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The interaction between pseudomonas syringae pv. tomato and the lectin from tomato (Lycopersicon esculentum) a possible role in determining pathogen specificity.

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THE INTERACTION BETWEEN *PSEUDOMONAS SYRINGAE* PV. *TOMATO* AND THE LECTIN FROM TOMATO (*LYCOPERSICON ESCULENTUM*): A POSSIBLE ROLE IN DETERMINING PATHOGEN SPECIFICITY

by

Jamie Seymour Pitts

A Thesis

submitted to the Faculty of Graduate Studies

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Windsor, Ontario, Canada

1985

Approved:

Hugh B Fockwell

David A. Cobb

N F Taylor

This thesis is dedicated to my parents

and the memory of the late Mrs. Stephanie Brown.

ABSTRACT

A lectin isolated from the tomato fruit using a 50% ammonium sulphate precipitation, was the same as that previously isolated by Nachbar *et al* (1980) and Kilpatrick (1980). The tomato lectin had a molecular weight of 140,000, as determined by gel filtration, and was specific for oligomers of N-acetylglucosamine. As well as being a hemagglutinin, the lectin was a bacterial agglutinin, which agglutinated a wide range of bacterial species, including *Pseudomonas syringae* pv.*tomato* (*P.tomato*). The specific activity of the lectin was 80 times greater for bacterial agglutination than hemagglutination. Bacterial agglutination titres were dramatically increased when divalent cations were present, and mimiaked the prozone phenomenon, known to occur between an antigen and antibody.

P.tomato produced an exopolysaccharide in addition to levan, which completely inhibited its agglutination by the tomato lectin. A lipopolysaccharide extract of *P.tomato* also inhibited bacterial agglutination, to the same degree.

In consideration of the lectin recognition model, developed by Sequeira and Graham (1977), it seems likely that *P.tomato* is pathogenic to the tomato plant at least partly because it avoids recognition (lectin binding to the bacterial lipopolysaccharide). The ability to avoid recognition is imparted by the exopolysaccharide, which competitively inhibits the lectin from binding the bacterial lipopolysaccharide.

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LIST OF ABBREVIATIONS

AG, agglutination (buffer); BA, bacterial agglutination; BSA, bovine serum albumin; EPS, exopolysaccharide; HA, hemagglutination; HR, hypersensitive response; IgG immunoglobulin G; LPS, lipopolysaccharide; OA, ovalbumin; PBS, phosphate– buffered saline; YSM, yeast salts medium; YSMG, yeast salts medium supplemented with 5% (w/v) glucose; YSMS, yeast salts medium supplemented with 5% (w/v) sucrose.

INTRODUCTION

Since first reported by Okabe (1933), research on bacterial speck of tomatoes has progressed from the mere reporting of the causative organism, *Pseudomonas syringae* pv. *tomato* in the field, to the development of tomato plant cultivars resistant to the pathogen. However, the specific interaction that occurs between the tomato plant cells and *Pseudomonas syringae* pv. *tomato* is still poorly understood even though the initial confrontation between a potential pathogen and a host cell plays an important role in determining the direction in which the interaction will proceed. Studies conducted so far, have focused mainly on the offensive tactics of the bacterium, the results of which can be summarized briefly. *Pseudomonas syringae* pv. *tomato* is capable of entering the tomato plant via wounds in the leaves or open stomata (Bashan, Okon and Henis, 1978). Having breeched the cuticle of the leaf surface, a primary defensive barrier of the plant, *Pseudomonas syringae* pv. *tomato* causes a disease condition characterized by small necrotic lesions on fruit with encircling chlorotic halos when present on leaves. *Pseudomonas syringae* pv. *tomato* produces a thermostabile, extracellular toxin, capable of causing chlorotic halo formation (Garber and Schaffer, 1963) and ammonia which, at levels of 100 ug/ml produces necrosis on leaves (Bashan, Okon and Henis, 1980).

We are still ignorant of the interaction between the plant and pathogen in the time between entrance of the bacterium into the plant and the appearance of disease symptoms, a time span of approximately 100 hours (Bashan *et al*, 1981). Some of the questions which have therefore been left unanswered are:

1) Does the tomato plant offer any resistance at the subsurface cell layer, to invading microorganisms?
2) If some defensive measure is taken by the tomato plant, what is its nature?

3) How does a pathogen such as *Pseudomonas syringae* pv. *tomato* circumvent this defensive tactic of the plant?

Studies by Sing and Schroth (1977), Sequeira *et al* (1977) and others (see literature review) indicate that a specific recognition system operates in many plants. In those plant systems studied, lectins present on the surface of leaf mesophyll cells were capable of binding to a specific carbohydrate that may be present on the surface of the invading bacterium. The presence of this specific receptor for the lectin resulted in the binding of the bacterium to the mesophyll cell, followed by the immobilization of the bacterium by a dense granular material, as observed by electron microscopy. This immobilization prevented the multiplication of the bacteria and therefore its further spread throughout the plant. Some bacteria were capable of circumventing this recognition either by the absence of this specific receptor on their surface or by the masking of this receptor. *Pseudomonas syringae* pv. *solanacearum*, a pathogen of the potato plant, avoids recognition by masking its specific receptor for the potato lectin with an exopolysaccharide (EPS) which competitively inhibits the binding of the bacteria by the lectin (Sequeira and Graham, 1977). This defensive measure taken by the bacteria allow them to continue multiplying and spreading throughout the plant.

The intention of this thesis was to study the possibility of such a recognition system operating between the tomato plant and *Pseudomonas syringae* pv. *tomato* and determine its significance in the disease process.

LITERATURE REVIEW

Bacterial Speck of Tomato

The incidence of bacterial speck of tomato (*Lycopersicon esculentum* Mill.), caused by the bacterium *Pseudomonas syringae* pv. *tomato,* herein referred to as *P.tomato,* has steadily increased over recent years. Since first reported by Okabe (1933) in Taihoku Japan, the disease has become recognized as a worldwide problem. Figure 1 illustrates the global distribution of the incidence of bacterial speck and was prepared by collecting all known published occurrences of the disease.

The symptoms of the disease appear as lesions on the aerial portions of the tomato plant, spreading from the leaves near the ground to the younger leaves near the top of the plant, and finally the fruit. Early leaf lesions appear as small water soaked areas, which later become dark and necrotic, reaching a size of approximately 2–5 mm, and develop encircling chlorotic halos (figure 2). Lesions on the fruit are somewhat smaller, approximately 1 mm is size, dark in color and rarely develop any deeper than the skin of the tomato (figure 3). For this reason, economic losses in tomato crops are confined mainly to fresh market tomatoes although severe infection may also decrease the quality of processing tomatoes (Schneider *et al,* 1975).

The close resemblance of the symptoms of bacterial speck and bacterial spot, caused by the bacterium *Xanthomonas campestris* pv. *vesicatoria,* has lead to confusion in early disease diagnosis, thereby delaying control measures. A better understanding of the ecology and epidemiology of bacterial speck of tomato has therefore become a study of utmost importance.

The bacterium *P.tomato,* is a Gram negative, strictly aerobic, motile rod, capable of levan production in the presence of sucrose. It is negative for starch hydrolysis, oxidase activity, trehalose utilization, arginine dihydrolase and growth at

FIGURE 1

The global distribution of bacterial speck of tomatoes.

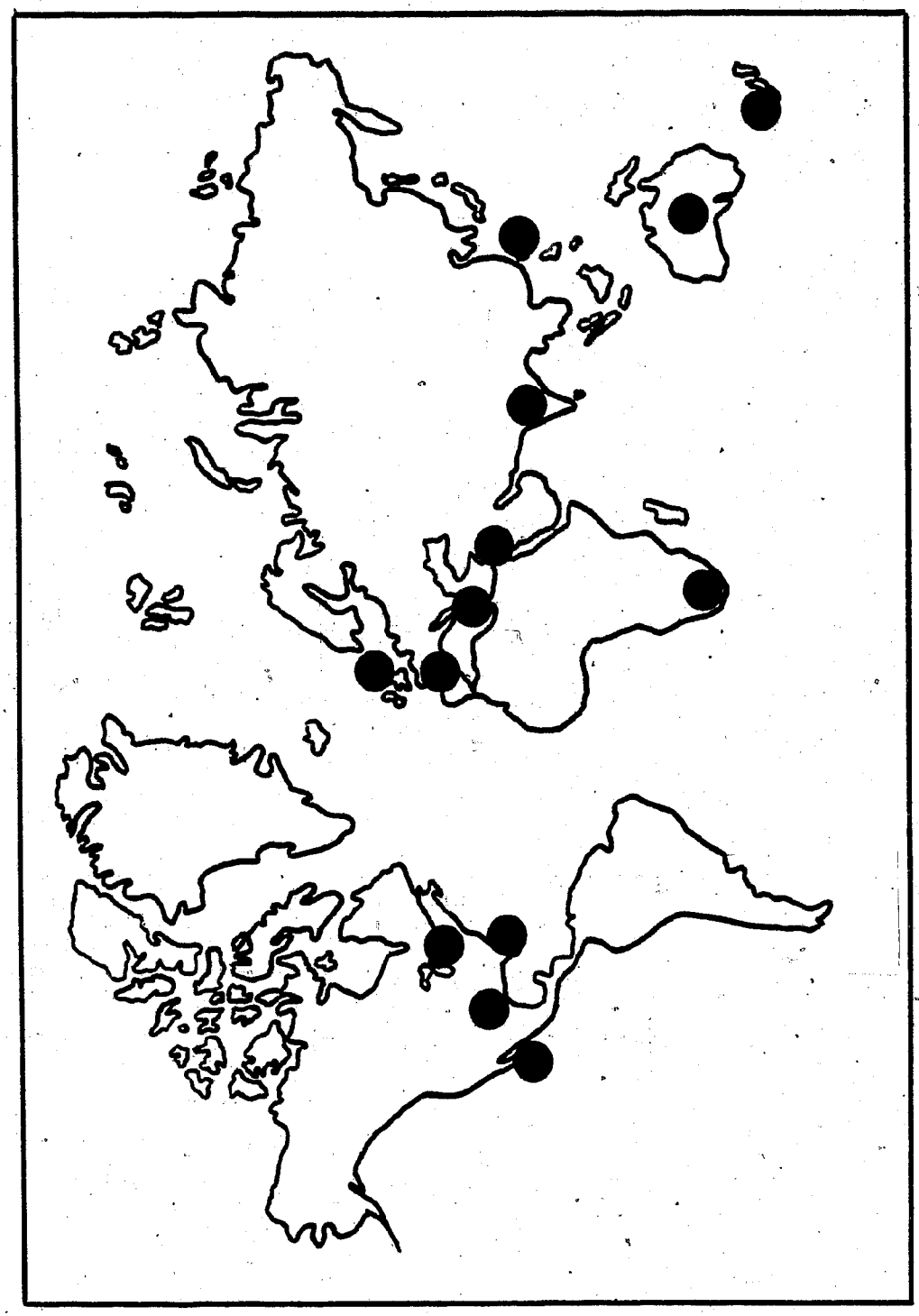
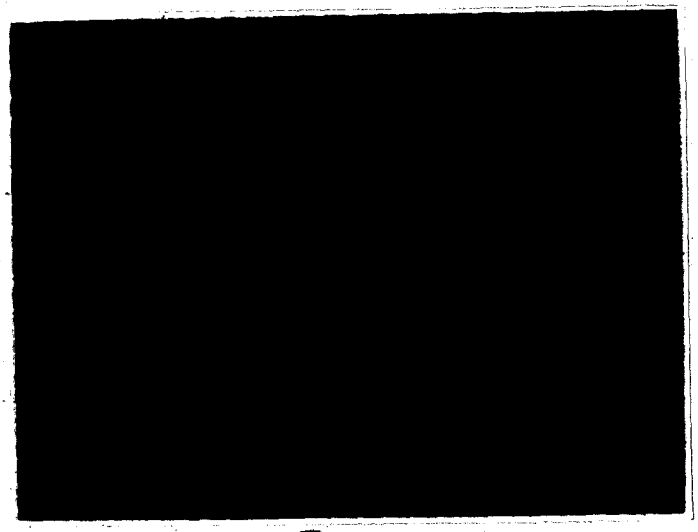


FIGURE 2

Typical speck lesion found on leaves of tomato plants infected with *Pseudomonas syringae* pv. *tomato*.



COLOURED PICTURES
Images en couleur

FIGURE 3

Typical speck lesions found on fruit of tomato plants infected with *Pseudomónas syringae* pv. *tomato*.

COLOURED PICTURES
Images en couleur

41°C (Doudoroff and Palleroni, 1974). Levan, the exopolysaccharide produced by *P.tomato,* requires sucrose as a precursor in its synthesis. The synthesis of levan occurs by the successive addition of β–fructosyl residues in a 2–6 linkage to the terminal glucosyl residue of the sucrose molecule (Sutherland, 1972). Since in the absence of sucrose, the growth of *P.tomato* is unaffected, the EPS in not necessary for the survival of the bacterium (Sutherland, 1972)

The several pathovars (pv.) of *Pseudomonas syringae*, of which pv. *tomato* is one, have been named on the basis of the plant they were isolated from and are pathogenic to, although the entire host range of each pathovar has yet to be determined (Doudoroff and Palleroni, 1974). *P.tomato* belongs to the fluorescent group of phytopathogenic pseudomonads, which produce a fluorescent pigment on Kings B medium (King *et al*, 1954). The flurocscent pigment, fluorescein, is a green, water soluble, chloroform insoluble, pteridine pigment (Stanier *et. al*, 1966). Colonies of phytopathogenic pseudomonads are examined for fluorescence with a 366 nm ultraviolet lamp, after growth at 27°C for 48 hours.

P.tomato produces an extracellular, low molecular weight, thermostabile toxin which causes chlorotic halo formation but not necrosis, on leaves (Garber and Schaffer, 1963). High yields of toxin are obtained after 48 or 72 hours of growth at 18°C, but much lower levels at 25°C to 30°C, the optimal growth temperature for *P.tomato* (Okon, Bashan and Henis, 1978). Okon and coworkers found that the toxin of *P.tomato* was produced when cells were grown in nutrient broth, yeast peptone broth and on synthetic media using galactose, sucrose, lactose, maltose, mannitol or peptone as the single carbon source.

P.tomato produces a second toxic factor which is responsible for the necrotic lesions typical of bacterial speck. Upon purification of filtrates of *P.tomato* grown in broth culture, ammonia was detected at levels of 120ug/ml, which when applied

to leaf surfaces, caused necrosis (Bashan, Okon and Henis, 1980). High levels of ammonia were also detected in extracts of tomato leaves infected with *P.tomato*. The authors noted a very high electrolyte leakage from the infected plant cells at a time (120 hours following inoculation of *P.tomato*), coinciding with the highest point of ammonia accumulation.

Studies by the same authors (Bashan, Okon and Henis, 1978), involved the factors affecting the infection and spread of *P.tomato* on the tomato plant. Minimal suspensions of 10^4–10^5 cells/ml were required to produce disease symptoms, which could be seen five days after inoculation into tomato leaves. Lesion formation was enhanced by wounding the plant leaves before inoculation with carborundum powder or by spraying with dilute wax solvents. Incubation of tomato plants under mist for 24 hours or at 40°C for 30 minutes prior to inoculation also increased lesion formation. Scanning electron micrographs, taken following wounding and misting, showed damage to epidermal cells and open stomata. Further electron microscopy studies (Bashan et al, 1981) traced the infection path of *P.tomato* in the tomato plant. Large microcolonies of *P.tomato* first congregated in specific areas such as the stomata and base of leaf trichomes. Following incubation, the bacteria moved from the leaf surface to the intercellular spaces and necrotic lesions were visible 100 hours after inoculation. All tissues which border the necrotic lesions were filled with bacteria but the necrotic area itself was free of bacteria.

Field studies conducted over a two year period (Yunis *et al*, 1980b) demonstrated that bacterial speck develops and spreads only at temperatures between 13°C and 28°C under high humidity. Plants inoculated with *P.tomato* and maintained under dry conditions in greenhouses, showed no spread of symptoms to new leaves (Schneider and Grogan, 1977a). Increased humidity and/or frequent

misting resulted in the spread of lesions to new leaves, demonstrating the involvement of open stomata and possibly free moisture in leaves, in disease development.

Uninjured tomato fruit are most susceptible to infection by *P.tomato* in the period after anthesis and before the fruit reaches a diameter of 3 mm (Getz *et al*, 1983). These authors were the first to demonstrate that entry of *P.tomato* into the tomato plant need not occur through stomata or leaf wounds. Scanning electron micrographs provided evidence that, until the cuticle develops, openings that remain after trichomes are shed, provide natural entry points for *P.tomato*.

For several years, attention focused on the means of transmission of *P.tomato* from plant to plant and resulted in several conflicting reports. Chambers and Merrriman (1975) reported that *P.tomato* survived in plant debris on or 2 cm below the soil surface for a period of up to 25 weeks in Austrailia. Seeds which had been subjected to the commercial extraction process were free of contamination.

Of 10 commercial seed lots studied by Okon *et al* (1979), all showed signs of contamination and when grown in pasteurized soil under controlled conditions, gave rise to infected plants. The pathogen survived on seeds which were dried at 40°C and stored at room temperature for one year. Seed borne *P.tomato* was erradicated when seeds were heated above 40°C for 30 minutes. The authors also noted that in greenhouse studies, *P.tomato* inoculated into pasteurized soil, survived there for periods of up to seven months. All plants grown from surface sterilized seeds in that soil, developed bacterial speck.

In contrast to the findings of Okon *et al* (1979), Schneider and Grogan (1977a) could find no *P.tomato* contamination in 10 lots of commercial seed tested by them, but found *P.tomato* to be ubiquitous in soil, occurring in the rhizosphere of

many weed and crop plants in California. To determine whether or not soil borne *P.tomato* could move to leaves of the tomato plant, infected leaves were dried and buried in pots of pasteurized soil. Either surface sterilized seeds or uninfected seedlings were planted in this soil and maintained under controlled (greenhouse) conditions. All plants developed bacterial speck even though infecton of tomato plants by *P.tomato* has been previously shown not to occur through the roots or stem of the tomato plant (Bashan *et al*, 1978).

During hot, dry periods of up to 14 days, *P.tomato* often resides on the tomato leaf surface without producing disease symptoms (Schneider and Grogan, 1977a). Schneider and Grogan refer to this phase as the "resident" phase and have shown that this resident phase produces disease symptoms on these leaves upon rewetting of the leaves and a decrease in temperature. Microscopic examination on the leaf surface demonstrated that intact, non-living trichomes serve as the habitat for this resident phase during periods of drying (Schneider and Grogan, 1977b).

Goode and Sasser (1980) believe that ineffective seed treatment by commercial suppliers has been one of the major reasons for the spread of bacterial speck. *P.tomato* can be erradicated from the the contaminated seeds by immersion in water at 52°C for one hour (Devash *et al*, 1980). If *P.tomato* is a ubiquitous soil organism though, as Schneider and Grogan (1977a) suggest, seed treatment alone would be ineffective. Okon and coworkers have found that weekly spraying of tomato plants during all stages of growth, with $Cu(OH)_2$ completely prevents disease development, however spraying plants already infected with *P.tomato* could only reduce disease symptoms. Conlin and McCarter (1983) confirmed the effectiveness of $Cu(OH)_2$ in controlling bacterial speck, but also suggest the use of streptomycin alone or in combination with the fungicides

mancozeb and chlorothalonil.

Yunis *et al* (1980a) reported on the development of 2 cultivar of tomato, resistant to bacterial speck. Based on field tests, cultivar Rehovot–B is highly resistant to *P.tomato* and cultivar Hosen–Eilon is moderately resistant. Pilowsky and Zutra (1982) have found that 10 of 19 wild strains of tomato studied, were resistant to their isolate of *P.tomato*, and that cultivars Rehovot–B and Hosen–Eilon said to be *P.tomato* resistant (Yunis *et al*, 1980), were susceptible to their *P.tomato* isolate.

The primary concern in the control of bacterial speck, as with any other plant disease, is clearly, the early detection of the causative organism. While weekly sprayings with $Cu(OH)_2$ or streptomycin may be effective in preventing bacterial speck, it can be a tremendous and often unnecessary expense. A rapid serological technique for the early detection of *P.tomato* in the soil or on the plant may provide a useful screening system before control measures are taken. In consideration of such a system, Fackrell and Sinha (1983) have made several observations concerning the serology of *P.tomato*. The authors employed three serological methods; bacterial agglutination, double diffusion and immunofluorescence, to study the antisera of the soluble and insoluble antigens of *P.tomato*. The strains of *P.tomato*, PTD and PT–7 appear to be serologically very similar, with respect to the insoluble somatic (O) and flagellar (H) antigens. Cross reactivity with other Gram negative bacteria could be reduced by employing long injection schedules. Of the several soluble antigens of *P.tomato*, five were extracellular and cross reactive with the supernatants of other Gram negative bacteria. Extracts of *P.tomato* infected and uninfected leaves were diffused against the various antisera developed, but precipitin lines were observed with both. Fackrell and Sinha suggest that only 1 serotye of *P.tomato* exists, but do so

cautiously since only 2 strains (PTD and PT-7) were tested.

Serological studies by Jones *et al* (1983), on 26 strains of *P.tomato* isolated from within North America, resulted in their suggestion of 2 distinct serovars. By employing Ouchterlony double diffusion, bacterial agglutination and indirect immunofluorescence, 20 of the *P.tomato* isolates were classed as serovar I, and 5 as serovar II. One *P.tomato* isolate did not fit into either serovar classification.

Plant Lectins

Lectins obtained primarily from plant extracts are "sugar binding proteins or glycoconjugates of non-immune origin, which agglutinate cells and/or precipitate glycoconjugates" (Goldstein *et al*, 1978). The existence of these carbohydrate binding proteins in various plant species has long been recognized, but their physiological role in the plant has until recently, been unknown. The majority of lectin studies have dealt with the exploitation of their carbohydrate binding specificity to characterize the surface of cells they bind. Several reviews have been published which deal specifically with this aspect of lectin research (Sharon, 1977; Liener, 1976; Sharon and Lis, 1972).

One of the first groups to study the interaction between a lectin and bacterium, was Sumner and Howell (1936), who discovered that a jack bean extract agglutinated species of *Mycobacterium*. Since then, many lectins have been characterized according to their ability to agglutinate bacteria (Pistole, 1981).

The interaction that occurs between the lectins of leguminous plants and species of *Rhizobium* in the symbiotic development of nitrogen fixing root nodules, is a familiar one. This interaction is a specific one, in which the host range of each *Rhizobium* species is very limited and mediated by the lectin of the host (Bhuvaneswari *et al*, 1977). A lectin named trifolin, that has been isolated from

clover, occurs in highest concentrations near the end of root hairs , the infection site of rhizobia (Brill, 1977). This lectin specifically agglutinates ***Rhizobium trifolii***, the species responsible for root nodulation in clover. Similar situations exist for several other legume–rhizobium systems (Hamblin and Kent, 1973; Bohol and Schmidt, 1974). The binding site for the lectin on the ***Rhizobium*** cell surface is as yet not certain, but is thought to be the capsule of the bacterium (Calvert *et al*, 1978).

This situation represents a type of lectin–bacterium interaction in which the binding of the bacterium by the plant lectin results in the formation of a symbiotic relationship between plant and bacterium. The result of a lectin–bacterium interaction though, is not always so favourable for the bacterium. Most meetings between potential pathogens and host plant are incompatible ones, in that a resistant, hypersensitive response (HR) is elicited by the plant (Klement, 1963). A hypersensitive response is characterized by rapid tissue necrosis around the infection site, as a result of rapid death of invaded cells, as well as cells in the immediate area. Compatible pathogens are those which either avoid or inhibit the hypersensitive response. Klement (1963) noted that saprophytic bacteria when introduced into the tobacco leaf, do not induce the hypersensitive response.

Goodman and coworkers (1976) were among the first groups to provide evidence for a type of recognition system operating in tobacco leaves. Saprophytic (*Pseudomonas fluorescens*), incompatible (*Pseudomonas syringae* pv. *pisi*) and compatible (*Pseudomonas syringae* pv. *tabaci*) bacteria were inoculated separately into tobacco leaf tissue and observations made electronmicroscopically, 2, 4 and 6 hours later. Both incompatible and saprophytic bacterial cells were immobilized, by attachment to mesophyll cell walls, but the hypersensitive response was induced only by the incompatible cells. Ultrastructural changes in the plant cell leading up

to the hypersensitive response included a rapid separation of the cuticle from the cell wall where bacteria were attached, which later became progressively thicker and surrounded the bacteria. The detachment and thickening of the cuticle was accompanied by the movement of vesicles to the plant cell surface and the development of fibrils which appeared to aid in immobilization of the bacteria. The tobacco pathogen did not attach to plant cell walls and remained free in the intercellular spaces and multiplied.

The attachment of saprophytic bacteria to mesophyll cell walls without the induction of the hypersensitive response is a common occurrence in the plant recognition sytems studied so far (Stall and Cook, 1979; Sing and Schroth, 1977; Sequeira *et al*, 1977). Apparently, the attachment of bacteria to the cell wall does not necessarily cause the hypersensitive response, but may be necessary for it.

A series of detailed studies of *Pseudomonas syringae* pv. *solanacearum* (= *P. solanacearum*), pathogen of several plant species including tobacco, potato and tomato, revealed some of the molecular aspects involved in recognition. Virulent strains of *P.solanacearum* produce copious amounts of an exopolysaccharide (EPS), while avirulent strains produce little or none at all (Husain and Kelman, 1958). Lozano and Sequeira (1970) observed that avirulent strains of *P.solanacearum*, when introduced into tobacco leaves, induce the hypersensitive response, but no hypersensitive response was elicited by the virulent strains. Electron microscopy studies revealed that attachment of avirulent strains to tobacco leaf mesophyll cells accompanied the hypersensitive response and as expected, virulent strains remained unattached, multiplied in the leaf tissue and did not elicit the hypersensitive response (Sequeira *et al*, 1977). Surprisingly, not all avirulent strains of *P.solanacearum* attached to cell walls.

The lectin of tobacco (Lamport, 1980) is identical to the potato lectin (Allen

and Neuberger, 1973) and both lectins are specific for oligomers containing N–acetylglucosamine.

The EPS of *P.solanacearum* is a heteropolysaccharide rich in N–acetylglucosamine (Sequeira, 1980). Avirulent (non EPS producing) strains of *P.solanacearum* are readily agglutinated by the potato lectin but all virulent (EPS) producing strains are not (Sequeira and Graham, 1977). When an EPS extract of the virulent cells was added to a suspension of avirulent cells, agglutination by the potato lectin was inhibited. Some avirulent strains of *P.solanacearum* are agglutinated by the potato lectin more readily than others (Sequeira and Graham, 1977). Those avirulent strains which agglutinate more readily are "rough" mutants which lack a portion of the O specific chain in their LPS and produce a hypersensitive response in tobacco leaves. Avirulent strains which do not readily agglutinate are "smooth" in that they possess at least a portion of the O specific side chain and these strains do no elicit the hypersensitive response in tobacco leaves (Whatley *et al*, 1979). Apparently, the presence of the O specific side chain hinders the binding of the lectin to some other portion of the LPS, either the core or lipid A regions. The core and lipid A regions of the LPS are fairly constant amongst those Gram negative bacteria so far studied (Luderitz *et al*, 1971). Of the five basal sugars which comprise the core region of Gram negative bacteria, N–acetylglucosamine is the most common.

These results prompted Sequeira and Graham (1977) to propose a recognition model for *P.solanacearum* in the potato plant. Three elements appear to be involved in the recognition of *P.solanacearum*, the bacterial LPS, bacterial EPS and the potato lectin. Virulent strains of *P.solanacearum* escape recogniton (hypersensitive response) by producing an EPS which competitively inhibits the potato lectin from binding the bacteria via its LPS. The induction of the

hypersensitive response by avirulent cells is at least partly due to the binding of the lectin to the core or lipid A region of the bacterial LPS. Although this recognition model seems to be a sound one, Sequeira points out several inconsistencies which warrant consideration (Sequeira, 1980). Some isolates of *P.solanacearum* produce a hypersensitive response in tobacco leaves even though they produce an EPS which inhibits agglutination of avirulent cells by the lectin. In addition, while LPS serves as a potent inhibitor of the hypersensitive response (Graham *et al*, 1977), EPS does not.

Preliminary studies by others (Romeiro *et al*, 1981; Goodman *et al*, 1976) provide further evidence for this type of recognition system (for review see also Pistole, 1981; Callow, 1977; Lippincott and Lippincott, 1980). Fett and Sequeira (1980b) have demonstrated though, that not all plant lectins exhibit such a specific recogniton behavior. A bacterial agglutinin isolated from seeds of the soybean cultivar Clark (Fett and Sequeira, 1980a) does not agglutinate avirulent strains of the pathogen *Xanthomonas phaseoli* var. *sonjensis*, even though ultrastructural studies show that it attaches to cell walls of soybean leaves.

A lectin recently isolated from tomato (Kilpatrick, 1980; Nachbar *et al*, 1980) has a specific affinity for oligomers containing N-acetylglucosamine, but different molecular weight estimates have been reported. Kilpatrick (1980) found the tomato lectin to be approximately 200,000 daltons in non-denaturing conditions and possibly composed of subunits of 74,000 daltons as determined by polyacrilamide gel electrophoresis using sodium dodecyl sulphate. Nachbar and coworkers determined their tomato lectin had a molecular weight of 71,000 daltons and found no evidence of any other form. The tomato lectin is a glycoprotein, stable at 65°C and immunologically cross reactive with the lectin isolated from thorn apple, another solanaceous plant (Kilpatrick, 1980). Nachbar *et al* (1980) reported that

the tomato lectin is stable in PBS at –20°C for 5–6 months, at 4°C for several weeks, at room temperature for 96 hours and in lyophilized form indefinitely. Although the tomato lectin is antimitogenic for mouse and chicken lymphocytes (Nachbar *et al*, 1980), no physiological role for the tomato lectin in the plant has been given.

MATERIALS AND METHODS

Bacterial Cultures

Cultures of *P.tomato* (PT–7 Ohio) were obtained from Dr. D.L. Coplin (Ohio Agricultural Research and Development Center, Wooster, Ohio, 44691, U.S.A.). The 11 isolates of various phytopathogens used for bacterial agglutina tion assays were obtained from Dr. G. Bonn (Agriculture Canada, Harrow Research Station, Harrow, Ontario, CANADA, NOR 1G0). Cultures of *Escherichia coli*, *Agrobacterium tumefasciens* and *Rhizobium leguminosarum* were obtained from the Departmental culture collection. All bacterial cultures were maintained at 4°C on slants prepared with Lowe agar (Holder–Franklin, 1981): proteose peptone, 3 g/L; $MgSO_4.7H_2O$, 50mg/L; $FeCl_3.H_2O$, 5 mg/L; K_2HPO_4, 200mg/L and Bacto agar (Difco Laboratories, Detroit, Michigan, 48232, U.S.A.), 15.0 g/L. In preparation for experiments, bacteria were transferred to plates prepared with yeast salts medium (YSM): NaCl, 0.5 g/L; $MgSO_4.7H_2O$, 200 mg/L; K_2HPO_4, 500 mg/L; $NH_4H_2PO_4$, 200 mg/L; yeast extract (Difco Laboratories), 5.0 g/L and Bacto agar (Difco Laboratories), 15.0 g/L. In experiments which required exopolysaccharide (EPS) production, the YSM medium was supplemented with 5% w/v sucrose and the cells incubated for 96 hours at room temperature. The effect of the addition of 5% w/v glucose was also studied with respect to EPS production.

The cells used for agglutination assays were grown in the YSM medium described above without agar. The liquid medium was inoculated by loop transfer from cultures growing on YSM plates and was shaken in a rotating shaking incubator for 24 hours at room temperature. The cells were harvested by centrifugation at 10,000 x *g* for 30 minutes and the supernatant discarded. The cell pellet was resuspended in 10 volumes of an agglutination (AG) buffer (pH 7.4) consisting of 0.1 M NaCl, 0.7 mM $CaCl_2$, 0.7 mM $MnCl_2$, 0.7 mM $MgCl_2$ and 10 mM Tris. The suspension was recentrifuged at 10,000 x *g* for 30 minutes and the

pellet washed twice more with the AG budfer. The final pellet was resuspended in the buffer at approximately 2.0 x 10^9 cells/ml. Cell numbers were estimated spectrophotometrically from a standard curve prepared using McFarland standards (McFarland, 1970).

Isolation of Lectin from the Tomato Fruit

Five kilograms of ripe fruit from the tomato plant (*Lycopersicon esculentum* Mill.) cultivars "Beefsteak" and "Best Boy" were purchased from a local fresh fruit market. Lectin isolation from the fruit of each cultivar was conducted separately using the method of Kilpatrick (1980). The fruit was rinsed in distilled water and homogenized in a Waring blender. The homogenate was then centrifuged at 1000 x *g* for 20 minutes and the supernatant passed through cheesecloth to remove all the pulp. An equal volume of saturated ammonium sulphate was added to the supernatant and the mixture left to sit overnight at 4°C. The precipitate that resulted was collected by centrifugation at 40,000 x *g* for 1 hour at 4°C and resuspended in 100 millilitres of phosphate–buffered saline (PBS). Phosphate–buffered saline consisted of 145 mM NaCl and 10 mM sodium phosphate buffer (pH 7.2) The crude extract was then dialysed against PBS for 48 hours at 4°C, with a change of buffer after 24 hours. The undissolved precipitate was discarded and the lectin extract frozen.

Gel Filtration of Crude Lectin Extract

Bio–Gel P–200 (mesh 100–200) beads (Bio–Rad Laboratories, Richmond, CA., 94804, U.S.A.) were used to construct a gel filtration column according to the manufacturers recommended procedure (Bio–Rad Laboratories, 1980). Twenty–five milligrams of dry gel were hydrated in 600 millilitres of PBS, heated to 90°C, and

then allowed to cool to room temperature. Following hydration for one hour, the gel suspension was deaerated under reduced pressure overnight. The column was prepared at 4°C with the following specifications: total volume (Vt) = 450 ml, void volume (Vo) = 115 ml, column diameter = 2.5 cm, bed height = 92 cm and flow rate = 18ms/hr. The molecular weight markers used were rabbit IgG (Sigma Chemical Company, St. Louis, MO., 63178, U.S.A.), bovine serum albumin (BSA) (Sigma) and ovalbumin (OA) (Sigma). The 3 molecular weight markers were prepared to 1.0 mg/ml in distilled water and the 3 mixed 1:1:1 and applied to the column as a 9 millilitre sample. The elution buffer used was PBS and 4 millilitre fractions were collected. Fractions collected were analysed for protein concentration using the Bio-Rad One Step protein assay (Bradford, 1976), which utilizes Coomassie Blue G-250 dye. The standard protein used was IgG.

A 9 millilitre sample of the crude lectin was applied to the column and using PBS as the elution buffer, 4 millilitre fractions were collected and the protein concentrations as well as hemagglutination (HA) titre of each fraction was determined.

Lectin Concentration in Crude Extract

To estimate the relative concentration of the crude extract, a protein determination of the extract using the Bio-Rad One Step protein assay, was done before and after complete absorption of all lectin activity. Ten millilitres of a 2% erythrocyte suspension was centrifuged at 500 x *g* for 10 minutes and the supernatant discarded. Two millilitres of the crude lectin extract was added to the packed erythrocytes, the cells were resuspended in the extract and the suspension was incubated at room temperature for 30 minutes. The erythrocyte suspension was then centrifuged at 500 x *g* for 10 minutes and the supernatant removed. The

supernatant was then assayed for protein concentration (as done previously) and, as well, assayed for HA titre to ensure all the lectin was removed by absorption. The original crude lectin extract was assayed similarly. The protein concentration determined for the absorbed extract was subtracted from that of the original crude extract to establish the lectin concentration.

Hemagglutination (HA) Assay

Human blood (types A, B and O) was collected from volunteers in sterile 5 millilitre vacutainers containing 7.5 mg EDTA (Becton Dickinson and Co., Mississauga, Ontario, CANADA, L5J 2M8). Prior to use, the erythrocytes were washed three times in 6 to 10 volumes of PBS. The concentration of the erythrocytes is expressed as a percentage, indicating the volume of packed cells (ml) (500 x *g* for 10 min.) per 100 millilitres of final suspension. The washed erythrocytes were resuspended to 2% v/v in PBS.

All agglutination assays were carried out in Cooke Microtitre plates (Dynatech Laboratory Products, Alexandria, Virginia, 22314, U.S.A.) with U–shaped wells.

A twofold dilution of an initial 100 microlitre volume of tomato lectin solution was prepared in PBS. To each well, 50 microlitres of a 2% suspension of erythrocytes, type A, B or O, were added. A control for the assay consisted of 50 microlitres of the 2% erythrocyte suspension in 50 microlitres of PBS. The assay was performed in duplicate and plates were incubated at 4°C for one hour. The assay was rejected if spontaneous agglutination occurred in the control wells.

The hemagglutination titre for each blood type was taken to be the reciprocal of the highest dilution of lectin showing hemagglutination.

Carbohydrate Specificity

Various carbohydrates were tested for their ability to inhibit hemagglutination. A twofold dilution series of an initial 100 microlitre volume of tomato lectin was prepared with PBS. To each well, 50 microlitres of a 2% v/v suspension of erythrocytes was added, followed by the addition to each row of a 50 microlitre solution of either glucose (50mM), sucrose (50 mM), galactose (50 mM), fructose (50mM), N–acetylglucosamine (50mM) or N,N',N"–triacetylchitotriose (10mM). The control for the assay consisted of a twofold dilution series of the lectin to which was added 50 microlitres of the 2% erythrocyte suspension and 50 microlitres of PBS. The assay was performed in duplicate and the plates were incubated at 4°C for 1 hour. The assay was rejected is no agglutination occurred in the control wells.

Bacterial Agglutination (BA) Assay

All bacterial cultures, including *P.tomato*, were grown in YSM broth and washed three times with the AG buffer and resuspended in that buffer at 2.0 x 10^9 cells/ml.

A twofold dilution of an initial 100 microlitre volume of tomato lectin solution was prepared in the AG buffer. To each well, 50 microlitres of a 2.0 x 10^9 cells/ml suspension of each bacterial isolate was added separately. A control for the assay consisted of 50 microlitres of each bacterial suspension added separately to 50 microlitres of the AG buffer. The assay was performed in duplicate and plates were then incubated at room temperature overnight. The assay was rejected if spontaneous agglutination occurred in the control.

To determine if the bacterial agglutinin and hemagglutinin were synonymous, all the HA activity of the lectin solution was adsorbed with increasing numbers of

PT–7 Ohio cells. Bacteria (PT–7 Ohio) grown in YSM broth for 48 hours at room temperature were washed 3 times in the AG buffer (10,000 x *g* for 30 minutes) and resuspended in the buffer. Several cell suspensions of increasing cell numbers were added to 1 millilitre of lectin solution and left at room temperature overnight. The mixture was then centrifuged at 10,000 x *g* for 30 minutes to pellet the bacteria and the supernatant assayed for HA activity. To discount the possibility of lectin denaturation at room temperature overnight, a sample of the lectin solution was set out overnight as a control, and the HA titre taken before and after incubation.

Exopolysaccharide Extraction

Bacterial cells (PT–7 Ohio) were grown on plates of YSM, YSM with 5% glucose or YSM with 5% sucrose plates at room temperature for 96 hours. The plate surfaces were scraped and the cells suspended separately in 25 millilitres of the AG buffer. The cell suspensions were shaken vigorously to loosen the EPS from the cells and then centrifuged at 10,000 x *g* for 15 minutes. The 3 supernatants were assayed for carbohydrate content using the anthrone reaction (Shields and Barnett, 1974) and glucose as the standard carbohydrate and then retained at 4°C for later bacterial agglutination (BA) inhibition studies. The cell pellets were resuspended in distilled water and a total cell count taken spectrophotometrically, using the previously described standard curve (McFarland, 1970). The amount of EPS/cell from the 3 different media was then determined.

Lipopolysaccharide Extraction

The extraction procedure for lipopolysaccharide (LPS) was done according to the method of Westphal and Jann (1965). Approximately 3 litres of a 96 hour culture of PT–7 Ohio cells was centrifuged at 10,000 x *g* for 15 minutes, the cell

pellet resuspended in 500 millilitres of ice cold PBS and recentrifuged. The bacterial pellet was then suspended in 350 millilitres of 68°C distilled water to which was added 350 millilitres of a 90% (w/v) phenol solution at the same temperature. The suspension was kept at 68°C for 15 minutes and stirred vigorously, then immediately transferred to an ice bath until the temperature of the mixture reached 10°C. The mixture was then centrifuged at 10,000 x *g* for 30 minutes. After removing the upper water layer, another 350 millilitres of distilled water was added to the remaining phenol layer and again maintained at a temperature of 68°C for 15 minutes while stirred. The mixture was centrifuged again and the aqueous layer removed and pooled with the first aqueous layer obtained. The pooled aqueous phases were dialysed against distilled water at 4°C for 3 days, with 3 changes of distilled water over that time. The dialysed solution was then concentrated from an initial volume of 400 millilitres to 100 millilitres using an Amicon ultrafiltration system fitted with a PM–30 membrane filter and the LPS concentrate then stored at 4°C until required.

Inhibition of Bacterial Agglutination

A) Exopolysaccharide Inhibition

A twofold dilution series of an initial 100 microlitre volume of the crude tomato lectin extract was prepared in AG buffer. To two rows of wells, 50 microlitres of EPS solution from bacteria grown on either YSM, YSM with 5% glucose or YSM with 5% sucrose was added. To each well, 50 microlitres of a 2.0 x 10^9 cells/ml suspension of PT–7 Ohio cells grown in YSM broth was added. A control for the assay consisted of a twofold dilution series of the tomato lectin to which was added 50 microlitres of AG buffer and 50 microlitres of the 2.0 x 10^9

cells/ml PT–7 Ohio cell suspension. The assay was performed in duplicate and the plates were incubated at room temperature overnight. The assay was rejected if no agglutination was visible in the control wells. The BA titre was taken to be the reciprocal value of the highest dilution of lectin showing agglutination.

A) Lipopolysaccharide Inhibition

A twofold dilution series of an initial 100 microlitre volume of tomato lectin crude extract was prepared with AG buffer. To each well, 50 microlitres of the LPS concentrate was added, followed by the addition of 50 microlitres of a 2.0 x 10^9 cells/ml PT–7 Ohio suspension. A control for the assay consisted of a twofold dilution series of lectin to which was added 50 microlitres of AG buffer and 50 microlitres of the PT–7 Ohio cell suspension. The assay was performed in duplicate and the plates were incubated overnight. The assay was rejected if no agglutination occurred in the control wells. The titres were taken to be the reciprocal of the highest dilution of lectin showing agglutination.

RESULTS

CHAPTER 1

CHARACTERIZATION OF THE TOMATO LECTIN

Erythrocyte Specificity

The crude lectin extract obtained from ammonium sulphate precipitation was dialysed against PBS (0.01 M, pH 6.8) for 48 hours at 4°C and then tested for hemagglutinin activity. Hemagglutination tests were carried out in duplicate by performing a 2 fold dilution series of the extract in microtitre plates, followed by the addition to each well, of an equal volume of a 2% v/v erythrocyte suspension, type A, B, or O. All 3 erythrocyte types were found to be agglutinated by a component of the extract, and as well, an HA titre of 128 was observed for each blood group (table 1). No spontaneous hemagglutination was observed in the control wells, which consisted of equal volumes (50uL) of the erythrocyte suspension and PBS (0.01 M). The protein concentration of the crude lectin extract was determined to be 2.75 mg/ml, using the Bio–Rad One Step protein assay. Using this value and that of the HA titre, the specific HA activity of the crude extract was 46.5 HA/mg for all 3 major blood types tested.

Molecular Weight Determination

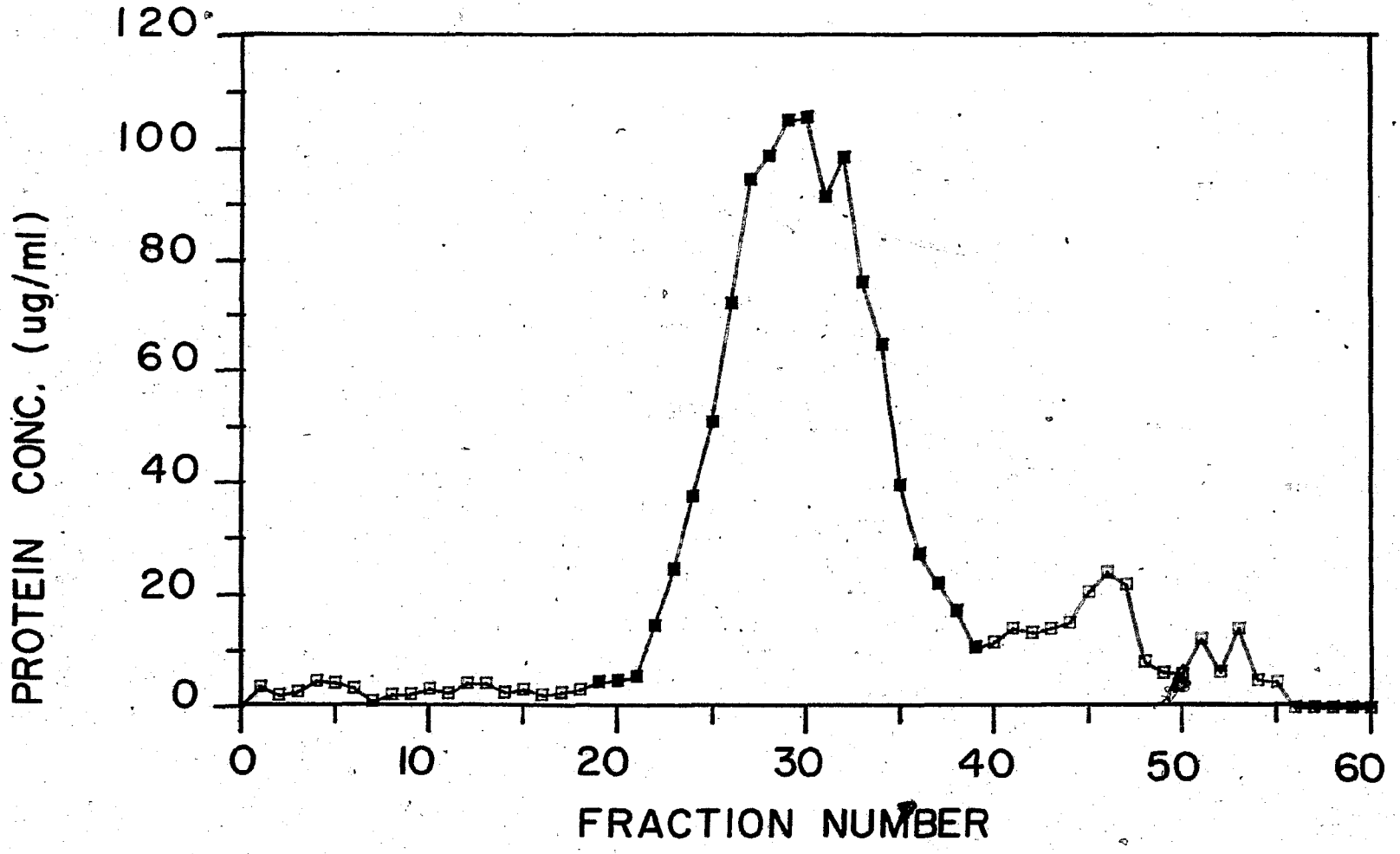
A 9 millilitre sample of the crude lectin extract from the ammonium sulphate precipitation was applied to a Bio–Gel P–200 column (100–200 mesh), running at a rate of 18 ml/hr at 4°C. Four millilitre fractions were collected, using PBS as the elution buffer, and each fraction was assayed for relative protein content using the Bio–Rad One Step protein assay (figure 4). The resulting elution pattern was found to contain 1 major peak at an elution volume of approximately 120 ml, and 3

TABLE 1
Erythrocyte Specificity of Tomato Lectin

Erythrocyte type	HA titre	crude lectin extract (mg/ml)	specific HA activity (HA/mg)
A	128	2.75	46.5
B	128	2.75	46.5
C	128	2.75	46.5

FIGURE 4

Elution profile of crude tomato lectin extract from P–200 gel filtration column. Darkened squares (■) represent fractions capable of agglutinating 2% type B erythrocytes. All methods used are described in the text.


120
100
80
60
40
20
0
PROTEIN CONC. (ug/ml)
0
10
20
30
40
50
60
FRACTION NUMBER

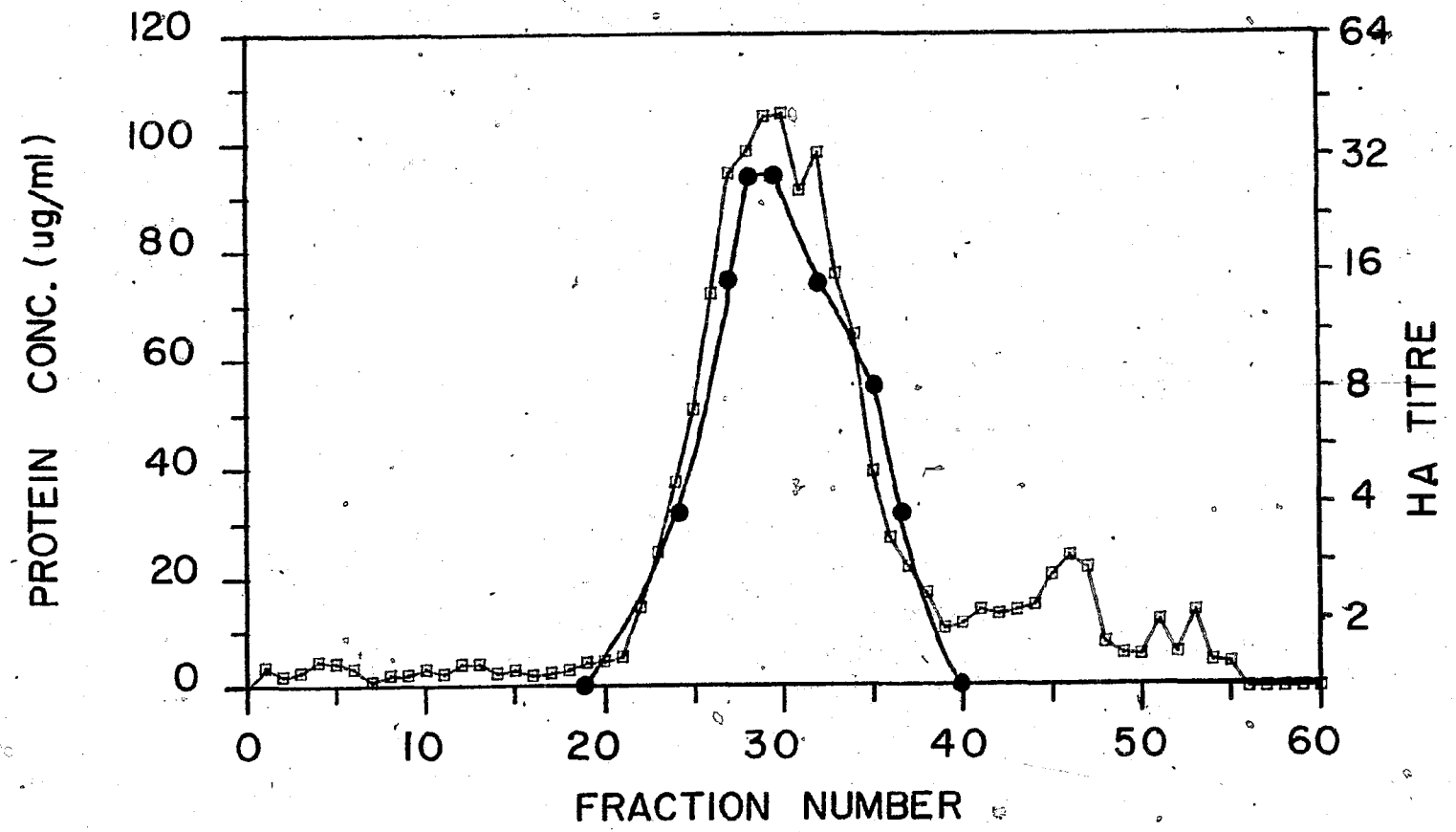
minor peaks eluting at volumes 180 ml, 205 ml and 216 ml.

As well as being assayed for relative protein content, each fraction was tested for hemagglutinin activity. A 50 uL sample of each fraction was added to an equal volume of a 2% v/v type B erythrocyte suspension in a microtitre plate and incubated at 4°C for 1 hour. All fractions eluted between volumes of 96 ml and 144 ml, the area of the major peak, showed HA activity (figure 4). These fractions were then further tested to find their hemagglutination titre. A 100 ul sample of each hemagglutinating fraction was diluted twofold in a microtitre plate using PBS and to each well was added 50 uL of a 2% v/v type B erythrocyte suspension. The plates were incubated at 4°C for 1 hour and the plate was then checked to determine the titre for each fraction. The hemagglutination titres, graphically, produced a pattern similar to that of the relative protein concentration, with the highest HA titre of 32 occurring at an elution volume of 120 ml and the titres declining to 0 on either side of that fraction (figure 5).

Having determined that the protein concentration of the fraction eluted at 120 ml was 0.1 mg/ml, using the Bio-Rad One Step protein assay, the specific HA activity of this lectin sample was 320 HA/mg. The gel filtration process therefore resulted in a 7 fold increase in specific HA activity. Following the gel filtration of the crude lectin, a series of 3 molecular weight markers were used to estimate the molecular weight of the isolated lectin. A 1:1:1 mixture of rabbit immunoglobulin G (IgG), bovine serum albumin (BSA) and ovalbumin (OA), in a total volume of 9 millilitres was applied to the column, again running at 18 mls/hr at 4°C and eluted with PBS. Four millilitre fractions were collected and each fraction was assayed for relative protein content using the Bio-Rad One Step protein assay. As expected, 3 major peaks were obtained, IgG eluted at a volume of 124 ml, BSA at 180 ml and OA at 220 ml. Using this data and the parameters of the column itself

FIGURE 5

Hemagglutination (HA) titres of the fractions eluted from the gel filtration column. Open squares (□) represent the relative protein concentration of each fraction. Closed circles (●) represent the HA titre of each fraction. All methods used are described in the text.


PROTEIN CONC. (ug/ml)
120
100
80
60
40
20
0
64
32
16
8
4
2
HA TITRE
0
10
20
30
40
50
60
FRACTION NUMBER

a standard curve was prepared to determine the molecular weight of the tomato lectin. A linear relationship exists between Ve/Vo and molecular weight of the markers. The Ve/Vo value for the tomato lectin was therefore calculated and its molecular weight, interpolated from the standard curve, was approximately 140,000 (figure 6).

Carbohydrate Specificity

In order to determine the carbohydrate specificity of the isolated tomato lectin, six carbohydrates were tested for their ability to inhibit hemagglutination. A twofold dilution series of the crude lectin extract (initial volume 100 uL) was prepared using AG buffer. To each row of wells was added 50 uL of one carbohydrate solution followed by 50 uL of a 2% v/v type B erythrocyte suspension. The plates were incubated at 4°C for 1 hour and then examined for hemagglutination inhibition. Table 2 demonstrates the ability of the six carbohydrates tested to inhibit hemagglutination. The only carbohydrate tested, capable of causing an HA titre decrease from the control was N,N',N''–triacetylchitotriose, an oligomer of N–acetylglucosamine. The carbohydrates chosen for this inhibition study, were tested by others previously (Kilpatrick, 1980; Nachbar, 1980). This assay was conducted to help determine whether or not the lectin isolated here was the same as that reported by previous authors (Kilpatrick, 1980; Nachbar,1980). At a 10 mM concentration N,N',N''–triacetylchitotriose caused complete inhibition of hemagglutination while at 50 mM concentrations no other carbohydrate tested, including N–acetylglucosamine, had any effect. These results confirm that the lectin isolated here is specific for carbohydrate oligomers containing N– acetylglucosamine.

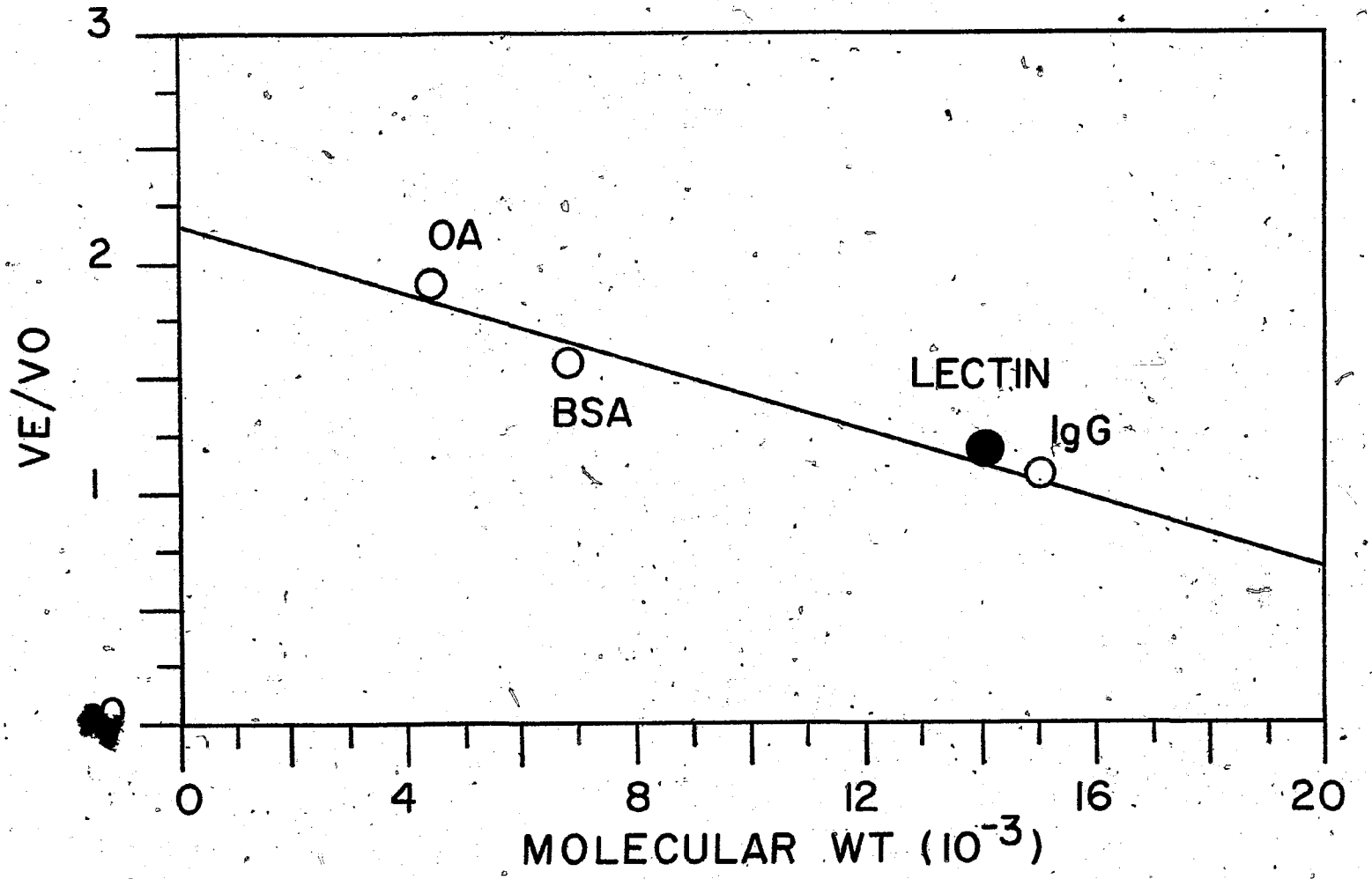
TABLE 2

CHO Inhibition of Hemagglutination

CHO conc. (mM)	HA titre
glucose (50)	64
sucrose (50)	64
galactose (50)	64
fructose (50)	64
N–acetylglucosamine (50)	64
N,N',N"–triacetylchitotriose (10)	0
CONTROL	64

FIGURE 6

Standard curve of Ve/Vo versus molecular weight of the markers used to determine the molecular weight of the tomato lectin by gel filtration. All methods used are described in the text.


3
2
1
0
VE/VO
OA
BSA
LECTIN
IgG
0
4
8
12
16
20
MOLECULAR WT (10-3)

Bacterial Agglutination (BA)

A) *Pseudomonas syringae* pv.*tomato*

The bacterial agglutinating activity of the lectin isolated from the tomato fruit was studied using *P.tomato* (strain PT–7 Ohio) cells, grown in a yeast salts medium broth (YSM). Broth culture was used in order to minimize the production of any exopolysaccharide, which would interfere with interaction of the lectin and the bacterial cell surface. The PT–7 Ohio cells were washed three times with the AG buffer and finally suspended in that buffer at 2.0 x 10^9 cells/ml . A twofold dilution series of the crude lectin extract (initial volume 100 uL) was prepared using AG buffer and to each well was added 50 uL of the bacterial suspension and the plate incubated overnight at room temperature. The PT–7 cells were found to be agglutinated at all dilutions of lectin greater than 1/128. To determine the highest dilution of lectin capable of agglutinating the PT–7 Ohio cells, the assay was repeated using an initial 1/10 dilution of the crude lectin extract. The bacterial cells were agglutinated up to and including a lectin dilution of 1/10,240 and the BA titre was therefore 10,240. The control assay which consisted of equal volumes (50 uL) of the PT–7 Ohio cell suspension and AG buffer, showed that no spontaneous bacterial agglutination had occurred. A comparison of the results obtained in both BA assays indicate that agglutination of the PT–7 Ohio cells begins at a dilution of the lectin between 1/80 and 1/128. It was not very surprising to find that high concentrations of lectin do not result in the agglutination of the bacteria since the interaction between a lectin and its carbohydrate receptor on a cell surface is analogous in several respects to the interaction between an antibody and its antigenic receptor. In this case, a dilution of lectin less than 1/128 mimics the response of an antibody when it is in great excess of its specific antigen, resulting

in the dissolution of the precipitate (Sharon and Lis, 1972). With respect to an antibody–antigen interaction, this situation is referred to as the prozone phenomenon. By carrying this analogy further, dilutions of lectin less than 1/128 would represent the lectin excess zone, dilutions greater than 1/10,240 the carbohydrate receptor excess zone and dilutions between 1/128 and 1/10,240 the zone of equivalence (figure 7).

Having determined both the BA titre of the crude lectin extract, as well as it protein concentration, the specific BA activity of the crude lectin extract was 3723.6 BA/mg. In comparison to the specific HA activity of the crude lectin sample, calculated to be 46 HA/mg, the bacteria are 80 times more sensitve to agglutination than the erythrocytes.

A) Other Bacteria Tested

The bacterial agglutinating activity of the lectin isolated from the tomato fruit was also studied using 15 isolates of pathogens of tomato, non–pathogens of tomato and saprophytes. The bacteria were grown in separate YSM broth cultures and washed three times with AG buffer before being suspended in that buffer at 2.0×10^9 cells/ml. A twofold dilution series of the crude lectin extract (initial volume 100 uL) was prepared using AG buffer and to each row of wells was added one of the bacterial suspensions before overnight incubation at room temperature. The results obtained showed no correlation between ability of the lectin to agglutinate any particular bacterial species and whether the species was pathogenic, non–pathogenic or saprophytic (table 3). One crucial factor that must be considered here is the absence of any loose EPS, that is eleviated by growing the bacteria in broth culture followed by repeated washings. Several of the bacterial species assayed for agglutination, may naturally produce an EPS that under

TABLE 3

Specificity of Bacterial Agglutination

Bacteria	BA titre
Escherichia coli	4
Agrobacterium tumefasciens	32
Rhizobium leguminoserum	2
Xanthomonas pruni	2
Erwinia herbicola	2048
Erwinia carotovora	4
Xanthomonas phaseoli	1
Pseudomonas maculicola	8
Pseudomonas tabaci	1
Pseudomonas cichorii	16
Pseudomonas marginalis	32
Xanthomonas vesicatoria	32
Pseudomonas phaseolicola	2048
Pseudomonas papulans	2
Pseudomonas tomato	128

natural conditions may inhibit lectin binding to the bacterial surface. Although all species of bacteria were agglutinated by the tomato lectin, the titres varied from 1 to 2048. The most significant result obtained here, is the presence of some carbohydrate oligomer containing N–acetylglucosamine on all bacterial species tested, which serves as a receptor for the tomato lectin under these test conditions.

Lectin Concentration in Crude Extract

The original protein concentration of the crude lectin extract was approximately 2.75 mg/ml, while that of crude extract from which all lectin was adsorbed, was 1.5 mg/ml, as determined using the Bio–Rad One Step protein assay. The subtraction of the second value from the first was considered to be the lectin concentration in the original crude lectin and was found to be 1.25 mg/ml. The HA titre of the adsorption treated extract was found to be 0 and therefore all the lectin had been removed by the absorption. This calculated value for actual lectin concentration in the crude extract allowed for a determination of specific activities, both HA and BA, of the lectin itself. The specific HA activity of the lectin was 102.4 HA/mg and the specific BA activity 8192 BA/mg.

Is the Bacterial Agglutinin the Hemagglutinin

To be sure that the bacterial agglutinating activity and the hemagglutinating activity are elicited by the same protein, an attempt was made to adsorb all hemagglutinating activity through pretreatment of a lectin solution with an excess amount of bacteria. To accomplish this, varying amounts of PT–7 Ohio cells ranging from 5.0×10^{10} cells/ml to 1.0×10^{12} cells/ml were pre– incubated in a 1 ml sample of the crude lectin extract overnight at room temperature. Following this

incubation, the cells were removed by centrifugation and the supernatants assayed for hemagglutinin activity using the typical HA titre assay. A twofold dilution (initial volume 100 uL), of each supernatant was prepared in a microtitre plate and to each well was added 50 uL of a 2.0% v/v suspension of type B erythrocytes. Following incubation at 4°C for 1 hour the HA titre of each supernatant was taken (table 4). To ensure that any decrease in HA titre was not due to the denaturation of the lectin from overnight incubation at room temperature, a control was run that consisted of equal volumes (50 uL) of lectin solution and PBS (0.01 M), left to sit overnight at room temperature. The HA titre of the control was taken before and after incubation and any drop in HA titre would have rendered the assay invalid. It was found that as the number of PT-7 Ohio cells increased from 5.0×10^{10} cells/ml to 1.0×10^{12} cells/ml the HA titres of the supernatants decreased from 16 to 0, with any further increase in cell numbers resulting in a titre of 0. No decrease in titre was observed in the control, the HA titre before and after the overnight incubation remained 32. Since all hemagglutinin activity could be removed by bacterial agglutination we could be sure that the hemagglutinin and bacterial agglutinin are one in the same.

TABLE 4
Adsorption of HA Activity

total # PT–7 Ohio cells	crude extract added (mls)	HA titre
5.0×10^{10}	1	16
1.0×10^{11}	1	8
5.0×10^{11}	1	0
1.0×10^{12}	1	0

CONTROL	before incubation	32 (HA)
	after incubation	32 (HA)

CHAPTER 2

LECTIN–BACTERIUM INTERACTIONS

Exopolysaccharide of *P.tomato*

To determine whether or not *P.tomato* produces an exopolysaccharide (EPS), and if so under what conditions, PT–7 Ohio cells were grown on 3 types of solid media considered to be conducive to EPS formation by many pseudomonad species (Schaad, 1980). The cells were grown on plates of either yeast salts medium (YSM), yeast salts medium supplemented with 5.0% w/v glucose (YSMG) or yeast salts medium supplemented with 5.0% w/v sucrose (YSMS) and incubated for 96 hours at room temperature. The bacteria were then washed from the plates using the AG buffer and suspended in 25 mls of that buffer. Each cell suspension was subjected to vigorous shaking using a vortex to loosen any EPS from the cells and then centrifuged at 10,000 x *g* for 15 minutes to remove the bacteria. A sample from each supernatant was then removed and assayed using the anthrone reaction to determine the relative carbohydrate content of each. The standard curve for carbohydrate concentration determination was prepared using glucose as the standard carbohydrate. Table 5 shows the results of the carbohydrate content determination of the supernatants of the PT–7 Ohio cells grown on the 3 different media. To discount the possibility that the difference in EPS production on the different media was due to different cell numbers, the bacterial pellet obtained from the centrifugation of each cell suspension was collected and the cell numbers estimated spectrophotometrically. The amount of EPS produced by the bacteria from different media was then expressed as ug/10^{11} cells. As expected, cells grown on YSMS media produced a relatively large amount of EPS. Schaad (1980) states

TABLE 5

EPS Production on 3 Different Media

growth medium	EPS (ug/ml)	total # cells	EPS (ug x 10^{11}/cell)
YSM	400	4.0 x 10^{10}/cell	1.0
YSMG	1800	6.4 x 10^{10}/cell	2.8
YSMG	72,000	5.2 x 10^{10}/cell	13.8

that P. tomato when grown in the presence of sucrose produces levan as an extracellular polysaccharide. The effect of glucose as a supplement to the YSM media also appeared to have an effect in the production of an EPS since its presence caused an increase in EPS production three times that of cells grown on YSM alone. These results indicated that in the absence of sucrose, PT–7 Ohio cells produce an EPS distinct from levan, since the formation of levan requires the presence of sucrose as a precursor (Sutherland, 1972).

Exopolysaccharide Inhibition of Bacterial Agglutination

The three EPS samples extracted were retained to determine their effect on the agglutination of the PT–7 Ohio cells by the tomato lectin. Since the growth of bacteria in broth culture inhibits or at least lessens to a great extent the production of EPS (Sutherland, 1972), PT–7 cells grown in a YSM broth for 24 hours at room temperature were considered to have produced only minimal amounts of loose EPS. The cells from broth were then washed three times with the AG buffer using centrifugation at 10,000 x *g* and then finally resuspended in the AG buffer at 2.0 x 10^9 cells/ml. Any loose EPS that may have been produced in broth culture would be removed by centrifugation.

A two fold dilution series of the tomato lectin was prepared in a microtitre plate using the agglutination (AG) buffer. An equal volume (50uL) of the supernatants of cells grown on either YSM, YSMG or YSMS was then added to each well. The addition of 50 uL of AG buffer, in place of a supernatant, to one row of wells served as a positive agglutination control. The mixtures were then allowed to incubate at room temperature for 15 minutes prior to the addition of 50 uL of the PT–7 Ohio cells grown in broth culture. Following overnight incubation at room temperature the plate was then examined for inhibition of bacterial

agglutination (table 6). The control wells demonstrated that the dilution of lectin required to agglutinate PT-7 Ohio cells was 1/64 and agglutination of the bacteria continued at all dilutions greater than that, as in other typical BA assays. The addition of any of the three supernatants containing EPS resulted in the complete inhibition of bacterial agglutination. Apparently, an EPS produced by *P.tomato* has a pronounced affect on its interaction with the tomato lectin. Since the supernatants of cells grown on all 3 media were capable of inhibiting bacterial agglutination, and levan is produced only in the presence of sucrose (Sutherland, 1972), levan was excluded as being the possible interfering EPS. The fact that levan is a polyfructose molecule (Sutherland, 1972) and the tomato lectin is specific for oligomers of N-acetylglucosamine also eliminated levan as a consideration. The interfering EPS must therefore be one produced by *P.tomato* whether sucrose is present or not.

Cell Surface Binding Site for the Tomato Lectin

Studies by Sequeira and Graham (1977), as well as others, suggest that a frequent binding site on the bacterial cell surface for a lectin is the lipopolysaccharide (LPS). In consideration of this finding, the LPS of PT-7 Ohio was extracted to study its possible role in the interaction between tomato lectin and PT-7 Ohio cells. The method of LPS extraction was essentially that of Westphal and Jann (1965). A twofold dilution series of the tomato lectin solution (initial volume 100 uL), was prepared in a microtitre plate using AG buffer, followed by the addition of an 50 microlitres of the LPS extract. A 50 uL aliquot of a 2.0 x 10^9 cells/ml suspension of PT-7 Ohio cells grown in YSM broth was then added to each well and the plate incubated at room temperature overnight. A positive agglutination control for the assay consisted of equal volumes (50 ul) of

TABLE 6

EPS Inhibition of Bacterial Agglutination

medium EPS produced on	BA titre
YSM	0
YSMG	0
YSMS	0
CONTROL	128

the tomato lectin solution, AG buffer and the PT–7 Ohio bacterial suspension. Following the overnight incubation, the plate was examined for BA inhibition. The control row demonstrated that the lectin dilution required to agglutinate the bacteria was 1/64 and agglutination was observed at all dilutions greater than that. The wells to which the LPS extract had been added showed complete inhibition of bacterial agglutination. It was shown here then that the LPS of the PT–7 Ohio cells in extracted form was capable of binding the tomato lectin.

DISCUSSION

The identification and characterization of many plant lectins has long been reported and many reviews have been written concerning those so far studied (Boyd, 1970; Sharon and Lis, 1972; Nicolson, 1974; Sharon, 1977; Goldstein *et al*, 1978). Until recently though, the most striking lack of understanding of plant lectins has been their physiological role in the plant. One most recent consideration is that they may serve as recognition factors that aid in determining the response of a plant to a foreign invader.

At the onset of this study, two reports were published (Kilpatrick, 1980; Nachbar *et al*, 1980), both of which discussed the isolation of a lectin from tomato fruit. Studies by Kilpatrick (1980) suggest that the lectin is approximately 200,000 daltons in its native form, with subunits of approximately 63,000 daltons in a dissociated form. Nachbars group (1980) found the molecular weight to be approximately 71,000 with no evidence of a polymeric form. The 140,000 dalton estimate presented in this thesis, indicates that the lectin may indeed exist functionally in non-denaturing conditions as a complex of 2 subunits, each of approximately 70,000 daltons. This would explain the finding of such a high molecular weight form by Kilpatrick as well as, the 71,000 dalton species by Nachbars group. Differences in molecular weight estimates may be attributed to the different isolation and purification methods used here and by the other authors.

Both authors agree that the tomato lectin exhibits a specificity for oligosaccharides containing N-acetylglucosamine, as does the lectin isolated here. These similarities in molecular weight and carbohydrate specificity between the lectin studied here and that studied by the previous authors indicate that the lectins are one and the same. This was an important fact to develop, since several plant species possess more than one lectin form, these different forms being called isolectins (Howard *et al*, 1971; Lis *et al*, 1966; Gould and Scheinberg, 1970). Some

plant species such as *Ulex europaeus* and *Bandeiraea simplicifolia* possess two isolectin forms with different carbohydrate specificity. No evidence of a second lectin form in tomato fruit, was found here or in the reports of Kilpatrick and Nachbar.

It was determined in this thesis that as well as being a hemagglutinin, the tomato lectin was also a bacterial agglutinin. Although the addition of divalent cations was not required for hemagglutination, their presence had a profound effect on bacterial agglutination. Initially, the BA assay was attempted using bacterial suspensions in distilled water. The titres obtained were either very low or absent, and certainly inconsistent. When BA assays were conducted using AG buffer, containing a variety of divalent cations (see materials and methods), the titres obtained were 500 fold higher and consistent. The requirement for bound divalent cations such as Mg^{2+} and Ca^{2+} has been reported for both proper lipopolysaccharide (LPS) physical structure on the bacterial surface (Gray and Wilkinson, 1965; Olins and Warner, 1967) as well as for many lectins to exhibit biological activity (Yariv et al, 1968; Paulova *et al*, 1971). Generally, the removal of bound cations results in complete loss of biological activity for both the LPS and the lectin. It seems likely that the addition of these divalent cations to the BA assay system may have resulted in the proper conformation for agglutination.

The fact that the tomato lectin agglutinates *P.tomato* cells indicates that a binding site must exist on the bacterial surface which contains an oligosaccharide containing N– acetylglucosamine. Nachbar (1980) found that the binding site for the tomato lectin need not be composed entirely of N– acetylglucosamine, since peptidoglycan was also capable of inhibiting hemagglutination. In consideration of the carbohydrate requirement for the lectin binding site the LPS was considered as the most likely. An extract of the LPS of *P.tomato* completely inhibited the

agglutination of the bacterial cells by the tomato lectin. Since the exact structure of the LPS of *P.tomato* is not known, the portion which is responsible for lectin binding cannot be stated conclusively, although a suggestion can be made. Agglutination studies conducted here, using 15 different bacterial species demonstrated that the tomato lectin exhibits little BA specificity, in the absence of any loose EPS. This indicates that the receptor on the bacterial cell surface is a common one, and therefore the highly variable "O" side chain is an unlikely consideration. It is possible though, that a limited number of bacterial isolates were agglutinated by the tomato lectin, due to the presence of N-acetylglucosamine in their "O" specific chain, but the high carbohydrate variability of this region amongst gram negative bacteria does not allow it to be accountable for all bacteria agglutinated. The core region of the LPS contains five basal sugars which are said to be common ones amongst all species of any given bacterial genus so far studied (Luderitz *et al*, 1971). One of the common basal sugars of the core region of *Pseudomonas* LPS is N-acetylglucosamine (Wilkinson *et al*, 1983). This N-acetylglucosamine residue may be a possible binding site for the tomato lectin on the bacterial surface.

Results obtained here, on exopolysaccharide (EPS) production indicate that the presence of yet another factor involved in recognition.

The exact nature of an "EPS" has recently received some attention by many in the field of microbiology. There appears to be some confusion in the terminology used to describe the extracellular substances which are produced by, and surround many bacteria. In fact, several terms have been coined which describe this extracellular material to varying degrees, ranging from slime and capsule to glycocalyx and EPS. Each term implies a different structure which may or may not be appropriate. The term EPS has been used here to describe the

material which is exuded by the bacterial cell and is at least partly composed of polysaccharide.

When PT-7 Ohio cells were grown on solid media, they produced at least two types of EPS. In the presence of sucrose, which serves as a necessary precursor for levan production (Sutherland, 1972), virtually all pathovars of *Pseudomonas syringae* including pv. *tomato*, produce levan as an EPS (Doudoroff and Palleroni, 1974). Levan is a polyfructose molecule in which the predominant linkage is -fructosyl 2 6 D- fructose (Sutherland, 1972) and being as such, does not represent a possible binding site for the lectin. When PT-7 Ohio cells were grown on solid media in the absence of sucrose however, supernatants of the cell suspensions still contained considerable amounts of carbohydrate. Those grown on YSM plates supplemented with glucose produced approximately three times the amount of EPS, of cells grown on YSM alone. This EPS, which must be distinct from levan since sucrose was not present, appears to be produced by *P.tomato* in the presence or absence of glucose, although the presence of glucose does increase EPS production. The EPS produced by the PT-7 Ohio cells grown on all 3 media inhibited the agglutination of PT-7 Ohio cells grown in broth culture. A similar situation was seen when initial BA assays were conducted by growing PT-7 Ohio cells on these three different media and suspending them directly into the AG buffer, without prior washings. The typical BA assay failed to demonstrate agglutination of any of the three cell suspensions, when the EPS produced by each was present. No actual distinction has been made here as to whether the EPS of *P.tomato* is of the tight capsular form which adheres to the cell wall or a loose slime which is released from the cell. It appears though that the EPS produced by *P.tomato* is more likely to be of the loose slime form, for two main reasons. Firstly, the EPS could be removed from the cells using moderate centrifugation and

secondly, numerous attempts to observe a capsular form, using negative staining procedures, failed.

As a point of comparison, the related bacterium *P.solanacearum*, a pathogen of several plants including potato and tomato, produces an EPS which is a heteropolysaccharide rich in N–acetylglucosamine (Sequeira, 1980). Sequeira and Graham (1977) demonstrated that only avirulent strains of *P.solanacearum* are agglutinated by the lectin of the potato plant and that the LPS of the bacteria possibly served as the binding site for the lectin. Virulent strains of *P.solanacearum* differ from the avirulent forms in that the former produces an EPS which inhibits its agglutination by the potato lectin. Previous electron microscopy studies by the Sequeira and coworkers (1977), revealed that in the potato plant, all virulent (slime forming) strains of *P.solanacearum* introduced into the host leaves, remained free in the intercellular spaces and continued to multiply with no induction of the hypersensitive response (HR). The introduction of avirulent (slimeless) strains resulted in the attachment of the bacteria to the mesophyll cells, followed by the formation of a surrounding granular material and a hypersensitive response by the plant. Not all avirulent forms were found to be capable of inducing the HR. Those which did not were agglutinated by the lectin to a lesser degree and found to possess an "O" side chain of greater length than the avirulent cells which induced the HR. It was suspected that the presence of the entire "O" side chain in these select mutants prevented the binding of the lectin to the core or lipid A or core region of the LPS.

Husain and Kelman (1958) believed that the wilting of the tomato plant when injected with *P.solanacearum*, was directly related to the amount of EPS produced by the bacterium. Their results indicated that mutant strains of *P.solanacearum* which did not produce an EPS were avirulent while those that did, were virulent.

They also found that the amount of EPS produced by different strains showed a high positive corelation with pathogenicity. The authors reported that the primary pathogenic factor of *P.solanacearum* was EPS and that it merely served to block xylem vessels and hence cause the characteristic wilting. Although this report was not directly pertinent to this thesis, it serves to strengthen the argument for a lectin recognition system in the tomato plant. It is likely that the presence of the EPS of *P.solanacearum* does affect pathogenicity, but does so because it inhibits the lectin from binding to the bacterial surface.

The similarities between *P.tomato* and pv. solanacearum are obvious ones, and ones which help to strengthen the lectin recognition model for the tomato plant. Since the tomato plant apparently produces only one lectin form, which is considered here to play a primary role in recognition, it must be able to interact with more than one bacterial species to be effective. Conversely, the manner in which actual pathogens escape recognition should be similar. *P.solanacearum* is one pathogen of tomato, from which all reports indicate, may escape recognition by the tomato plant in much the same manner as *P.tomato*.

From the results obtained in this study it appears that *P.tomato* is pathogenic to tomato at least partly because it avoids recognition by the tomato lectin. Recognition is avoided by the production of an EPS which competitively inhibits the lectin from binding to the LPS of the bacteria. This statement is made cautiously though since several other factors are known to be involved in the pathogenicity of bacteria and disease resistance in plants. Studies indicate that both sparophytes and non-pathogens are bound by the lectin of many plants (Sing and Scroth, 1977; Sequeira *et al*, 1977), yet only the non- pathogens elicit the HR by those plants. Attachment alone must therefore not necessarily be the cause of the HR, and some other factor must be involved. Although attachment of bacteria

to the plant cell wall does not always result in the HR, attachment is a necessary prerequisite for the HR.

Electron microscopy studies conducted by McCurdy and Berlie (unpublished data) provide additional evidence for the occurrence of lectin recognition in the tomato plant. They observed that leaves infected with *P.tomato* demonstrated a large number of bacterial cells free in the intercellular spaces of the mesophyll. On the other hand, nonpathogens known to elicit the HR in tomato leaves, became immobilized by a dense granular material and were attached to the mesophyll cells.

As yet, a few gaps need to be filled in our understanding of the recognition of bacteria by plant lectins, but the evidence presented here warrants its consideration in the tomato plant. Furthermore, a better understanding of the interaction between the tomato lectin and *P.tomato* may provide key information to aid in the development of tomato cultivars resistant to the pathogen.

SUMMARY

1. A lectin was isolated from tomato fruit and found to have molecular weight of approximately 140,000.

2. The tomato lectin was specific for N,N',N"– triacetylchitotriose, an oligomer of N–acetylglucosamine and was therefore considered to be the same as the one previously isolated (Kilpatrick, 1980; Nachbar, 1980).

3. The tomato lectin agglutinated a wide variety of bacterial species, including *Pseudomonas syringae* pv. *tomato* (PT–7 Ohio) cells at a concentration of approximately 6 ug/ml.

4. The agglutination of *Pseudomonas syringae* pv. *tomato* (PT–7 Ohio) cells by the tomato lectin mimicked the prozone phenomenon, which occurs between an antibody and antigen.

5. The agglutination of bacterial cells by the tomato lectin required the presence of at least one of the cations, Ca^{2+}, Mn^{2+}, Mg^{2+} and Na^{2+}.

6. *Pseudomonas syringae* pv. *tomato* (PT–7 Ohio) produced at least 2 distinct exopolysaccharides, one of which did not require the presence of sucrose as a precursor for its synthesis.

7. The exopolysaccharide produced which did not require sucrose as a precursor completely inhibited the bacterial agglutination of *Pseudomonas syringae* pv. *tomato* (PT–7 Ohio) cells by the tomato lectin.

8. The tomato lectin bound to the LPS of *Pseudomonas syringae* pv. *tomato*.

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Thesis topic: Interaction between the tomato lectin and *Pseudomonas syringae* pv. *tomato*.

ACADEMIC AWARDS:

1982 University of Windsor Summer Bursary

1983 University of Windsor Summer Bursary

1984 University of Windsor Summer Bursary

COMMITTEE AND COUNCIL SERVICE:

1979–1980 Elected third year student representative to the Biology Departmental Council.

Elected Biology Club vice–president.

1980–1981	Elected Biology Club president.
1982–1983	Elected graduate student representative to the Faculty Council of Graduate Studies.