1987). A katal

(kat) was defined as the enzyme activity catalyzing the formation of 1 mol of N-acetyl-D-glucosamine (GlcNAc)(or its equivalent) per sec.

## **2.8. Preparative Isoelectric Focusing Electrophoresis (IEF)**

Preparative isoelectric focusing was carried out on an LKB Multiphor II IEF unit at 4 °C as described by Haunerland and Chisholm (1990). Briefly, chitinase samples extracted from powdery mildew-infected cucumber (cv. Calypso) plants were loaded into a flat bed of pre-focused granulated Sephadex IEF (Pharmacia-LKB Biotechnology, Baie D'Urfé, Canada) (containing Pharmalyte 3-10) at constant power of 4 W, to a total of 5000 Vh. Proteins were visualized by the paper print technique. The gel was fractionated according to the protein positions visualized on the filter paper, and proteins were eluted with 15 ml of 20 mM sodium acetate and concentrated to 1 ml through ultrafiltration. The resulting protein fractions were loaded onto a native PAGE gel and analyzed for chitinase activity as described above.

## **2.9. Antifungal activity**

Chitinases extracted from powdery mildew infected plants (cv. Calypso) were partially purified by IEF. Spores of *Thielaviopsis basicola* (provided by P. Morris, Simon Fraser University, Canada) were suspended in distilled water at  $10^5$  spores/ml. The purified chitinase was added to a protein concentration of 0.2 mg/ml. Distilled water was added to the spore suspension to serve as a control. The rate and extent of spore germination were examined under the microscope and photographed. Growth inhibition of *Trichoderma* sp. (isolated from soil) was conducted in Petri

dishes containing 10 ml of 2% malt extract agar at the bottom and 15 ml of 1.5% malt extract agar at the top as described by Schlumbaum et al. (1986). Spores were mixed with chitinase extracts from control and powdery mildew-infected plants (cv. PSX) and then placed into wells (5 mm-diameter) punched into the 1.5% medium layer. The extent of radial growth from the wells was recorded, and the plates were photographed after 2 weeks of incubation at room temperature. Distilled water and autoclaved protein samples were used as controls.

## **2.10. Chemicals**

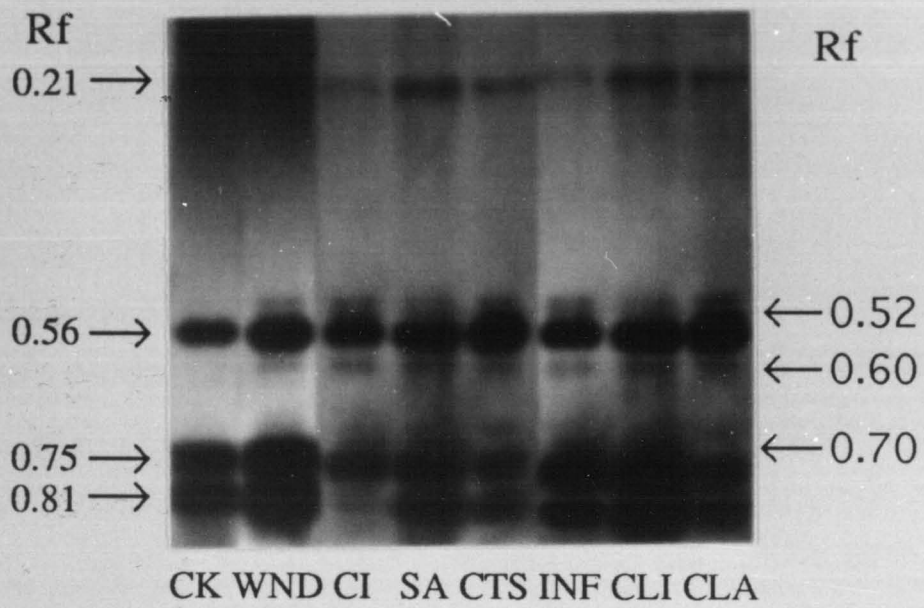
All chemicals for enzyme extraction, electrophoresis, standard protein markers, Coomassie Brilliant Blue R-250 were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA); chitosan (practical grade from crab shells) was supplied by Sigma; glycol chitin was synthesized from glycol chitosan as previously described (Trudel and Asselin 1989).

## **3. Results**

### **3.1. Effect of treatments on chitinase induction**

Proteins extracted from cucumber tissues were analyzed in the non-denaturing Davis buffer system used for separation of acidic or neutral isoforms of chitinase. Control (nontreated) cotyledonary tissues of cucumber cv. Calypso revealed the presence of four bands ( $R_f$  0.21, 0.56, 0.75, and 0.81) with chitinolytic activity (Fig. 2.1). Plants infected with *S.*

**Fig. 2.1. Chitinase isoform banding patterns in cucumber (cv. Calypso) after different treatments. Native PAGE was followed by incubation with an overlay gel containing glycol chitin. The overlay gel was stained with fluorescent brightener 28 and photographed under UV. CK: control; WND: wounding; CI: tissue culture calli; SA: salicylic acid treatment; CTS: chitosan treatment; INF: powdery mildew infection; CLI: *Colletotrichum lindemuthianum* inoculation; CLA: *C. lagenarium* inoculation. Arrows on left depict constitutive bands; arrows on right are induced bands. 50 µg of protein were loaded.**



*fuliginea* or *C. lagenarium* had three additional bands (Rf. 0.52, 0.60, and 0.70) not seen in the control plants. Plants treated with chitosan, or salicylic acid, or that were wounded or inoculated with *C. lindemuthianum*, had similar isoform patterns to those infected with the two fungal pathogens (Fig. 2.1). A total of seven bands with chitinase activity were apparent, four of which were identical to those in the control, and three of which were induced by the treatments. The isoforms found in healthy tissue cultured calli were similar to those induced by the treatments (Fig. 2.1).

### **3.2. Effect of cultivars on chitinase induction**

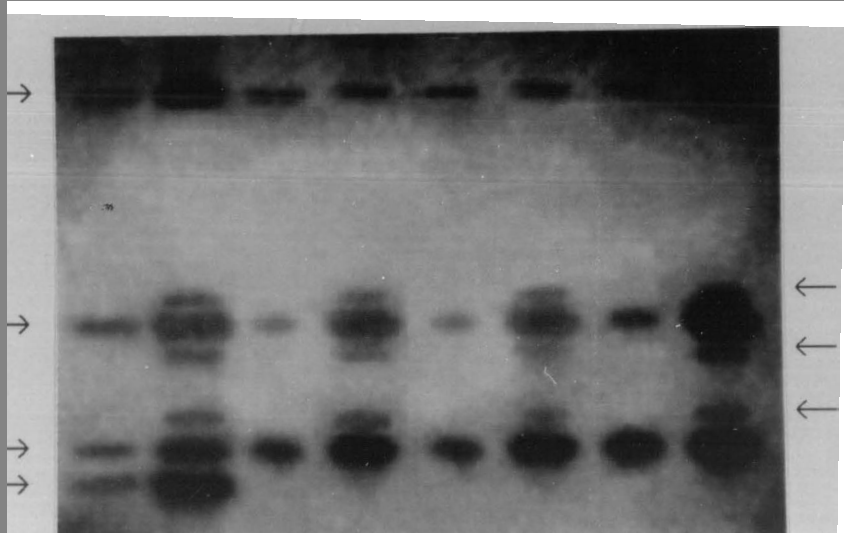
Four isoforms were detected in non-infected cotyledons of Calypso, while three bands were seen in each of Fidelio F1, Alaska and PSX; the Rf. 0.81 band was absent (Fig. 2.2). When these cultivars were inoculated with the powdery mildew pathogen, three additional bands were observed in all cultivars regardless of whether they were resistant or susceptible to powdery mildew (Fig. 2.2). The intensity of the constitutive bands was increased in all cultivars following infection.

### **3.3. Chitinase induction in different tissues**

Chitinase samples were extracted from cotyledons, true leaves, and roots of non-infected and powdery mildew infected plants. Similar banding patterns were seen in the samples from all tissues (Fig. 2.3) and the banding patterns were similar to those observed previously following treatment. The intensity of the constitutive bands was enhanced following

**Fig. 2.2. Chitinase isoform banding patterns in four cucumber cultivars following native PAGE and activity staining on the overlay gel. CK: control; INF: powdery mildew infection. Arrows on left indicate the position of constitutive bands; arrows on right indicate induced bands. 50  $\mu$ g protein was loaded in each lane.**

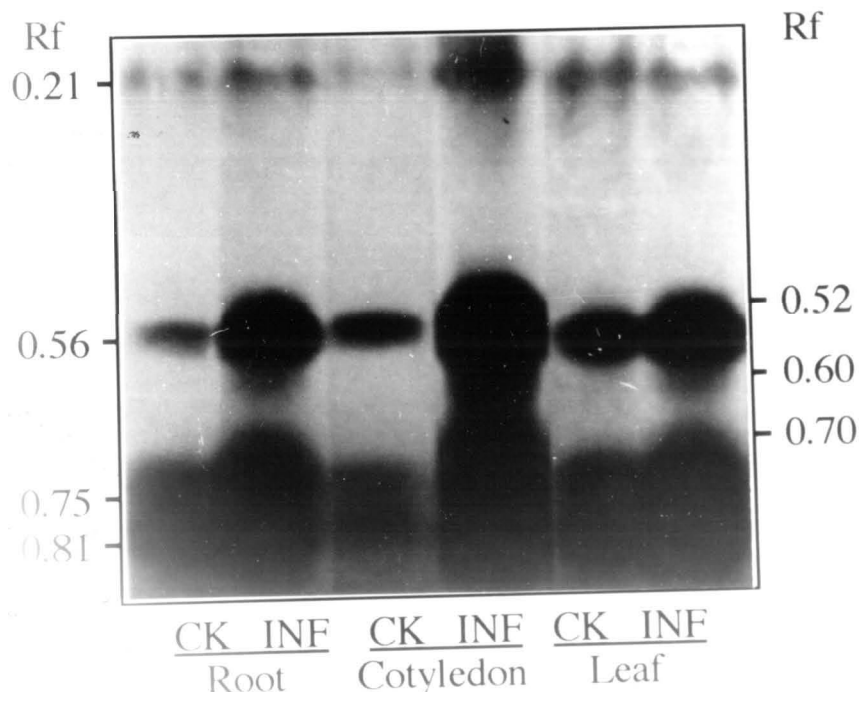




CK INF    CK INF    CK INF    CK INF  
 Calypso    Fidelio    Alaska    PSX



**Fig. 2.3. Chitinase isoform banding patterns in different tissues of cucumber. Native PAGE was followed by incubation with an overlay gel containing glycol chitin. The overlay gel was stained with fluorescent brightener 28 and photographed under UV. All samples were from two-week-old plants of cv. Calypso. CK: control; INF: powdery mildew infection. Arrows on left indicate the position of constitutive bands; arrows on right are induced bands. 50  $\mu$ g protein was loaded in each lane.**



infection. There were no tissue-specific chitinase isoforms at this stage of plant development.

### **3.4. Molecular mass**

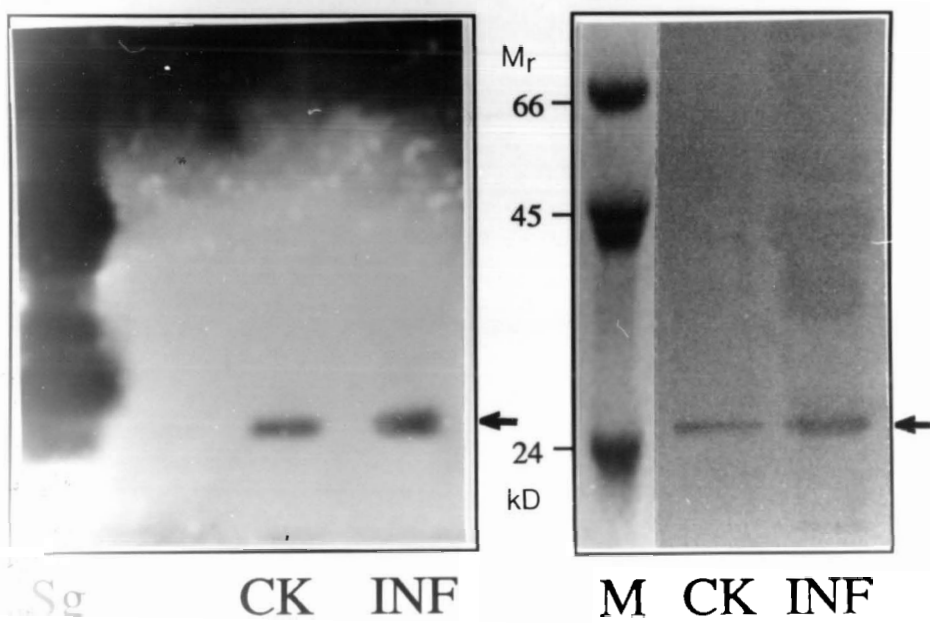
Only one band with chitinase activity was visualized on the SDS-PAGE gel whether the samples were from control plants, following treatment with chitosan, or infected with powdery mildew. By comparing with the Coomassie blue-stained protein standards which were loaded on the same SDS-PAGE gel using a standard curve, the molecular mass was estimated at 25.6 kDa (Fig. 2.4), which although different from previous estimates of 27 kDa (Métraux et al. 1989) and 28 kDa (Majeau et al. 1990), respectively, is well within the range.

One band was visualized on the nitrocellulose sheet after Western blot with protein samples from the control or powdery mildew-infected plants. The band was in the same position when compared with the band on SDS-PAGE gel (Fig. 2.4).

### **3.5. Time course of chitinase induction**

The increase in chitinase activity in both control and powdery mildew-infected plants over time is shown in Fig. 2.5. In infected plants, the activity increased much more rapidly within the first 3 days of infection (1.5 X the control) and then stabilized to the same degree as that in the control plants. The activity in the control increased slightly over time. Tissue samples from control and infected cotyledons were assayed on native-PAGE gel at various time intervals. The intensity of two constitutive lower bands ( $R_f$  0.75 and 0.81) was enhanced over time and

**Fig. 2.4. Cucumber chitinase SDS-PAGE and Western blot. Left: SDS-PAGE followed by in situ activity staining. Right: Western blot with antiserum raised against cucumber chitinase. Numbers in the middle indicate the molecular mass of protein markers in kDa. Sg: chitinase from *Streptomyces griseus* (positive control); CK: control plant; INF: powdery mildew infected plant. 50  $\mu$ g of protein were loaded for SDS-PAGE and 10  $\mu$ g for Western blot.**



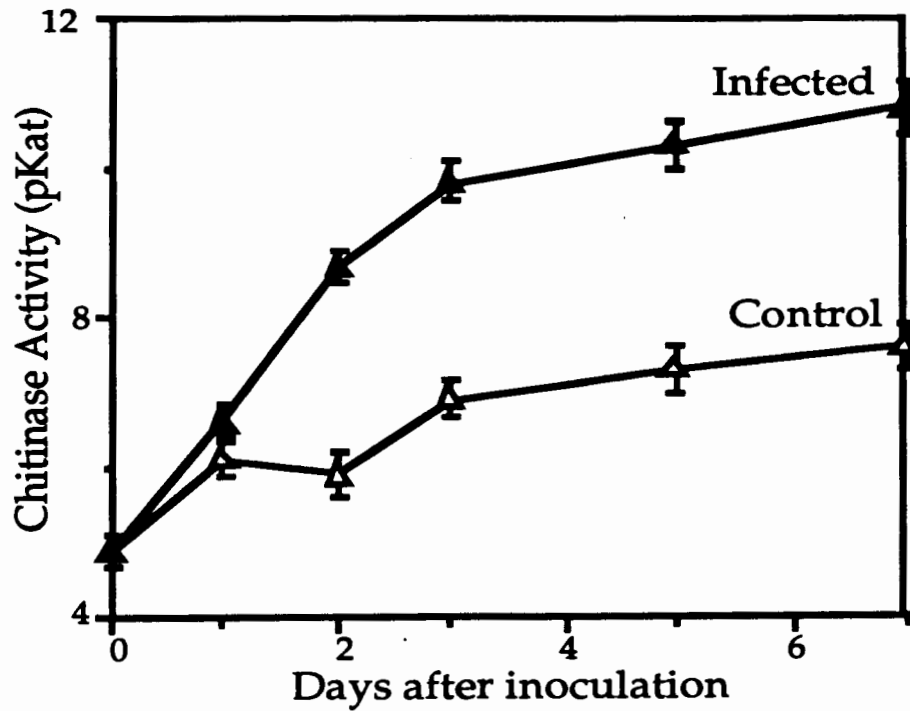


Fig. 2.5. Time-course of development of chitinase activity in cucumber (cv. Calypso) cotyledons in control and powdery mildew infected plants.

following infection (Fig. 2.6A). The induced bands were seen 5 days after inoculation. In order to determine the earlier expression of induced isoforms, the samples were concentrated by lyophilisation and then rerun; the induced bands could subsequently be detected within 2 days after treatment (Fig. 2.6B).

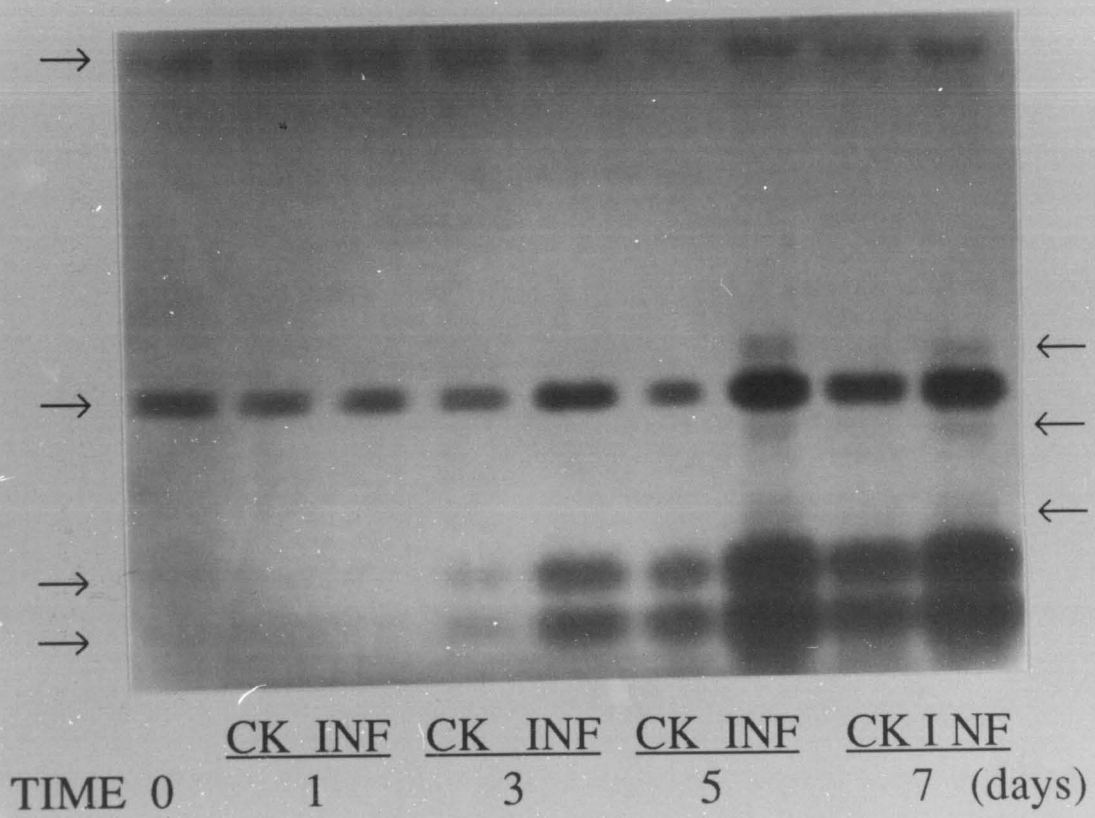
Based on these results, the expression of cucumber isoforms was characterized into three groups: I-constitutive isoforms (Rf. 0.21 and 0.56), the expression of which was somewhat constant over the course of seedling development; II-constitutive isoforms (Rf. 0.76 and 0.81), whose expression increases over the course of plant development; III-induced isoforms (Rf. 0.52, 0.60, and 0.70), the expression of which was activated by induction (or they may be expressed at undetectable levels and then increase upon induction). Although groups I and II are constitutive isoforms, their expression was also increased upon induction.

### **3.6. Preparative isoelectric focusing**

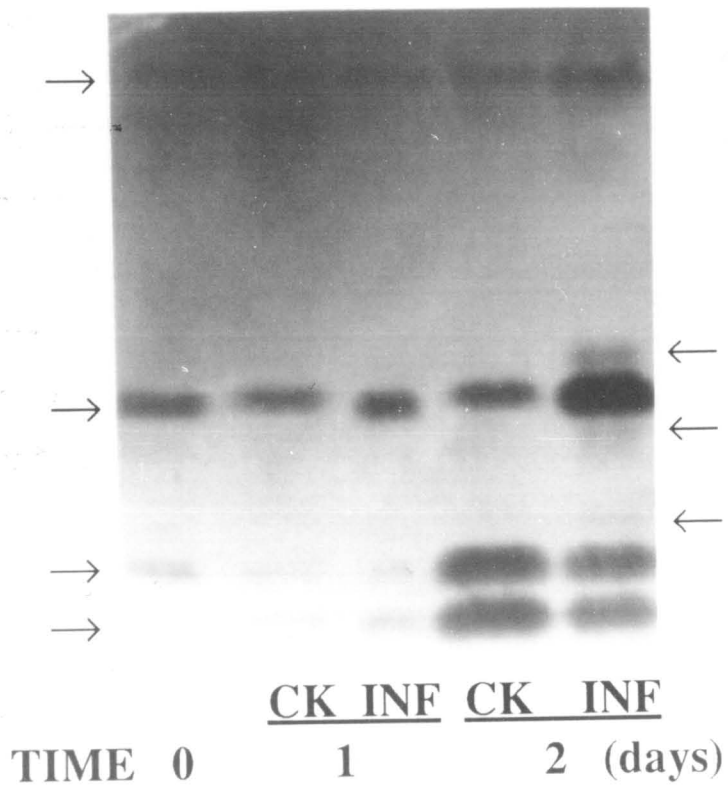
Preparative isoelectric focusing was used to separate the isoforms in the sample from infected cucumber (cv. Calypso). Protein bands were visualized, cut out, eluted, and loaded onto a native-PAGE gel to detect the isoforms (Fig. 2.7). Their pIs were estimated according to their position on the IEF gel. The bottom two bands (Rf 0.75 and 0.81) were visualized as the proteins collected from pH 4 and 4.5 positions. The Rf 0.56 band appeared at pH 4, 4.5 and 5.5 positions, with the vast majority at pH 4.5. The Rf 0.21 band appeared at pH 6. All the induced bands were observed at pH 4.5. The majority of the chitinase activity was observed around pH 4.2 when using a pH gradient from 4 to 6.5 for IEF (not shown). The



**Fig. 2.6A. Time-course of chitinase induction in cucumber (cv. Calypso). Samples were extracted with sodium acetate buffer (1ml/g) and loaded onto native PAGE gel without protein concentration adjustment. Bands were visualized on an overlay gel after fluorescent staining. Time indicates days after inoculation. CK: control; INF: powdery mildew infection. Arrows on left indicate the position of constitutive bands; arrows on right are induced bands.**

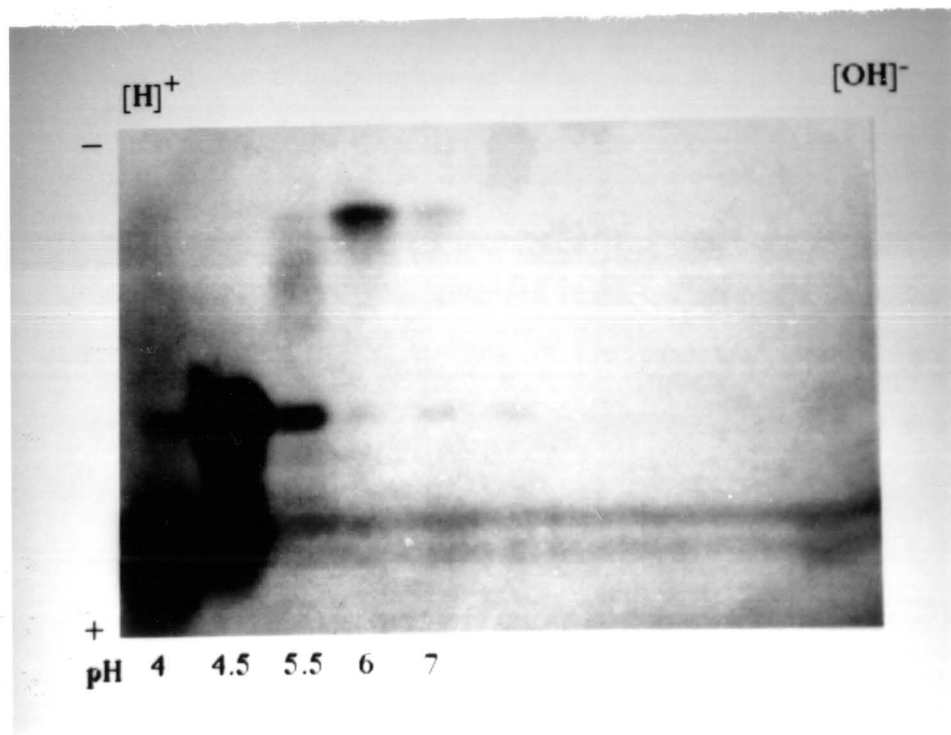


**Fig. 2.6B. Time-course of chitinase induction in cucumber (cv. Calypso) using five-fold concentrated samples loaded onto native PAGE. Bands visualized on an overlay gel after fluorescent staining. Time indicates days after inoculation. CK: control; INF: powdery mildew infection. Arrows on left indicate the position of constitutive bands; arrows on right are induced bands.**



**Fig. 2.7. Isoelectric focusing of cucumber (cv. Calypso) chitinases.**

**Protein (2 mg) extracted from powdery mildew infected plants was fractionated by preparative IEF. Protein bands were eluted with 15 mL 20 mM Na acetate and concentrated to 1 mL. 50  $\mu$ L aliquots was loaded onto native PAGE gel and chitinase isoforms were visualized on the overlay gel after fluorescent staining. All proteins with chitinolytic activities had pI's from 4 to 6.**



protein sample collected from pH 4.2 was sufficiently enriched in chitinases that Coomassie Blue staining of SDS-PAGE revealed the 25.6 kDa chitinase (Fig. 2.8). This sample was used as partially purified chitinase in the antifungal activity test.

### **3.7. Antifungal activity**

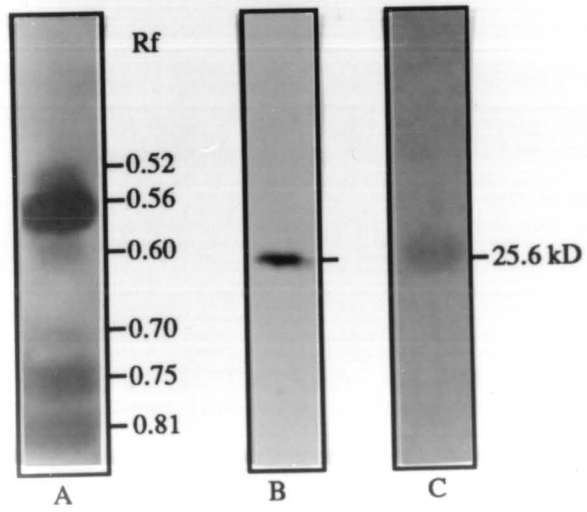
When spores of *Thielaviopsis* were incubated with chitinase extract from infected cucumber plants, many germ tubes were lysed and the cell contents were released. By comparison, the water control showed normal germ tube development with no lysis (Fig. 2.9 A, B). The colony growth of *Trichoderma* was reduced by extracts from both uninfected and infected cotyledons of PSX compared to the controls (Fig. 2.10). However, the chitinase extract from infected seedlings had more of an inhibitory effect to colony growth than the extract from healthy control plants (Fig. 2.10 D).

## **4. Discussion**

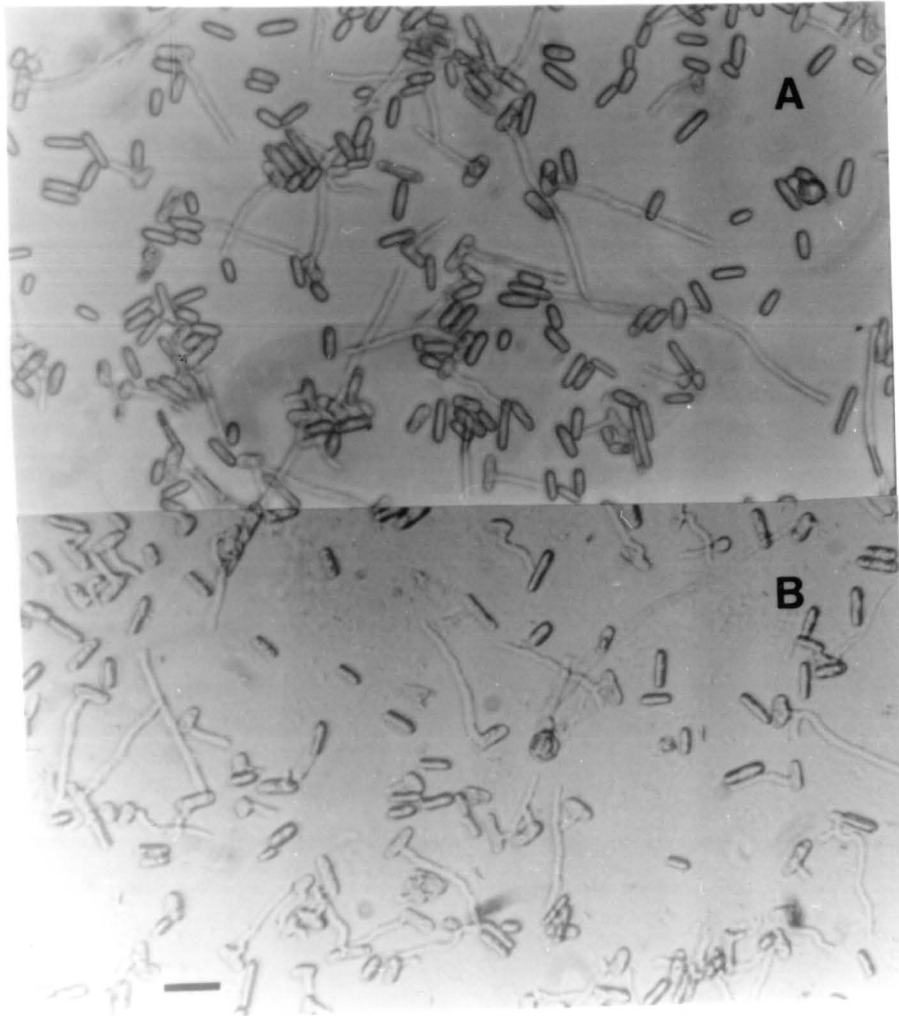
Chitinase has been shown to be expressed constitutively in cucumber (Majeau et al. 1990) and was induced by ethylene, salicylic acid, and by pathogens (Métraux and Boller 1986; Pan et al. 1992). In this study, we have demonstrated that chitinase was induced also by wounding, by application of chitosan, and by a non pathogenic species that induced a hypersensitive response. Thus, the induction of chitinase in cucumber

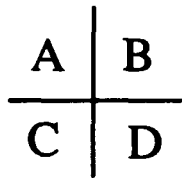
**Fig 2.8. Chitinases were enriched and partially purified through IEF. Protein sample was eluted from pH 4.2 on IEF and loaded on native PAGE and SDS-PAGE. A; Native PAGE/ overlay gel after fluorescent staining; B: SDS-PAGE/ in situ chitinase activity staining; and C: SDS-PAGE/Coomassie blue staining. 20  $\mu$ g of protein were loaded.**



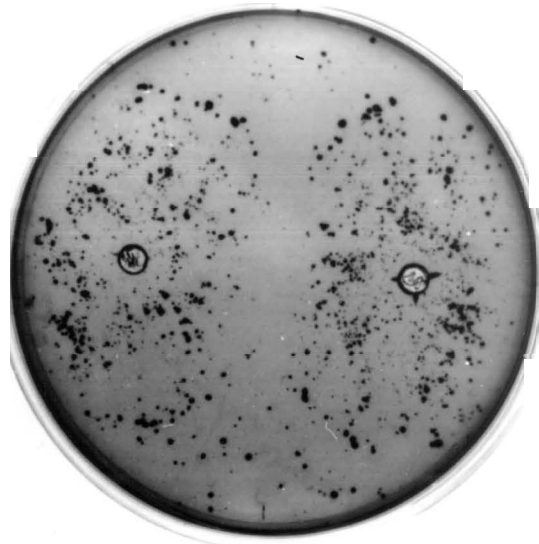


**Fig. 2.9. Effect of chitinase from cucumber on the spore germination of *Thielaviopsis basicola*. A) water control, B) exposure to the cucumber chitinase. Note the lysis and reduced growth of germ tubes. Photo was taken after 8 h. Scale bar = 20  $\mu$ m.**

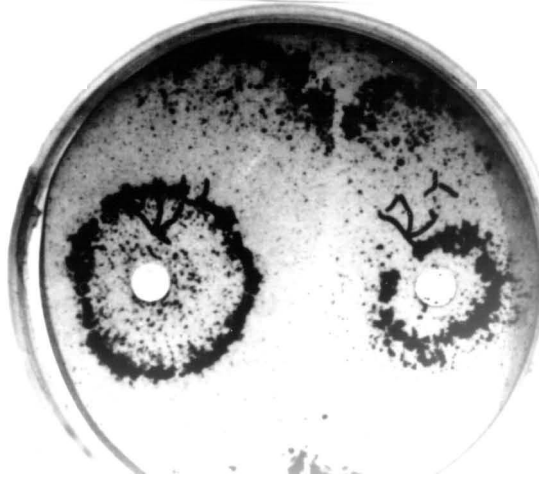




**Fig. 2.10. Antifungal activity of cucumber (cv. Calypso) chitinase against the growth of *Trichoderma* sp. A: water control; B: autoclaved sample from control plants; C: healthy plants; D: powdery mildew infected plant. 20  $\mu$ L of spore suspension was mixed with 10  $\mu$ g of partially purified IEF protein samples and placed in a well cut out in 1.5% malt extract agar. Plates were photographed after incubation at 25 °C for 2 weeks.**



8



appears to be non-specific to pathogen infection, which is similar to reports from other plant species (Punja and Zhang 1993).

Chitinases have been shown to occur in calli of several plant species, such as bent-grass (Koga et al. 1992), carrot (Kurosaki et al. 1987) and onion (Williams and Leung 1993). We observed the induction of chitinase during the growth of healthy calli of cucumber. This could explain previous reports where tissue cultured cells were found to have higher chitinase activity than intact plant tissues (Sauter and Hager 1989). This observation is also consistent with the report that cultivated tobacco protoplasts synthesized and accumulated typical stress proteins including chitinases (Grosset et al. 1990). Therefore, the use of tissue cultured materials for studies on chitinase induction should take into account the preinduction due to the tissue culture process (either due to stress or growth regulators).

There is one previous report describing constitutive chitinase isoforms in cucumber (Majeau et al. 1990). Using native-PAGE gel under Davis buffer system, three bands were found in cotyledons of cucumber (cv. SMR-18) 11 days after germination and five bands were observed in roots. However,  $R_f$  values of the isoforms were not indicated (Majeau et al. 1990). From our calculation, the constitutive chitinase isoform banding patterns we have described in cv. Fidelio, Alaska, and PMX are comparable to those reported in SMR-18 (Majeau et al. 1990). In roots, the patterns were similar except that the No. 3 band (from the top) in cv. SMR-18 (Majeau et al. 1990) was found to be induced in our study. We observed no root specific isoforms since the bands corresponding to the

reported root specific isoforms (Majeau et al. 1990) were seen in cotyledons and true leaves.

The induction of chitinase isoforms and subsequent changes in different tissues following induction have not been previously studied in cucumber. Inoculation of cotyledons in this study resulted in similar patterns of chitinase isoforms in roots, cotyledons, and true leaves. The banding patterns after various treatments (abiotic or biotic, pathogen or nonpathogen) were very similar. No tissue specific isoform was seen and the induction was systemic. In other plant species, e.g. tobacco, seven chitinase isoforms were expressed constitutively, and one of these and a new isoform were induced by fungal infection (Pan et al. 1992). Four chitinases were induced in peanut by treatments of elicitors, yeast extract, and UV irradiation, and one chitinase was differentially induced by pathogen inoculation (Herget et al. 1990). Four chitinase isoforms were reported in celery and both fungal infection and chitosan treatment induced the same isoforms (Krebs and Grumet 1991).

It is likely that the increase in total chitinase activity in cucumber tissues was due to enhanced expression of constitutive and induced chitinases isoforms. The time course study clearly indicated that the intensity of all constitutive bands (except band R<sub>f</sub> 0.21) was increased, and additional bands were observed after induction. This conclusion is supported by the observation that the increase in enzyme activity is at least partially due to increased chitinase mRNA abundance (Lawton et al. 1994).

In comparing different cucumber cultivars, an additional isoform (R<sub>f</sub> 0.81) was seen in cv. Calypso that was absent in the other three cultivars. There was no correlation between isoform banding patterns and genetic

resistance to powdery mildew among the cultivars tested, since the induction of three chitinase isoforms was similar to all cultivars irrespective of whether they were susceptible or resistant. However, the resistant cultivar PSX appeared to have a much higher intensity of induction. It has been shown that chitinase activity may accumulate faster and to a higher level in incompatible host-pathogen interactions than in compatible ones (Daugrois et al. 1990), but no unambiguous correlation was found between the enzyme activity and the host resistance (Fric and Huttova 1993). The magnitude of chitinase induction following infection in our study is also not as high as that reported (Métraux and Boller 1986). This may be due to the obligate parasite we used. The powdery mildew pathogen *S. fuliginea* has an obligatory parasitic relationship with the cucumber host, it tends to co-survive with the host and causes mild and chronic damage. It is conceivable that the chitinases may have less of a role in defense than against necrotrophic pathogens (Mauch and Staehelin 1989). The induction of chitinases was a response to active infection rather than the factors responsible for powdery mildew resistance in near-isogenic lines of barley (Ignatius et al. 1994). The young cotyledonary tissues we used may also be an attributing factor.

It is not clear how many chitinase isoforms exist in cucumber. We observed a total of 7 isoforms in Calypso, while previous reports indicated as few as two and as many as 13 isoforms (Majeau et al. 1990). The 13 isoforms (Majeau et al. 1990) were the total number of chitinolytic bands observed on native PAGE gel with both Davis and Reisfeld buffer systems, which might have included isoforms that migrated in both buffer systems. Results from native-PAGE after preparative IEF indicated that only acidic



chitinases were observed in this study with no basic ones being detected. These acidic chitinases had antifungal activity, similar to that reported for class I chitinases (basic) from other plant species (Schlumbaum et al. 1986; Broekaert et al. 1988). Upon pathogen infection increased chitinase production enhanced the inhibitory effect on the test fungi. Although the chitinase extracts were not totally purified, biological activity was demonstrated. The induction of chitinases may therefore contribute to induced resistance commonly reported in cucumber (Irving and Kuc 1990; Métraux and Boller 1986).

Three groups of chitinase isoforms were observed in this study, which could have different functions in defense. Group one isoforms were produced constitutively at a low level, possibly in response to general stress factors or environmental stimuli. Upon fungal infection, increased stress or signals could activate the host defense system, with increased expression of constitutive isoforms (group 2 chitinases) and also activation of inducible isoforms (group 3 chitinases). A number of isoforms seem to be developmentally regulated and increasingly expressed in the cotyledonary tissues over the course of the time. The systemic induction of these chitinases throughout the plant in addition to a localized induction could play a role in induced host resistance in cucumber (Irving and Kuc 1990; Métraux and Boller 1986). Recently, the class III chitinase coding region has been characterized to be a gene triplication, three genes with complete open reading frames which are highly conserved, and the expression of cucumber chitinase is developmentally regulated (Lawton et al. 1994). However, the relationship between the regulation of these chitinase encoding genes and chitinase isoform patterns still remains unclear.

## Chapter III

### **Chitinase profiles in mature carrot (*Daucus carota* L.) roots and purification and characterization of a novel isoform**

#### ***1. Introduction***

Endochitinases (E. C. 3.2.1.14) are expressed in many plant species in response to pathogen infection or to other environmental stresses (Punja and Zhang 1993). Higher plants do not have any known endogenous substrate for the enzyme, whereas chitin is an important component of the cell wall of many fungal pathogens. Chitinases can degrade the cell walls of many pathogens, and have been shown to inhibit hyphal growth of several fungi *in vitro* (Collinge et al. 1993). Thus, one of the speculated functions of chitinases in plants is in defense against fungal infection (Punja and Zhang, 1993).

In cultured carrot cells, chitinase activity was found to be expressed constitutively at a low level, but was significantly enhanced when cells were incubated with the fungus *Chaetomium globosum* or other fungal cell wall components (Kurosaki et al. 1987). Multiple isozymes were observed, and the whole fungal culture induced more isoenzymes than the culture filtrate only (Kurosaki et al 1990). De Jong et al. (1992) reported that a temperature sensitive embryo mutant was rescued by a 32 kDa endochitinase, and suggested that chitinase may have a role in carrot somatic embryo

development. The 32 kDa chitinase was recently characterized as a class IV chitinase (Kragh et al, 1994). To date, all of the reports on chitinases from carrot have investigated the occurrence of the enzyme in suspension cultured cells (Kurosaki et al. 1987, 1990; De Jong et al. 1992). There are no reports of the occurrence of chitinases in intact plant tissues of carrot, such as in the roots or leaves.

We were interested in purifying and characterizing chitinases from carrot roots to investigate their biological function and their relationship to chitinases from other plant species. We selected mature carrot root tissues to avoid reported differential induction in cultured plant cells containing plant growth regulators (Zhang and Punja 1994).

## ***2. Materials and methods***

### **2.1. Protein extraction**

Harvested mature carrot roots (*Daucus carota* L. cv. Eagle) were cut into small pieces (about 1 cm<sup>3</sup>) and homogenized with ice cold 0.1 M sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 20,000 g for 45 min. at 4 °C. The supernatant was incubated at 37 °C for 30 min. and quickly cooled to 4 °C. Ammonium sulfate was added to the supernatant to 25% saturation and stirred at 4 °C for 4 h; the precipitate was removed by centrifugation. Additional ammonium sulfate was added to 75% saturation and stirred at 4 °C overnight. The precipitate was collected after centrifugation and then redissolved in the acetate buffer and dialyzed overnight.

## **2.2 Ion exchange chromatography**

The protein sample was passed through columns containing celite (Sigma, USA) and CM-Sephadex (Pharmacia, Sweden). The non-absorbed proteins were collected and loaded onto a DEAE-cellulose (Sigma, USA) column (2.5 x 50 cm) equilibrated with 0.1 M Tris-HCl (pH 7.5). Proteins were eluted with a linear gradient of 0-0.4 M NaCl in 0.1 M Tris-HCl at a flow rate of 2.0 ml/min. delivered with the Econo system (Bio-Rad, USA). Fractions with chitinase activity were pooled separately and concentrated by ultrafiltration in a stirred cell with a YM-10 membrane (Amicon, USA). Buffer exchange and concentration adjustment were performed on the ultrafiltration unit with agitation device.

## **2.3. Hydroxylapatite chromatography**

The sample from DEAE-cellulose was loaded onto a hydroxylapatite (Behring Diagnostics, USA) column (1.5 x 10 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). Proteins were eluted and fractionated at a flow rate of 0.25 ml/min. Fractions with chitinase activity were pooled and further concentrated as described above.

## **2.4. Gel filtration chromatography**

The above samples were loaded onto the Protein Pak 125 (Millipore, USA) column (7.8 mm x 30 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7). Proteins were eluted with the same buffer at a flow of 0.6 ml/min controlled with a Waters' model 510 HPLC system (Waters, USA). Fractions with chitinase activity were pooled and concentrated.

## **2.5. Gel electrophoresis**

SDS-PAGE and native PAGE gel electrophoresis and subsequent enzymatic activity test on the gels were carried out as described in Chapter II (Section 2.4 and 2.5). Prestained protein markers (low range, Bio-Rad, USA) were used to calculate the Mr. Analytical isoelectric focusing (IEF) was performed on ultrathin (0.4 mm) acrylamide gels according the manufacturer's instructions with Phamalyte 3-11 (Pharmacia, USA). Western blots were performed as previously described in Chapter II (Section 2.6). Antisera raised against chitinases from petunia (L. S. Melchers, M $\ddot{o}$ gen, The Netherlands), cucumber (S. J. Stewart, Ciba-Geigy Co., USA), tobacco (both basic and acidic) (B. Fritig, IBMP, France), and *Arabidopsis* (D. A. Samac, U of Minnesota, USA) were used at dilutions of 1:2000, except for the petunia chitinase antiserum which was used at a dilution of 1:5000.

## **2.6. Amino acid composition and N-terminal sequencing**

The protein sample was separated on a PAGE gel and transferred on a polyvinylidene difluoride (PVDF) membrane with 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 11) and 10% methanol as transfer buffer. The band was visualized with Commassie Blue and cut out. Micro amino acid analysis was carried out on an Applied Biosystems model 120H derivatizer-analyser system. Sequence analysis was carried out on an Applied Biosystems 473 pulsed liquid protein sequencer (Micropartite Sequencing Centre, University of Victoria, Canada).

## **2.7. Physical and biochemical characterization**

Chitinase activity was assayed using the method described by Yamagami and Funatsu (1993) using glycochitin as the substrate. One unit of enzyme was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of GlcNAc per min at 37°C. Chitinase activity was also detected on denatured and native PAGE gels according to the method described in Chapter II (Section 2.4 and 2.5).

The effect of pH on chitinase activity was tested by adding 0.1 M buffers at different pHs (Na acetate buffer, pH 3.5 - 5.5, Na phosphate, pH 6.0 - 8, Na carbonate, pH 8.5 - 10.5) into the enzymatic reaction mixture and incubating it at 37 °C for 30 min. The effect of pH on chitinase stability was examined by incubating the enzyme solution with the above pH buffers for 24 h at 37 °C. The remaining chitinase activity was measured at pH 5.0.

The effect of temperature on the enzymatic activity was tested by incubating the enzymatic reaction mixture in a water bath maintained at various temperatures. The effect of temperature on chitinase stability was tested by heating the enzyme solution at various temperatures for 1 h. After addition of glycochitin to the enzyme solution, the mixture was incubating at 37 °C for 30 min and the remaining activity was measured.

Chitin binding activity was determined on a regenerated chitin column according to Molano et al. (1979).

## **2.8. Lysozyme activity and antifungal activity**

Lysozyme activity was assayed by using the lyophilized cells of *Micrococcus lysodeikticus* as the substrate as described by Audy et al. (1990), lysozyme purified from chicken egg white (Sigma, USA) was used

as a positive control. Antifungal activity was tested on *Trichoderma* sp. as described in Chapter II (Section 2.9).

### **3. Results**

#### **3.1. Chitinase profiles**

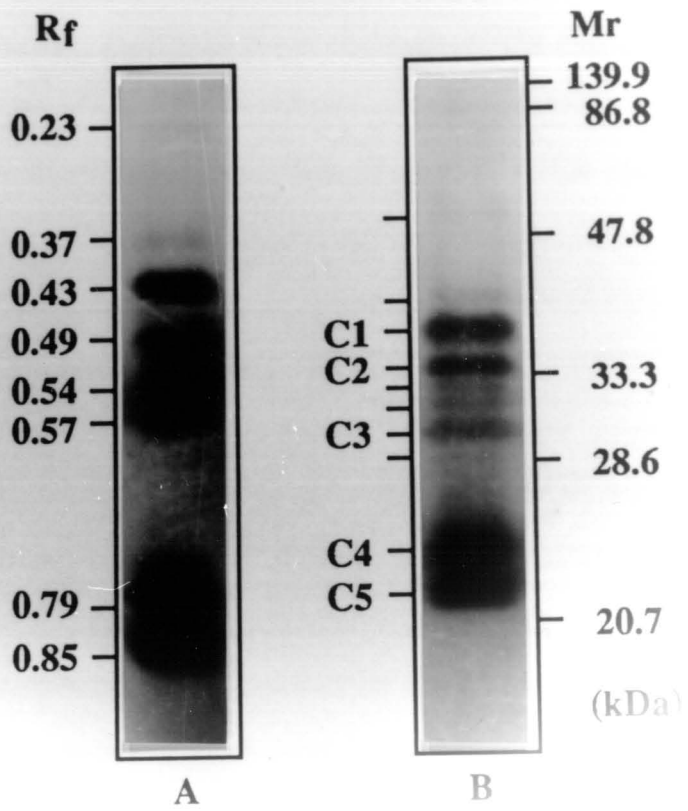
Proteins collected after  $(\text{NH}_4)_2\text{SO}_4$  precipitation were separated on native PAGE gel and SDS-PAGE gels. Bands with chitinolytic activity were visualized on a native PAGE gel with the overlay gel (containing glycol chitin as the substrate) technique, or on an SDS-PAGE gel (containing glycol chitin) then incubated with Triton X-100, after which the proteins were renatured and reacted with the substrate in the gel.

Multiple bands with chitinolytic activity were observed on both native and SDS-PAGE gels (Fig. 3.1). On the native PAGE/overlay gel (Fig. 3.1, A), a total of eight bands were visualized ranging from  $R_f$  (relative mobility to the dye front) 0.23 to 0.85. There were four ( $R_f$  0.43, 0.49, 0.79, and 0.85) major bands, two ( $R_f$  0.54 and 0.56) intermediate, and two ( $R_f$  0.23 and 0.37) weak bands, based on the visual intensity of the bands.

There were 10 chitinolytic bands visualized on the SDS-PAGE gel (Fig. 3.1, B), with  $M_r$  estimated from 25 to 51 kDa, with the majority of the bands at  $M_r$  from 20 to 40 kDa. Five bands, named C1, C2, C3, C4, and C5, respectively, were major bands with  $M_r$  at 39.5, 35, 30.5, 24.3, and 22.2 kDa, respectively. The remaining five bands were weak and considered to be minor bands.

**Fig. 3.1. Chitinase profiles on native PAGE and SDS-PAGE. Bands with chitinolytic activity were visualized after fluorescent staining on native PAGE/overlay gel (A) and SDS-PAGE gel containing glycol chitin as the substrate (B). 8 and 10 bands were detected on native PAGE and SDS-PAGE gels, respectively.**





### 3.2. Immunoblot

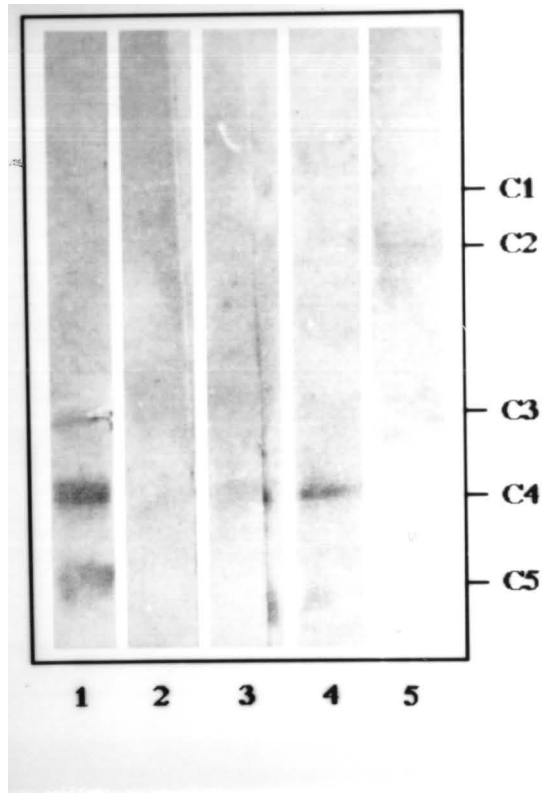
Chitinases in crude extracts of carrot roots were separated on SDS-PAGE and blotted onto a nitrocellulose membrane. The blots were probed with antisera raised against petunia chitinase (class II), *Arabidopsis* chitinase (class I), tobacco basic (class I) and acidic chitinase (class II), and cucumber chitinase (class III) (Fig. 3.2). Only the major bands were visible on the blot. C1 did not react with any of the antisera tested. C2 reacted weakly with antiserum raised against class III chitinase from cucumber. C3 reacted weakly with antiserum against class I basic chitinase from tobacco, class II chitinase from petunia, and class III chitinase from cucumber. C4 reacted strongly with class II chitinase from petunia and tobacco and weakly with class I chitinase from tobacco, but not class I chitinase from *Arabidopsis*. C5 reacted strongly with class II chitinase from petunia and weakly with class II chitinase from tobacco.

The minor bands with chitinolytic activity seen on SDS-PAGE were not seen on the immunoblot, perhaps due to the absence of  $\beta$ -mercaptoethanol in the sample buffer for SDS-PAGE/activity gel, which could result in the incomplete breakage of disulphide bonds, or because they were not related to any of the chitinases to which antisera were raised.

### 3.3. Purification

Since chitinase C1 did not react with the antisera against class I-III chitinases, efforts were made to purify and further characterize it. The purification process consisted of temperature treatment,  $(\text{NH}_4)_2\text{SO}_4$  precipitation, CM-Sephadex and DEAE-cellulose chromatography (Fig. 3.3),

**Fig. 3.2. Immunoblotting of chitinases from mature carrot roots after SDS-PAGE. The blots were probed with antisera raised against petunia chitinase (1), *Arabidopsis* chitinase (2), tobacco basic chitinase (3), tobacco chitinase Q (4), and cucumber chitinase (5). 25  $\mu$ g of protein was loaded in each lane.**



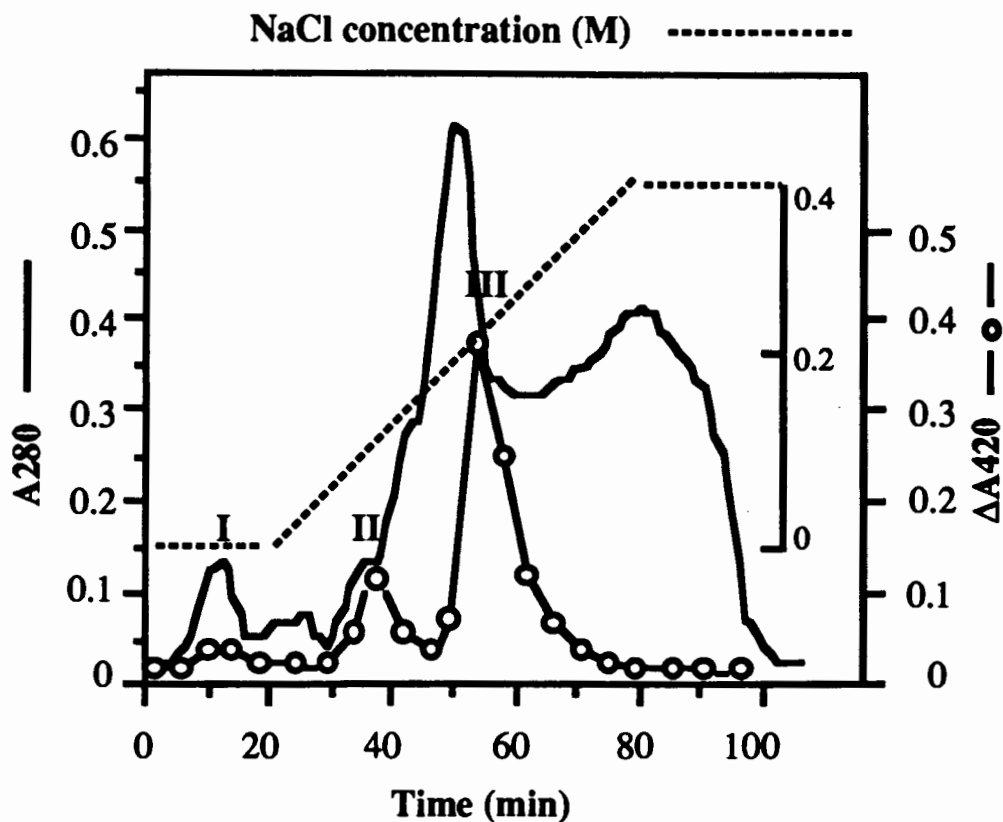


Fig. 3.3. DEAE-cellulose column chromatography. The protein sample from CM-Sephadex was put onto a DEAE-cellulose column previously equilibrated with 0.1 M Tris-HCl, pH 7.5. The elution was carried out with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. Fractions corresponding to peaks I, II, and III were pooled separately.

hydroxylapatite chromatography (Fig. 3.4), and Protein Pak 125 HPLC (Fig. 3.5). Samples collected after each purification step were loaded onto an SDS-PAGE gel and proteins were visualized after Coomassie blue staining (Fig. 3.6). The efficiency of the purification procedure is shown in Table 3.1.

Since chitinases are stable at relatively high temperature, the treatment at 37 °C for 30 min, together with 25% saturated  $(\text{NH}_4)_2\text{SO}_4$ , significantly reduced the amount of proteins in the preparation and still retained most of the chitinase activity. Ion exchange chromatography with DEAE-cellulose resulted in three peaks (I, II and III) with chitinase activity. Peak I and II are relative minor peaks compare to peak III. Since peak III contains not only the majority of chitinase activity, but also the C1 chitinase, the fractions corresponding to peak III were concentrated and subject to further purification. The purification with hydroxylapatite chromatography not only reduced the number of proteins but also selectively retained some chitinase isoforms in the column. Size exclusion HPLC with Protein Pak 125 was able to further purify the chitinase from contamination. The final recovery of 6.3% of chitinase activity represents only the activity of purified chitinase C1 of the total chitinase activity in the carrot roots which comprised several chitinases.

### **3.4. Physical properties**

The enzyme preparation after HPLC separation was homogeneous, as judged by HPLC gel filtration chromatography (Fig. 3.7, A), SDS-gel electrophoresis (Fig. 3.7, B) and analytical IEF (Fig. 3.7, C). It was an acidic protein with an isoelectric point of 4.3 (Fig. 3.7, C), and a molecular weight

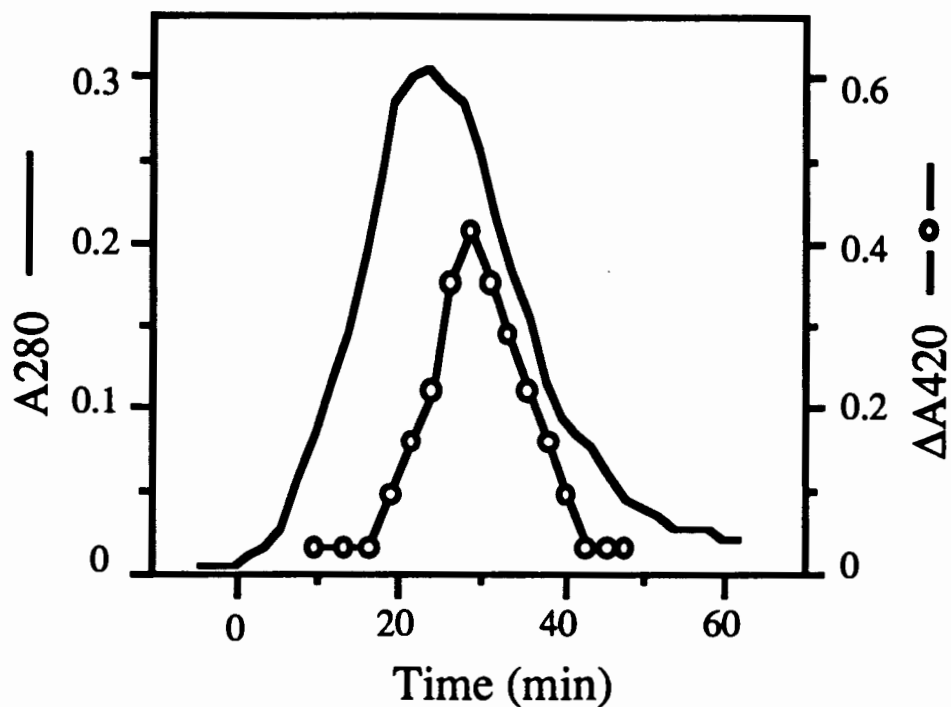


Fig. 3.4. Hydroxylapatite chromatography. Chitinase fractions from DEAE-cellulose chromatography corresponding to peak III were put onto a hydroxylapatite column equilibrated with 50 mM Na phosphate buffer (pH 7), and eluted with the same buffer. Fractions containing chitinase activity were pooled.

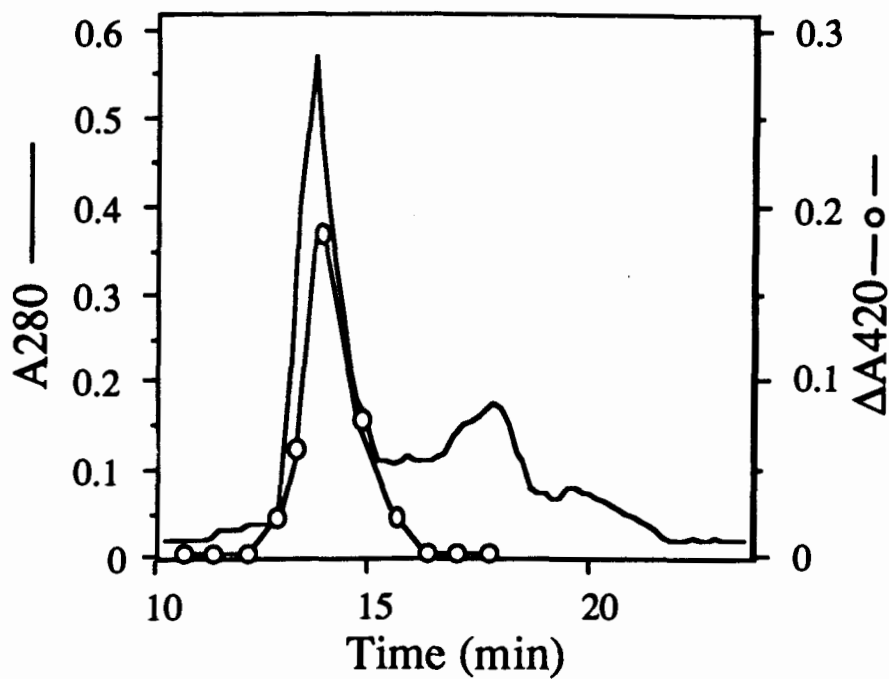


Fig. 3.5. Protein Pak 125 HPLC. Chitinase samples obtained from hydroxylapatite chromatography were put onto a Protein Pak 125 HPLC column previously equilibrated with 20 mM Na phosphate buffer (pH 7), and eluted with the same buffer. Fractions containing chitinase activity were pooled.



**Fig. 3.6. Purification profile on SDS-PAGE. Lane 1:  $(\text{NH}_4)_2\text{SO}_4$  precipitation (25  $\mu\text{g}$ ); lane 2: CM-Sephadex (25  $\mu\text{g}$ ); lane 3: DEAE-cellulose (25  $\mu\text{g}$ ); lane 4: hydroxylapatite (5  $\mu\text{g}$ ), and lane 5: Protein Pak HPLC (1  $\mu\text{g}$ ). M: prestained molecular mass markers.**

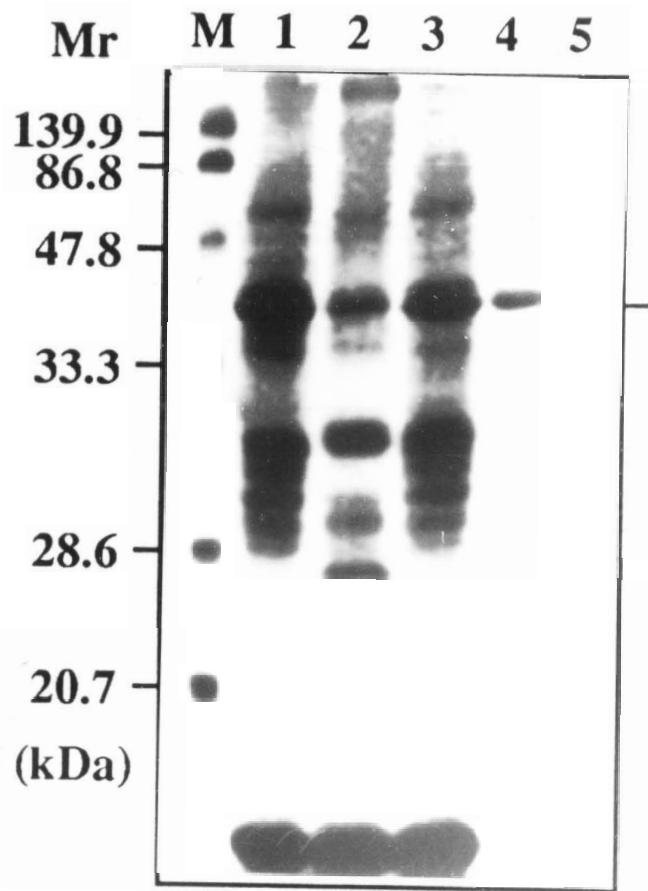
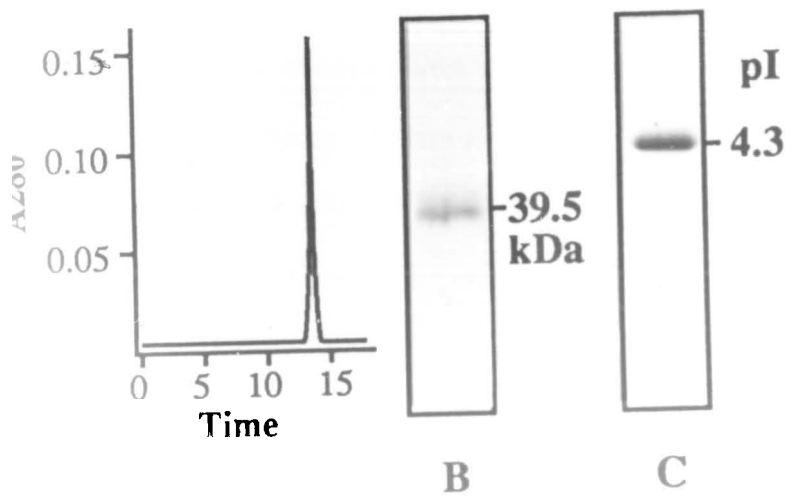


Table 3.1. Purification steps for a chitinase from carrot root.

Purification step	Total protein (mg)	Total units	Specific activity (U/mg protein)	Chitinase recovered(%)	Purification (-fold)
Crude extract	7346	6380	0.9	100	1
Heat + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1114	3560	3.2	55.8	4
CM-Sephadex	624	2720	4.4	42.6	5
DEAE-cellulose	152	1960	12.8	30.7	14
Hydroxylapatite	4	460	115.0	7.2	128
Protein Pak 125	2.4	400	166.7	6.3	185

**Fig. 3.7. Purity test. The purity of the purified chitinase was tested on HPLC (A, 1 $\mu$ g), SDS-PAGE with silver stain (B, 0.1  $\mu$ g), and analytical IEF (C, 1 $\mu$ g).**



of 39,500 (Fig. 3.6). The enzymatic activity of the purified chitinase was shown on a glycol chitin containing gel overlaid on a gradient gel after electrophoresis (Fig. 3.8).

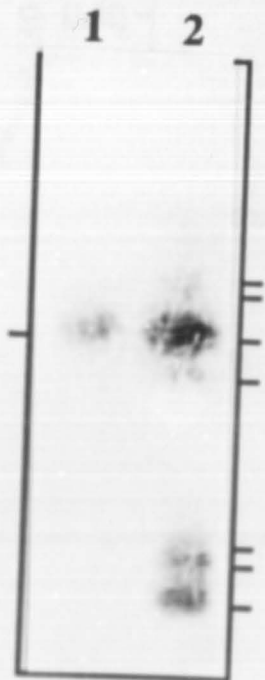
### **3.5. Effect of pH**

The C1 chitinase had a broad pH range between pH 4 to 6 with an optimum at pH 5 (Fig. 3.9). Less than 50% of maximal enzyme activity (at pH 5) was detected above pH 8. The effect of pH on the stability of the enzyme was tested by incubating the enzyme with buffers at different pHs for 24 h at 37 °C and the remaining activity was measured after adjusting to pH 5 (Fig. 3.10). The remaining activity of protein incubated at pH 5.5 was used as 100% to calculate the relative activity. The enzyme was relatively stable at pH below 8. At higher pH (> 8), the enzyme was unstable and drastically lost its enzymatic activity.

### **3.6. Effect of temperature**

C1 chitinase was most active at temperatures between 10 °C to 41 °C, with the optimum temperature for enzymatic activity assay at around 25 °C (Fig. 3.11). The enzyme was incubated at pH 5 for 30 min. at various temperatures ranging from 4 °C to 80 °C and the remaining activity was measured at 37 °C using glycol chitin (Fig. 3.12). The enzyme activity of the sample kept at 4 °C was used as 100% to calculate the relative activity. The enzyme was relatively stable at temperatures below 60 °C, and lost more than 90% activity at 80 °C.

**Fig. 3.8. Enzymatic activity of the purified chitinase. Purified protein (lane 1, 1  $\mu\text{g}$ ) and sample from DEAE-cellulose (lane 2, 25  $\mu\text{g}$ ) were loaded onto a gradient gel (10-15%). Bands with chitinolytic activity were visualized on a glycol chitin containing overlay gel after fluorescent staining. Bars indicating the position of the bands.**





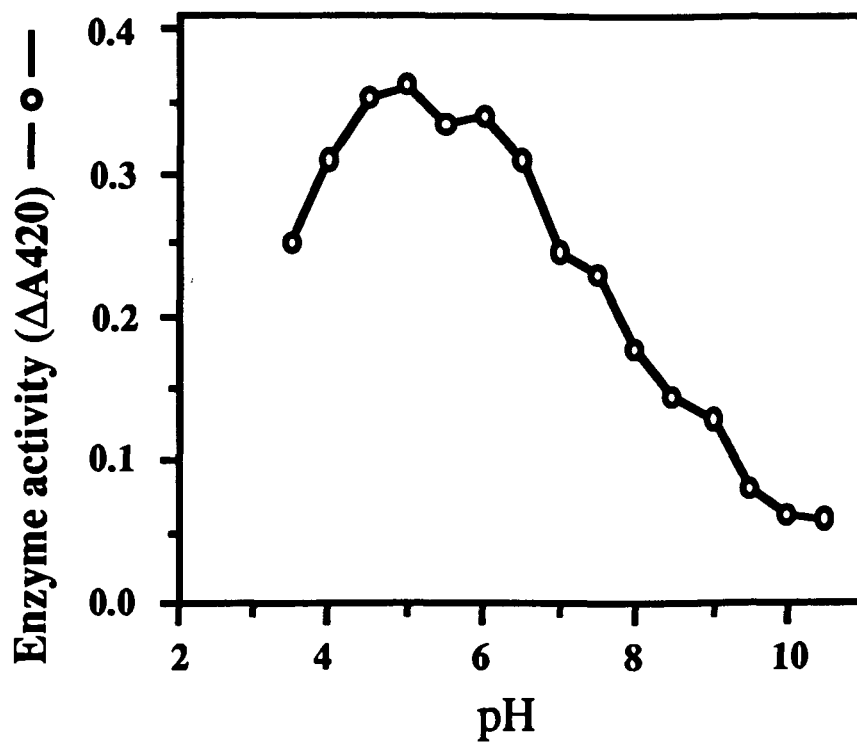


Fig. 3.9. The effect of pH on chitinase activity. A mixture of the purified chitinase and glycol chitin in buffers at different pH was incubated at 37 °C for 30 min, and the activity was measured at 420 nm.

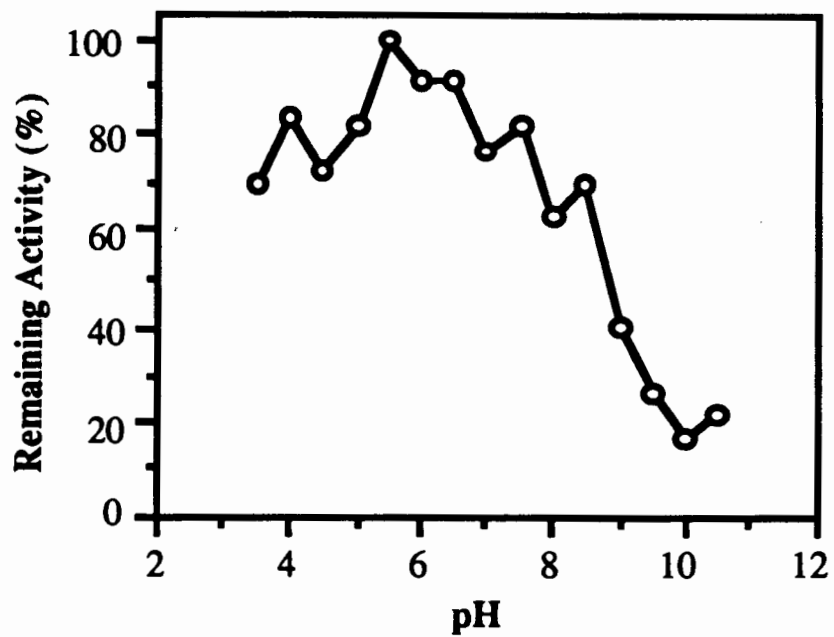


Fig. 3.10. The effect of pH on chitinase stability. The enzyme was incubated at 37°C for 24 h in buffers at different pH. The remaining activity was measured at pH 5.

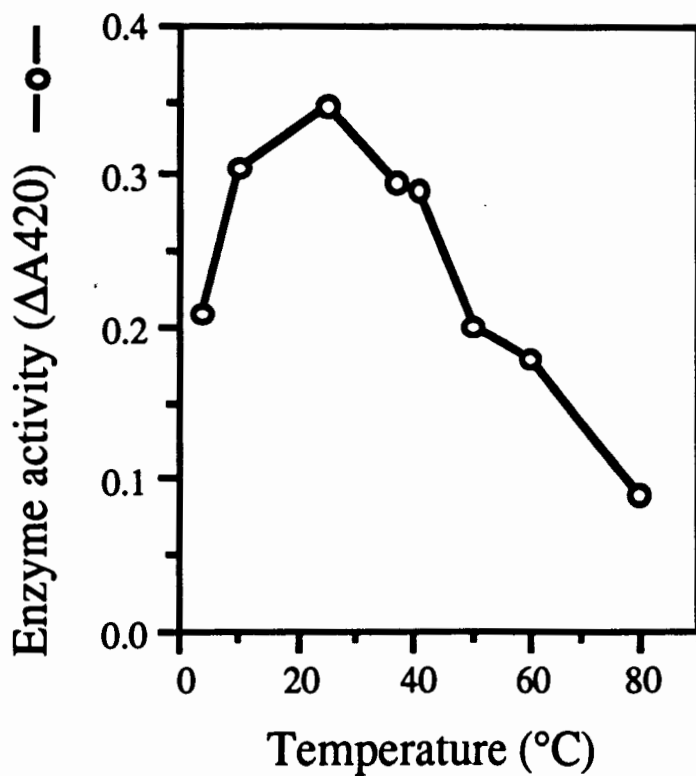


Fig. 3.11. The effect of temperature on chitinase activity. The enzyme reaction mixture was incubated at different temperatures for 30 min, and the activity was measured at 420 nm.

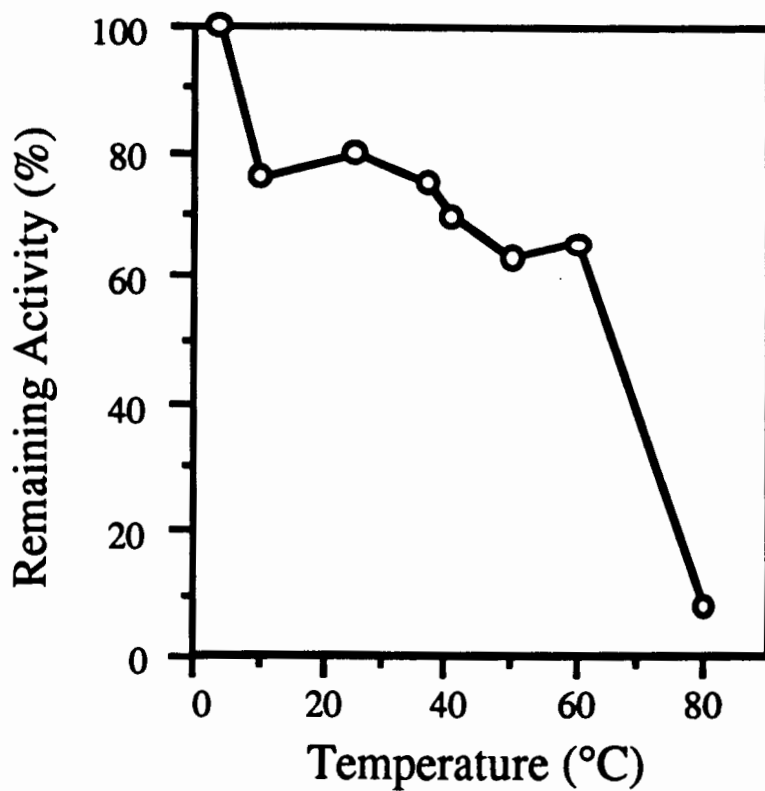


Fig. 3.12. The effect of temperature on chitinase stability. The enzyme solution in 0.1 M Na acetate (pH 5) was heated at various temperatures for 1 h. After addition of glycol chitin, the mixture was incubated at 37 °C for 30 min and the remaining activity was measured.

### **3.7. Regenerated chitin affinity chromatography**

The protein did not have a chitin binding domain, since it was not retained on the regenerated chitin column (data not shown).

### **3.8. Amino acid composition and N-terminal sequence**

The amino acid composition of chitinase C1 is shown in Table 3.2. It was estimated that there are 320 amino acid residues. The amino acid composition (percentage) of chitinase C1 was similar to that of tobacco class I chitinase, with a high content of Asx/Gly and Cys/Pro, and was different from that of the 32 kDa protein from carrot which has a high Ser content and is low in Pro.

The N-terminal of this purified protein was blocked. The N-terminal was de-blocked with pyroglutamate aminopeptidase (Boehringer Mannheim, Canada) as described by LeGendre et al. (1993) and sequenced with Edman reaction. The N-terminal amino acid sequence of this protein did not resemble the N-terminal sequences of other known classes of chitinases (Table 3.3).

### **3.9. Lysozyme activity and antifungal activity**

No lysozyme activity was found when the protein sample was tested on native PAGE/overlay gel or directly on petri plates containing the lyophilized cells of *Micrococcus lysodeikticus* (data not shown).

The antifungal activity of chitinase C1 was tested on *Trichoderma* (Fig. 3.13). When the *Trichoderma* spores were mixed with the enzyme (Fig. 3.13

**Table 3.2. Amino acid composition of chitinase C1 from carrot compared with published sequences for different classes of plant chitinases.**

Amino Acid	Carrot C1 Residues	C1 mol%	Tobacco Class I <sup>1</sup>	Tobacco Class II <sup>2</sup>	Cucumber Class III <sup>3</sup>	Sugar beet Class IV <sup>4</sup>	Carrot 32 kD <sup>5</sup>
Asx	32	10.1	11.3	15.3	13.9	9.1	15.9
Thr	23	7.3	4.9	7.4	2.2	5.0	6.0
Ser	22	6.9	8.3	4.8	11.6	10.0	10.7
Glx	18	5.5	6.3	8.3	6.7	7.5	8.1
Pro	24	7.6	7.3	4.4	4.9	3.7	2.9
Gly	36	11.1	14.9	12.2	10.9	11.2	12.4
Ala	26	8.2	7.0	8.7	11.2	10.8	9.5
Cys	22	6.9	5.7	2.2	2.2	6.2	5.3
Val	16	5.1	2.7	3.9	5.2	5.8	5.0
Met	1	0.4	1.3	1.3	1.1	0.4	0.1
Ile	20	6.2	5.0	7.0	5.6	4.6	3.2
Leu	20	6.2	4.7	3.5	12.4	3.3	3.6
Tyr	12	3.8	3.7	4.8	3.0	5.0	5.2
Phe	19	5.8	4.7	5.2	5.2	7.1	5.3
His	3	1.0	1.3	0.9	0.7	1.7	1.8
Lys	17	6.9	2.3	2.2	3.4	1.2	3.3
Arg	9	2.8	6.0	6.6	0.7	6.2	2.5
Trp	n.d.	n.d.	2.7	1.3	2.6	1.2	n.d.

1. Shinshi et al. 1990.
  2. Payne et al. 1990.
  3. Métraux et al. 1989.
  4. Mikkelsen et al. 1992.
  5. De Jong et al. 1992.
- n.d.: not determined.

**Table 3.3. Published N-terminal amino acid sequences of chitinases from plants compared with C1 chitinase from carrot.**

<b>Class</b>	<b>Sequence</b>
Carrot C1	ELNNGLDKQA LLQI
I	:QCGRQAGG: :CPNGNCCSQ
II	QGIGSIVTND :FNEMLKNRN
III	GGIAIYWGQN GNEGNLSATC
IV	QNCG----- -CASNLCCSR
V	QRCGSQGGGG TCPALWCCSI
VI	QNVIKGGYWF KDSGLALNNI

**Class I:** bean (Broglie et al. 1986).

**Class II:** *Petunia* (Linthorst 1990).

**Class III:** *Arabidopsis* (Samac et al. 1990).

**Class IV:** sugar beet (Mikkelsen et al. 1992).

**Class V:** *Urtica dioica* (Lerner and Raikhel 1992).

**Class VI:** tobacco (Melchers et al. 1994).

: indicates an identical amino acid in the same position as in chitinase C1.

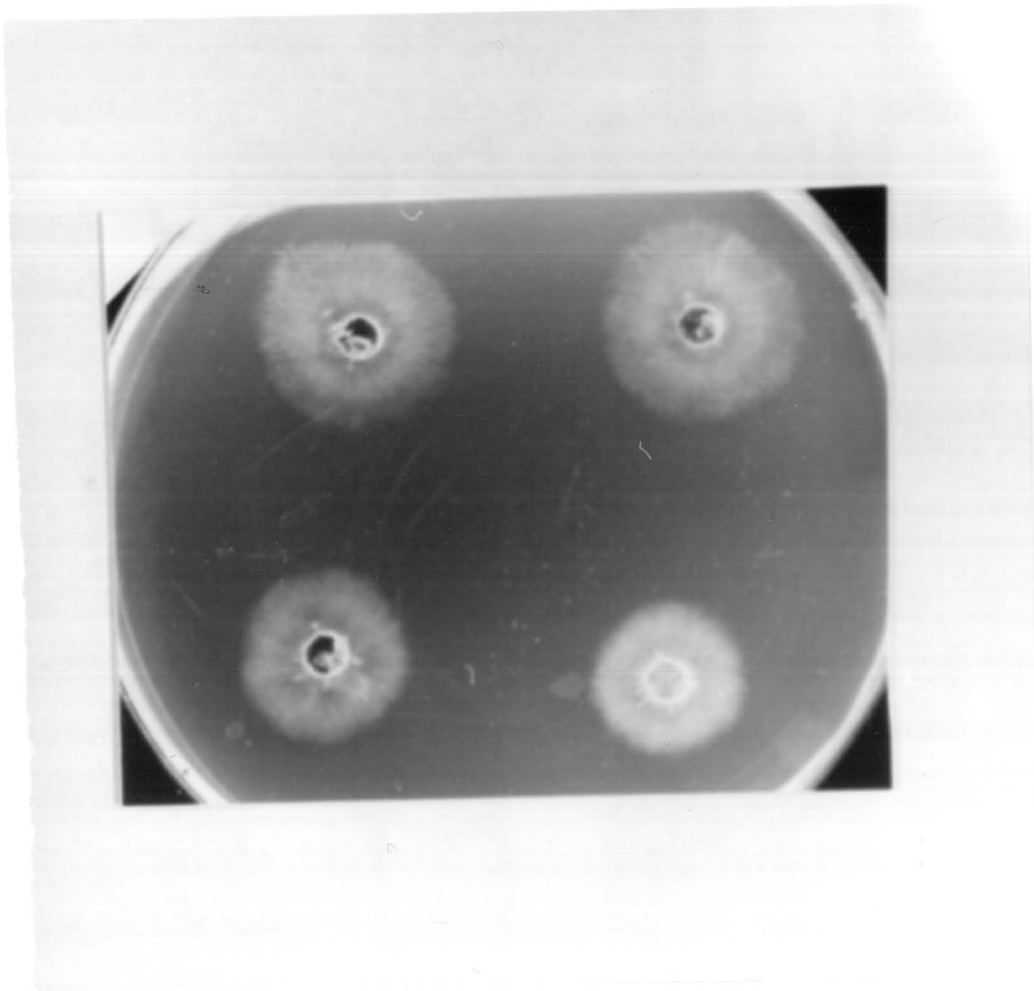
- indicates a deletion.

**A|B**  
**C|D**

**Fig 3.13. Antifungal activity of the purified chitinase from carrot roots.**

The purified chitinase was mixed with *Trichoderma* spores and put into wells in a petri dish containing 1.5% and 2% malt extract agar. The petri dish was incubated at 30 °C for 3 days and photographed. A: water control; B: boiled chitinase, 5 µg; C: chitinase, 2 µg; D: chitinase, 5 µg.





101B

C, D), smaller colonies were produced compared with those of the controls treated with dH<sub>2</sub>O or boiled chitinase (Fig. 3.13. A, B), which indicated an inhibitory effect on mycelium growth of *Trichoderma* (Fig. 3.13).

#### **4. Discussion**

It has been shown that higher plant species can produce several chitinases which may belong to different classes (Collinge et al. 1993). In tobacco, chitinases belonging to classes I, II, III, and VI have been reported so far. Here, we report that multiple isozymes also occur in carrot roots. It is likely that both constitutive and induced isozymes were detected in this study since the roots were harvested and injury during harvesting may induce chitinases (Punja and Zhang, 1993). The molecular weights of the major chitinases reported here, estimated at 22,200 to 39,500 by SDS-PAGE, were comparable to those measured by other investigators (Kurosaki et al. 1990) in tissue cultured cells (24,500 to 43,500 estimated by gel filtration). Carrot chitinases may belong to different classes since they showed different cross-reactivity profiles with class I to III antisera in this study. However, additional work is needed to further classify these chitinase isoforms in carrot.

The chitinase C1 from carrot showed no reaction to the antisera raised against class I to III chitinases from other plant species. Purification of this chitinase was carried out with temperature treatment, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation, ion exchange, hydroxylapatite, and HPLC. The chitinase C1 is clearly different from the 32 kDa class IV chitinase isolated from carrot cell

suspensions (De Jong et al, 1992; Kragh et al, 1994) with respect to its different molecular weight, amino acid composition, and chitin-binding ability. The amino acid composition of this isoform revealed a high content of Cys/Pro, which is similar to the class I tobacco basic chitinase; however, it may not belong to class I since it did not react with the tobacco chitinase antiserum, and did not have a cysteine-rich chitin binding domain, which is essential for chitin binding activity (Iseli et al, 1993). The N-terminal sequence furthermore did not resemble any of the known classes of chitinases. Additional information, such as internal sequence, is needed to place this chitinase into the appropriate class.

The optimum temperature for chitinase C1 was around 25 °C, which is lower than that reported for most of the chitinases (Nehra et al, 1994; Osswald et al. 1994). This may be due to the different origins of plant tissues and environmental conditions under which the tissues grow. Carrot roots in the soil would normally be exposed to lower ambient temperatures, and a lower optimal temperature for enzymatic activity would enable the enzyme to function properly. In leaves, the optimum temperature for activity would be expected to be higher, as has been reported for other plant chitinases (Nehra et al, 1994).

The biological function of the C1 isoform in carrot can only be speculated. No lysozyme activity was observed on lyophilized cells of *Micrococcus*. The purified enzyme was not contaminated by  $\beta$ -glucanase (data not shown) and had some antifungal activity against *Trichoderma*. It was not tested for embryogenesis rescue activity as reported by De Jong et al. (1992).

It is still far from clear what roles chitinases have in higher plants, since no substrate has been found to date, although a potential substrate occurs in fungal pathogens. The antifungal activity of these enzymes *in vitro*, the elicitive compounds released by their hydrolytic activity, and the induction and correlation with fungal infection and disease resistance, all point to the possible involvement of chitinases in plant-fungal interactions and plant defense (Punja and Zhang, 1993). In carrot, chitinases were induced by incubation with fungus and fungal filtrate (Kurosaki et al. 1990), and elicitor activity was found in the partial hydrolysates of mycelial walls released by chitinase (Kurosaki et al. 1988). Here we have shown that a chitinase from carrot roots has some antifungal activity *in vitro*. In the rhizosphere, carrot roots are constantly challenged by many soil-borne microorganisms, and the production and induction of chitinases may help to partially protect the roots. Multiple isoforms may confer an advantage, since fungal-specific isoforms may be present in the plant to distinguish a pathogen from other stress responses (Kurosaki et al, 1990). Additional work is required to elucidate the roles of the other chitinase isoforms detected in this study.

## Chapter IV

### Summary, General Discussion and Future Research

This research was aimed at characterizing chitinase isoforms in cucumber and carrot. In cucumber (*C. sativus*), multiple chitinase isoforms are expressed constitutively, and these may vary among different cultivars. The isoforms are induced by a number of biotic and abiotic factors, and the induction seems to be non-specific. At least three new isoforms were induced by various inducing agents, and the isoform banding patterns were similar to each other. It is not clear what role these chitinases have. Chitinase activity has been reported previously in cucumber seeds (Majeau et al. 1990), cotyledons, true leaves, and roots. Chitinase isoform patterns in true leaves and roots were found to be similar to those in cotyledons, and no tissue-specific isoforms were found in cotyledons, true leaves, and stems. All cucumber chitinases appear to be acidic isoforms in tissues such as roots, cotyledons, and true leaves; however, in seeds, basic isoforms have been reported (Majeau et al. 1990). The basic chitinase activity disappears after seed germination (Majeau et al. 1990). The expression of the different isoforms can be assigned to three groups (low constitutive, enhanced constitutive and newly induced) which may have different functions in plant defense.

A major cucumber chitinase has been shown to be an acidic, extracellular protein which also has lysozyme activity (Boller and Métraux

1988; Métraux et al. 1988). The molecular weight of this protein is around 26,000-28,000. A cDNA encoding this inducible, acidic chitinase has been isolated (Métraux et al. 1989). The deduced amino acid sequence of the encoded protein has no similarity to class I and class II chitinases, but has significant sequence homology to chitinases from *Parthenocissus*, *Hevea*, and *Arabidopsis*, and is classified as a class III chitinase. The the acidic class III chitinase coding region has been isolated and characterized (Lawton et al. 1994).

The class III acidic chitinase is the most abundant chitinase produced in cucumber leaves and roots, if not the only chitinase class produced. It contributes to about 90% of the chitinase activity in true leaves (Métraux and Boller 1986). Considerable basic chitinase activity has been detected in seeds (Majeau et al. 1990). The basic chitinase activity seems to be seed-specific and disappears after seed germination (Majeau et al. 1990). The acidic and basic proteins are speculated to be class I and III chitinase (Graham and Sticklen 1994), however, class III chitinases are found to be either acidic or basic (Lawton et al. 1992). What is the role of these basic chitinases and why would they be present in seeds specifically? Why would the basic chitinase activity vanish after seed germination and the acidic chitinase activity stay low in many plant tissues? How would the basic isoforms compare with those acidic isoforms in terms of effectiveness against fungal pathogens? Since a number of constitutive and induced chitinase isoforms are present in cucumber, it would be interesting to compare constitutive isoforms versus induced isoforms as to their

accumulation, distribution, enzymatic activity, substrate specificity, and antifungal activity.

It is still not clear why some plants express a particular class or a combination of classes of chitinases. Studies are still to be done on whether class III chitinase (such as cucumber chitinase) may be synergistic with other PR proteins, such as  $\beta$ -glucanase, or for other means of defense.

Chitinases from cucumber were found to have antifungal activity against some fungal pathogens. The induction of chitinase following pathogen infection is correlated with SAR (Métraux et al. 1989). However, conflicting results have been reported when studying *C. lagenarium* infected cucumber. Métraux and Boller (1986) found that the extent of disease resistance was correlated with the extent of chitinase induction in the second leaves, while Irving and Kuc (1990) observed no significant correlation between the two. How chitinase may be involved in defense in cucumber remains to be addressed.

Most studies on chitinases in carrot have been conducted with suspension cultured cells (De Jong et al. 1992; Kurosaki et al. 1987, 1990). We studied chitinases in carrot using whole plant material. Multiple isoforms of chitinases were detected from mature carrot roots on both native and SDS-PAGE. There were 8 bands detected on native PAGE (4 major bands, 2 intermediate, and 2 weak bands, based on the visual intensity of the bands) and 10 chitinolytic bands (5 major bands and 5 minor bands)

visualized on SDS-PAGE gel. The molecular weight of these major chitinases was estimated at 22,200 to 39,500 on SDS-PAGE, which is comparable with those identified by other investigators (Kurosaki et al. 1990) in tissue cultured cells (24,500 to 43,500 estimated by gel filtration).

Higher plants may produce several classes of chitinases in the same tissue (Collinge et al. 1993). Classes I, II, III, and VI chitinases have been reported so far in tobacco leaves. Since carrot chitinases have shown differential immuno-reaction towards antisera raised against class I, II, and III chitinases from other plant species, it is possible that these carrot chitinases may belong to several different classes (Chapter III, Section 3.2). However, more work is needed to characterize these isoforms and classify them into appropriate classes.

A major carrot chitinase, which showed no reaction to the antisera raised against class I to III chitinases, with a relatively high molecular weight, has been purified to homogeneity (Chapter III, Section 3.3). It is an acidic protein with pI at 4.3 and a molecular weight of 39,500. This protein does not have a chitin-binding domain, but is similar to tobacco class I chitinase in its amino acid composition. The N-terminal amino acid sequence did not resemble any of the described classes of chitinases. This chitinase did not possess any lysozyme activity. Additional information is needed to classify this chitinase, especially the amino acid sequence and the functional domains. Since it is estimated that this protein contains only one methionine in its amino acid composition, it may be a good start to obtain some internal amino acid sequence by using cyanogen bromide cleavage. It



may be very useful in cloning the encoding gene, because these internal sequences can be used to generate primers for polymerase chain reaction (PCR) or RACE.

In cultured carrot cells, chitinase activity has been reported to be expressed constitutively at a low level, but is significantly enhanced when incubated with fungi or other fungal cell wall components (Kurosaki et al. 1987). It is interesting to note that whole fungal culture induces more isoenzymes than the culture filtrate only (Kurosaki et al, 1990), which suggests that the presence of fungi is necessary to induce additional chitinase isoforms. The purified C1 chitinase has been showed to have antifungal activity against *Trichoderma* sp. However, the involvement of chitinases in carrot disease defense is still to be shown.

It has been reported that a chitinase from carrot is capable of rescuing a temperature sensitive embryo mutant and was suggested to have a role in carrot somatic embryo development (De Jong et al. 1992). Although the actual mechanism of the rescuing is not clear and there is no similar report from other plant species, the possibility of chitinase having a role in plant development is attracting some attention and is worthy of further study.

Since chitinases often exist as a multi-enzyme system in higher plants, it is very difficult to evaluate the role of chitinases in plant defense based upon the study of individual chitinases. The problem is also compounded by the fact that disease resistance is commonly contributed by multiple

factors. Chitinases may not be equally important in plant defense against a whole spectrum of fungal pathogens. It may be not as effective in combatting pathogens, such as *Pythium*, due to the fact that chitin is not an important component of the cell wall of this fungal genus which contrasts with the case in many other fungi.

Although there is strong evidence for the involvement of chitinase in plant defense, some key questions, such as what role it might play, at what extent the plant defense is dependent upon the production of chitinase, and what the mechanism for chitinase's involvement is in the defense, are still far from clear. Increasing the production of a certain class (or a combination) of chitinase in a particular plant-pathogen interaction might be more effective over the others. Chitinases alone are probably not enough to control many fungal diseases, and therefore, enhancing chitinase production alone in plants by gene transformation may not achieve the goal of protecting plants from an array of pathogens. Recently reports have shown that the introduction of a chitinase gene along with other defense genes, such as  $\beta$ -glucanase encoding genes, is more effective than chitinase alone (Cornelissen and Melchers 1993; Zhu et al. 1994). This change in strategy may illustrate a new direction for chitinase research and its potential application in disease resistance.

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