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QUANTITATION AND LOCALIZATION OF EXTENSIN BY IMMUNOCHEMICAL METHODS

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ABSTRACT

QUANTITATION AND LOCALIZATION OF EXTENSIN BY IMMUNOCHEMICAL METHODS

BY

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A competitive indirect enzyme-linked immunosorbent assay (ELISA) was developed for the rapid quantitation of the glycosylated and deglycosylated forms of the monomeric soluble extensin precursor subunits P1 and P2. Linear response range for each kind of precursor in the competition curve was between 1 ug/ml and 10 pg/ml. The cellular distribution of insoluble extensin was investigated in hypocotyls of cucumber (Cucumis sativus L.) seedlings subjected to a disease resistance inducing heat shock followed by inoculation with the fungal pathogen Cladosporium cucumerinum Ell and Arth., using antibodies to the soluble precursor forms of extensin. Fluorescence microscopy revealed that anti-precursor antibodies bound only to the upper and lower epidermal cell walls of sections from inoculated and non-inoculated heat-shocked plants.

To my parents

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LITERATURE REVIEW

I. Extensin

The first evidence for a protein which is an integral part of the primary cell wall, and which contains virtually all of the hydroxyproline of the cell was presented by Lamport and Northcote and independently by Dougal and Shimbayashi in 1960 (28,9). In 1965 (29), Lamport hypothesized that this hydroxyproline-rich protein must play a structural role in the cell wall and, therefore, must inevitably be involved in cell extension. This idea was referred to as the extensin hypothesis and led to the hydroxyproline-rich protein being given the name extensin (by analogy with the structural proteins collagen and elastin). In 1967 (30) Lamport reported the isolation of arabinosyl-hydroxyproline, a new type of carbohydrate-amino acid link, out of partial alkaline hydrolysates of tomato cell walls. Extensin was described as a polypeptide backbone with hydroxyproline residues involved in O-glycosidic links to short arabinosides. It was pointed out that these arabinose oligosaccharides might serve as attachments for other wall polysaccharides, enabling a small amount of extensin to cross-link a large amount of wall polysaccharide. Low concentrations of hydroxyproline residues could thus play an important part in determining the properties of the primary cell wall.

Analysis of enzymatic degradation products of cell walls from

cultured tomato cells confirmed the presence of hydroxyproline-arabinosides and demonstrated that galactose was an additional sugar component of the glycoprotein (31). In 1970 Lamport (32) suggested that the short arabinose side-chains might represent the beginning of much larger polysaccharide chains which were alkali sensitive and therefore not recovered from earlier alkaline treatments used to obtain the hydroxyproline-arabinosides. Lamport and Miller (33) later demonstrated that the arabinosyl-hydroxyproline linkage is widely distributed in the plant kingdom.

A second amino acid-sugar link, O-galactosyl serine, was subsequently identified in 1973 by Lamport et al. (35). Galactosyl-serine was identified in glycopeptides prepared from cell walls of cultured tomato cells by acid hydrolysis (to remove arabinosides) followed by trypsin digestion.

Lamport, et al. (35) and Lamport (34) developed a technique for estimating the degree of serine glycosylation in the wall. This technique was based on the finding that glycosylated serine residues were degraded when treated with hydrazine, whereas serine residues free of sugars were stable. They reported results of preliminary experiments which showed that there was a decrease in sugar-free serine residues in the cell wall with increasing age of the suspension culture. This was consistent with the extensin hypothesis which called for increased cross-linking as cell extension decreased (29). Esquerre-Tugaye and Lamport (14) and Esquerre-Tugaye and Mazau (15), after a study of the glycosylation patterns of the hydroxyproline-rich glycoprotein which accumulates in the cell wall of Colletotrichum lagenarium infected melon plants, suggested more specific functions for the carbohydrate side-chains. Subsequent work confirmed and expanded that the extent of

glycosylation of hydroxyproline was higher in infected plants (14). In contrast, the extent of serine glycosylation did not vary significantly upon infection, but decreased with age in healthy as well as infected plants. This led them to suggest that some of the galactosylated serine residues could provide temporary links for orienting other wall polymers, while the arabinosides attached to hydroxyproline could have a different role. This role may, perhaps, include an involvement in the disease response once the arabinose residues were known to protect the wall glycoprotein against proteolysis. In particular, proteolytic enzymes which may be involved in pathogenisis (by C. lagenarium) had been shown to be ineffective on cell walls without prior deglycosylation of the hydroxyproline residues (14).

Mort and Lamport (42) applied a new technique to the problem of studying extensin. They used anhydrous hydrogen fluoride which specifically hydrolyzes the polysaccharides of the cell wall and leaves peptide bonds intact. They found that the cell wall remained as an insoluble residue rather than completely dissolving as expected. This insoluble fraction, about 10% of the wall, consisted of equal amounts of wall protein and an unknown, possibly phenolic, component (37). They suggested that this insoluble residue must contain some other, as yet unidentified, cross-links. In addition, it may be cross-linked to itself independently of any possible links to cell wall polysaccharides. However, Lamport (37) pointed out that proof of this idea required the isolation of cross-linked peptides and the identification of components involved at the cross-link region. Based on additional results, Lamport presented (38) the concept of two semi-independent cell wall networks: protein and carbohydrate. He suggested that the tyrosine derivatives,

which he had earlier been unable to identify (36) could be possible candidates for the cross-links, but the isolation of cross-linked peptides was still needed for proof.

More recent evidence which supports the concept of an independent glycoprotein network was presented by Fry (19). He isolated a new phenolic amino acid from cell wall hydrolysates. The new amino acid was shown to be an oxidatively coupled dimer of tyrosine with the two tyrosine units linked by a diphenyl ether bridge. He proposed the name isodityrosine for this compound. Fry reported that the amount of isodityrosine in cell wall hydrolysate was proportional to the amount of hydroxyproline and suggested it was a component of extensin. He also suggested that isodityrosine might be the uncharacterized tyrosine derivative reported by Lamport (36) and that the glycoprotein is held in the cell wall by interchain isodityrosine cross-links.

Cooper and Varner (8) demonstrated that much of the extensin in wounded carrot tissue arrives at the wall in soluble form and gradually becomes insoluble. They were successful in isolating soluble extensin and speculated that an increase in the extractability of carrot extensin could be facilitated by inhibition of peroxidase mediated crosslinking of extensin. This finding is in agreement with Fry (19) who previously speculated that the crosslinking of extensin by isodityrosine could be controlled by peroxidase.

The ability to isolate soluble extensin from wounded carrot root tissue made that system ideal for studies on the biosynthesis of HRGPs (3). Soluble extensin was found to accumulate as a salt-extractable hydroxyproline-rich glycoprotein (HRGP) in the cell wall (49,51). The glycoprotein was found to have a molecular weight of approximately 86,000 D and consisted of approximately two-thirds carbohydrate and one-

third protein. The amino acid composition resembled the composition of the insoluble extensin peptides described by Lamport (36) as well as that of soluble extensin eluted from intact tomato cell suspensions (47) and a hydroxyproline-rich bacterial agglutinin (39,41). Smith et al. (41) further found that the soluble extensin eluted from the cell wall of intact tomato cell suspensions yielded two components (P1 and P2) that displayed kinetic and chemical properties which indicated their role as precursors of insoluble extensin. P1 and P2 were characterized by tryptic degradation of the HF-deglycosylated polypeptides, DP1 and DP2 (47).

Cooper and Varner (8) suggest that the lack of acidic amino acids and the abundance of lysine and histidine in soluble extensin give this macromolecule a high isoelectric point (in the range of 10 to 12). Such an isoelectric point explains its activity as a non-specific bacterial agglutinin (39) (by binding to acidic bacterial cell wall components), and makes it a likely candidate for interaction with acidic pectins.

The idea of a glycoprotein network in the plant cell wall has been extended to a possible glycoprotein-phenolic complex containing lignin. Whitmore (55) suggested that an early stage of lignification may involve cross-linking of the protein during polymerization of lignin monomers. In experiments with lignin precursors in combination with various proteins and polysaccharides, he observed peroxidase-mediated bonding of polyphenolic substances to proteins, especially those containing hydroxyproline. In further experiments, using cell walls from tissue culture which were incubated with coniferyl alcohol and hydrogen peroxide, Whitmore (56) deomonstrated the formation of lignin which was bound to carbohydrate and hydroxyproline-containing proteins. Whitmore

(57) extended his previous argument for lignin-protein structural bonds by comparing the amino acid distributions of proteins associated with lignin and those of the whole cell wall. He suggested that, when polymerizing, lignin links covalently with cell wall glycoprotein, and that the bonds may be formed preferentially with hydroxyproline. However, no evidence for this has subsequently been presented.

In conclusion, the concept of a glycoprotein, or perhaps glycoprotein-lignin network in some cell walls seems to be a credible explanation for the attachment of extensin in the cell wall. The evidence of a hydrogen fluoride-insoluble residue, the finding of a potential protein-protein link in isodityrosine, and the high isoelectric point supports this idea. The notion that an extensin-pectin network inercalated with xyloglucan coated cellulose microfibrils is the most general working model of cell wall architecture (7).

II. Involvement of extensin in disease resistance

Esquerre-Tugaye and Mazau (15) suggested the involvement of extensin in disease resistance. The levels of HRGPs have been shown to increase greatly in melon seedlings infected with the fungus Colletotrichum lagenarium, the causal agent of anthracnose (14). This increase in extensin was correlated with resistance to fungal infection (16). Esquerre-Tugaye et al. (13) concluded that the accumulation of this glycoprotein acts as a defense mechanism which becomes efficient if started early in the host. Toppan et al. (50) found indirect evidence that ethylene regulates this defense mechanism. They showed that both ethylene and ^{14}C-hydroxyproline deposition was significantly lowered in the cell wall of infected tissue. Further, treatment of healthy tissues with natural precursor of ethylene stimulated both the production of

ethylene and incorporation of ^{14}C-hydroxyproline into cell wall protein.

The increase in cell wall hydroxyproline content observed in aged carrot discs has also been suggested to occur as part of a wound response in excised tissue (2). Chrispeels et al. (3) suggested that the increase in extensin biosynthesis may be part of the plant's defense mechanism against invading pathogens. Fukuda and Kagimoto (20) similarly observed an increase in the cell wall and hydroxyproline levels on aging sections of sweet pepper fruits. They thought the increase in hydrdoxyproline during aging derived from the biosynthesis of the HRGPs and its precursor in response to wounding of the tissues. It is possible that production of HRGPs in each of these stress situations may be controlled by ethylene, because wounding, aging, and infections are all known to cause plants to release large amounts of ethylene (50). Stermer and Hammerschmidt (48) describe how a brief disease resistance inducing heat shock stimulates the synthesis of ethylene in cucumber seedlings, enhances peroxidase activity, and increases the accumulation of bound extensin in their cell walls. They suggested that perhaps a heat-shock-induced increase in ethylene production could stimulate the peroxidase mediated accumulation of bound extensin in the cell wall, and this increase in extensin may confer resistance to attack by a pathogen.

In view of the possible existence of a glycoprotein-phenolic cell wall network containing lignin, it is significant that phenolic compounds have also been observed to accumulate during infection. Glazener (21) demonstrated that lignin-like materials are synthesized by young tomato fruits after infection by Botrytis cineria, and has suggested that the formation of a polyphenolic layer around the

infection helps limit the spread of the fungus. Grand and Rossignol (22) and Hammerschmidt and Kuc (24) described changes in lignification involved in systemic protection of melons and cucumber, to Colletotrichum lagenarium and Cladosporium cucumerinum, respectively. An initial inoculation with the fungus appears to stimulate the enzymes required for lignin synthesis so that in a later exposure to the pathogen rapid lignification occurs and restricts the infection. Hammerschmidt et al. (23) showed hydroxyproline and lignin enhancement in cucumber cell walls is associated with resistance to Cladosporium cucumerinum. These results also suggested an association between lignin deposition and hydroxyproline enhancement.

Further evidence for the possible role of extensin in the defense mechanism of plants can be found in the structural similarity of extensin to potato lectin (46) which is putatively involved in disease resistance. Potato lectin was reported to strongly agglutinate avirulent strains of the bacterial pathogen Pseudomonas solanacearum. The virulent isolates, however, produce an extracellular polysaccharide which apparently protected the cells from binding with the lectin (46). Sequeira et al. (45) demonstrated a similar agglutination of avirulent cells of P. solanacearum in tobacco cells, and showed that the bacteria were specifically attached to the cell walls. These workers postulate that these glycoproteins may function in binding of bacteria to the plant cell wall. However, related work done more recently by Leach et al. (39) demonstrated that potato lectin was not responsible for the agglutination activity, but rather an HRGP was involved in agglutination.

III. Methods to quantitate and localize soluble extensin

A. Quantitation by enzyme-linked immunosorbent assay.

Immunoassays have replaced many other methods used to detect or quantitate substances with important biologic properties. The high levels of sensitivity and specificity achieved with immunoassays result from the specific, high affinity, reversible binding of antigens to antibodies, and from the existence of methods for attachment of sensitively detected labels such as isotopes, fluorescent compounds, and enzymes to antigens or antibodies.

Among the first applications of enzymes as labels was the use of enzyme-antibody conjugates to detect and localize antigenic cellular components by light and electron microscopy (1). Later, the use of enzyme-antigen and enzyme-antibody conjugates in immunoassays was reported by Engvall and Perlmann (12), and indepently by Van Weemem and Schuurs (52).

The enzyme-linked immunosorbent assay (ELISA) has been the subject of several reviews (10,11,54,55). The ELISA is based on the principle that the amount of an enzyme-labelled antigen bound by a fixed level of antibody is inversely proportional to the amount of unlabelled antigen present. The unlabelled antigen competes with the labelled antigen for antibody binding. Thus, a dose-response curve for serial dilutions of samples containing unknown levels of a particular antigen can be compared to a standard curve derived from dilutions of a known quantity of the purified antigen. The key to the sensitivity, specificity, and precision of the assay is the antibody. The radioimmunoassay (RIA) in which a radioactive tracer labelled antigen along with the ELISA are known as ligand-binding assays. The sensitivity of both these assays is determined by the specificity of the binding reagent and its affinity for the ligand. The extreme specificity and high affinity of antibodies

for antigenic determinants ideally suits them for this role.

The development of an ELISA for a particular antigen involves several aspects, all of which contribute to the precision, sensitivity, specificity, and reproducibility of the assay (25). These include the purification of the antigen to homogeneity under conditions which do not alter its immunological properties relevant to the samples to be tested. Secondly, a specific antiserum must be chosen which ideally will have higher titres of high avidity antibodies which at the same time are immunologically specific. Thirdly, reaction conditions must be established to provide precision and sensitivity for the assay. Finally, the data must be analyzed both with regard to the quantitative and qualitative patterns of inhibition of binding. An example of the use of these assays to quantify a plant protein was demonstrated by Saunders et al. (44). The assay was used to quantify phytochrome, a plant protein, from extracts of plant tissue.

B. Localization by immunofluorescence.

Immunofluorescence technology provides a very important connection between biochemical or immunological approaches and cytological methods in the study of many biological problems. Cellular products which can be isolated and characterized can also be localized on or in cells using immunofluorescence techniques. The method, first outlined by Coons in 1941 (4) and further refined in 1942 (5) and in 1950 (6), has been used extensively in the biomedical field. The technique has the advantage of being capable of detecting components on living cells.

Immunofluorescence analysis also has the advantages of immunochemical methodology such as extreme specificity and sensitivity. This technique has many of the same potential problems as any

immunological method such as non-specific artifacts and impure antibodies with which to work. Therefore, all experiments using the technique require strict controls for non-specific staining, and the use of the purest fluorescent tracer reagents available. Much more detailed information on the methodology involved in immunofluorescence, and its limitations, can be obtained from many excellent reviews on the subject (18,25,43). Immunofluorescence techniques have been used previously to localize glycoproteins in plant cells. For example, Kilpatrick et al. (27) demonstrated that hydroxyproline-rich glycoproteins from Datura stramonium were localized in the cytoplasm and presented immunological evidence for structural similarly within the HRGP lectins from the Solanaceae (26). In addition, Etzler et al. (17) demonstrated that a molecule which cross-reacts with arom the

Solanaceae (26). In addition, Etzler et al. (17) demonstrated that a molecule which cross-reacts with anti-Dolichos biflorus lectin is located in the cell walls of stems, leaves, and cotyledons. Leach et al. (40) demonstrated that proteins similar to the HRGP-bacterial agglutinin extracted from potatoes are located on or in many varying species of monocot and dicot cell walls.

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PART I

DETECTION OF GLYCOSYLATED AND DEGLYCOSYLATED EXTENSIN PRECURSORS BY INDIRECT COMPETITIVE ELISA

ABSTRACT

A competitive indirect enzyme-linked immunosorbent assay (ELISA) was developed for the rapid quantitation of the glycosylated and deglycosylated forms of the monomeric soluble extensin precursor subunits P1 and P2. Immunization of rabbits with each extensin precursor resulted in anti-precursor antibody titers of 40 to 1600 in 18 weeks. A competitive indirect ELISA was conducted by simultaneously incubating precursor with anti-precursor antiserum over precursor solid phase and then determining bound rabbit immunoglobulin with goat anti-rabbit peroxidase conjugate. Linear response range for each kind of precursor in the competition curve was between 1 ug/ml and 10 pg/ml.

INTRODUCTION

Extensin is a plant cell wall-bound hydroxyproline-rich glycoprotein (HRGP). Recently, Smith et al. (11) were able to isolate two different soluble forms of extensin out of salt eluates from tomato suspension cell walls. These HRGPs were shown to vary in amino acid composition. These two extensins, called P1 and P2, represent monomeric precursors to insoluble extensin. Although the evidence is correlative, extensin may function as part of the resistance mechanism against potentially pathogenic microorganisms (2,3,8,9,12). Extensin accumulation has been demonstrated to be correlated with wound and pathogen induced responses (2,3,8,13). These responses include the accumulation of extensin in the cell walls of wounded carrots (13), the localization of an HRGP as a bacterial agglutinin in potato cell walls (8), and the accumulation of HRGPs in the cell walls of diseased plants in response to fungal infection (2,3). Recent work by Stermer and Hammerschmidt has shown that extensin accumulates in the cell walls of cucumber in response to a disease resistance inducing heat shock (12). The accumulation of extensin in cell walls may make the wall more resistant to degradation by pathogen induced cell wall degrading enzymes (3). Leach, et al. suggest that the HRGP may act in resistance to bacteria as an agglutinin which acts by immobilizing incompatible bacteria (8,9). The precise function and role of extensin in disease resistance have, however, not been unequivocally proven. A principal question in ascertaining the role of extensin in disease resistance is

whether it accumulates to sufficient concentrations at infection sites at or before the time that pathogen growth stops. Satisfactory evidence has not been provided on this point, partly because of the inability to quantitate soluble extensin accurately in plant cell walls.

Current techniques for quantitation of the accumulation of HRGPs in cell walls involve spectrophotometric measurement of hydroxyproline released after acid hydrolysis (7). Such methods are, however, cumbersome and require two days to obtain the final results. We report here the development of a highly sensitive and rapid enzyme-linked immunosorbent assay (ELISA) for the precise quantitation of extensin precursors.

MATERIALS AND METHODS

Materials. All inorganic chemicals were reagent grade or better. Bovine serum albumin (BSA) (fatty acid free and fraction V), polyoxyethylenesorbitan monolaurate (Tween 20), and 2,2'-azino-di(3-ethylbenzthiozaline) sulfonic acid (ABTS) were obtained from Sigma Chemical Co., St. Louis, MO; Freund's complete and incomplete adjuvants, and goat antirabbit IgG conjugated to horseradish peroxidase (antirabbit-peroxidase) were from Cooper Biomedical, Malvem, PA; and immunoassay microtiter plates (immunoplates) were from Nunc Intermed, Roskilde, Denmark.

Preparation of Immunogen. The extensin precursors, P1 and P2, were isolated by direct elution of the cell surface of intact tomato cell suspension cultures and further purified by column chromatography according to the method of Smith, et al. (11).

Deglycosylation of P1 and P2 involved hydrolysis of the carbohydrate moieties from the glycoprotein with HF-MeOH according to the method of Smith, et al. (11).

Rabbit immunization. P1, P2, and deglycosylated P1 and P2 (DP1 and DP2) were used as immunogens. Initial injection of all four immunogens employed a modification of the multiple site method of Vaitukaitis, et al. (14). For the two glycosylated immunogens, P1 and P2, 0.5 mg precursor in 1.0 ml of 0.9% saline was emulsified with 2.0 ml Freund's complete adjuvant. For the two deglycosylated immunogens DP1 and DP2, 0.5mg precursor in 0.5ml of 0.9% saline was emulsified with 1.5ml

Freund's complete adjuvant. With all four precursors, the preparation was injected intradermally into 4 sites on the back of a New Zealand white doe rabbit. Subsequent injections were made at six week intervals using Freund's incomplete adjuvant emulsified in the same ratios and concentrations as described above, but at one-half the volume. Rabbits were bled through the marginal ear vein and sera purified by three ammonium sulfate (35% saturated) precipitations (4) followed by dialysis overnight at 4°C against 0.1M phosphate buffer in 0.15M saline (PBS, pH 7.5).

Antibody titration by indirect ELISA. Appendix A summarizes the indirect ELISA protocol used for antibody titration in a flow diagram. Wells in polystyrene immunoplates were coated with 0.2 ml of 50 mM carbonate-bicarbonate buffer, pH 9.6, containing precursor diluted to 10 ug/ml. The plates were incubated overnight at 4°C. They were then washed with tap water for 1 minute using an immunoplate washer. The plate washer consisted of a plexiglass box in which holes (1 mm in diameter) had been made corresponding to wells of a 96 well microtiter plate. Tap water was forced into the box and through the holes over which an immunoplate was inverted and washed. After the washing procedure, the plate was shaken dry and 200 ul of PBS containing 1% (wt/vol) BSA was added to each well to block unbound solid phase sites and minimize nonspecific binding. The plates were then incubated for 30 min at 37°C, and washed as described above. Fifty ul of antiserum homologous to the coating precursor was diluted in PBS containing 1.0% BSA and 0.1% Tween 20, pH 7.5, were added to each well, and the plate was incubated at 37°C for 60 min. In all titration procedures and in the indirect competitive procedures (to be described), the antibody used

was always homologous to the precursor that was used to coat the plate. The plate was washed as described above and 50 ul of goat anti-rabbit peroxidase conjugate (diluted 1:2000 (v/v) in PBS containing 0.1% BSA-Tween) was added to each well. After incubation at 37°C for one hour, the plate was washed for 2 min and 0.1 ml of peroxidase substrate containing 1.2 mM hydrogen peroxide and 0.4mM of ABTS was added. Thirty minutes after incubation at 37°C, the reaction was terminated by adding 0.1 ml of stopping reagent (hydrofluoric acid-ethylenediamine tetra-acetic acid (10). The absorbance at 410nm was determined in a Dynatech Minireader (Dynatech Instruments, Alexandria, Va.) after incubation at room temperature. There were three replications of each treatment.

Determination of precursor concentrations by indirect competitive ELISA. Appendix A summarizes the indirect ELISA protocols in flow diagrams. The indirect competitive ELISA was identical to the indirect titration procedure described above except for an added preincubation before adding the antibody to the polystyrene wells. Twenty-five ul of 1% BSA in PBS-Tween was first added to each well to reduce nonspecific antibody binding. To obtain reproducible competition data, it was necessary to preincubate 25 ul of antisera along with 25 ul of varying dilutions of free glycosylated precursor in glass test tubes at room temperature for 2 hours. After 2 hours, 50 ul of the preincubated antibody/antigen mixture was added to microtiter wells and the assay proceeded as in the antibody titration procedure described above. When antisera to deglycosylated precursor was used, the preincubation step described above was not used; rather, 25 ul of free precursor was added to the wells followed by immediate addition of 25 ul of diluted antisera. Antisera dilutions used were 1:1500 (v/v). Cross-reactivity of the various precursors in the indirect ELISA was determined as above

except that heterologous instead of homologous antigen was added along with homologous antibody. Antibodies against glycosylated and deglycosylated forms of each precursor were tested.

RESULTS AND DISCUSSION

Production of antibody against extensin precursors. Two glycosylated (P1 and P2) and two deglycosylated (DP1 and DP2) extensin precursors were used to elicit anti-extensin antibody responses in rabbits. Production of antibodies was similar to a previously described method (5). An indirect ELISA was developed to monitor titers of anti-extensin antiserum. In this assay, rabbit antiserum was incubated over a microtiter plate solid phase coated with 10 ug/ml precursor and total bound antibodies were subsequently detected with goat-antirabbit peroxidase conjugate. The serum dilution showing absorbance distinct from that of a preimmune serum control at the same dilution was arbitrarily designated as the titer. Figure 1 shows the titration curve for rabbit P-1 serum (18 wks after initial inoculation). Preimmune serum controls showed negligible absorbance at each dilution whereas this antiserum was diluted 6400-fold and still had significant absorbance. Antisera to the other three precursors could be diluted at least 6400-fold and produce a significant response.

Competitive indirect ELISA. A competitive indirect ELISA was carried out by simultaneously incubating precursor with an appropriate dilution (1:1000-1:1600) of homologous rabbit anti-precursor antiserum over a precursor solid phase and then determining bound rabbit antibody with a goat anti-rabbit peroxidase conjugate. A typical competition curve for deglycosylated antigen is shown in Figure 2. The response range for this curve was between 0.01 and 1000 ng/ml. Each precursor

produced a similar competition curve with minimum sensitivity of 10 pg/ml. For each curve a level of 1.0 ng/ml could be determined readily without the aid of a spectrophotometer.

An essential component in our indirect ELISA was the incubation of glycosylated precursor with diluted anti-precursor antiserum for 2 hours in glass test tubes at room temperature prior to determination of bound rabbit antibody. Reproducible competition curves could not be obtained for glycosylated precursor without preincubation. However, this preincubation step was not necessary when assaying deglycosylated precursor.

Specificity of anti-precursor antibody. In order to ascertain the specificity of the rabbit anti-precursor antiserum, the ability of each precursor to compete with each bound precursor was evaluated. Concentrations of the four precursors which resulted in 50% inhibition of anti-precursor antibody binding to the solid phase are presented in Table 1. The 50% inhibition value is a standard means of comparing cross-reactivities among similar antigens to an antiserum (1). These values were calculated as the amount (by weight) of free precursor required to reduce antibody binding by 50% (Table 1). Concentrations which resulted in 50% inhibition of anti-glycosylated P1 antibody binding to the solid phase were 0.03, 0.07, 0.03, and 0.16 ug/ml for glycosylated precursors 1 and 2, and deglycosylated precursors 1 and 2 respectively (Table 1). Thus, P1 antibody appears to react similarly with all four precursors. Concentrations which resulted in 50% inhibition of solid phase-bound anti-P2 antibody were 0.41, 0.17, 14.0, and 21.0 ug/ml for P1, P2, DP1, and DP2 respectively (Table 1). Anti-P2 antibody thus appears to react more strongly with the glycosylated

precursors than with the deglycosylated precursors. Therefore, it appears that this antiserum binds primarily to the carbohydrate moiety of the native glycosylated protein. Both the anti-DP1 and anti-DP2 antisera bound strongly and equivalently with both deglycosylated precursors but not at all (at 10 ug/ml) with the glycosylated forms (Table 1). These results suggest that the carbohydrate moiety on the native glycosylated precursors do not allow the molecule to react with the antibodies made to the deglycosylated precursors presumably because the protein portion of the glycosylated molecule is unavailable for antibody binding.

Smith, et al. (11) determined that the molar ratio of each glycosylated precursor form consisted of approximately two-thirds carbohydrate and one-third protein. Therefore, it would take three times as many moles of DP1 to inhibit antibody binding than it does P1 even though, by weight, it takes a similar amount of DP1 as P1 to inhibit binding of P1 antibody.

Extensin is a naturally occurring glycoprotein in plant cell walls (6). Soluble extensin, or HRGPs, isolated from carrots (13) and sycamore (7), as well as bacterial agglutinin (8), have similar amino acid composition. It is conceivable, therefore, that antibodies prepared against tomato extensin precursors will recognize similar sequences of precursors present in cucumber.

The differential precursor cross-reactivities of these four antisera can be used for determinations of the different precursor forms present in a plant extract. If an extract is mixed with each antiserum and then used in the competitive indirect ELISA, the amount of cross-reactivity (inhibition) with each antiserum should reveal the precursor profile of that sample. For example, cross-reaction with P1 antiserum

and not DP1 antiserum establishes the presence of glycosylated precursors in the sample (Table 1). This result can be confirmed by similar results with P2 antiserum. Cross-reaction with P1 antiserum and DP1 antiserum but minimally with P2 antiserum would be expected with a sample containing only deglycosylated forms of the precursor (Table 1). Other precursor profiles should give reactions as indicated in Table 1.

The antibodies prepared in this study have cross-reactivities similar to precursor antibodies previously characterized (5). As explained above, these differing antibody specificities could be useful in assaying for precursors present in biological systems.

In conclusion, we have described procedures for the production of anti-extensin precursor antibody in rabbits and for the use of these antibodies in the competitive indirect ELISA for extensin. Indirect competitive ELISA would have significant advantages over existing methods (7) for quantifying low levels of soluble extensin in plant extracts.

Figure 1. ELISA titration of rabbit anti-P1 antibody. Absorbance of preimmune serum at same dilutions were subtracted from absorbance using immune serum.

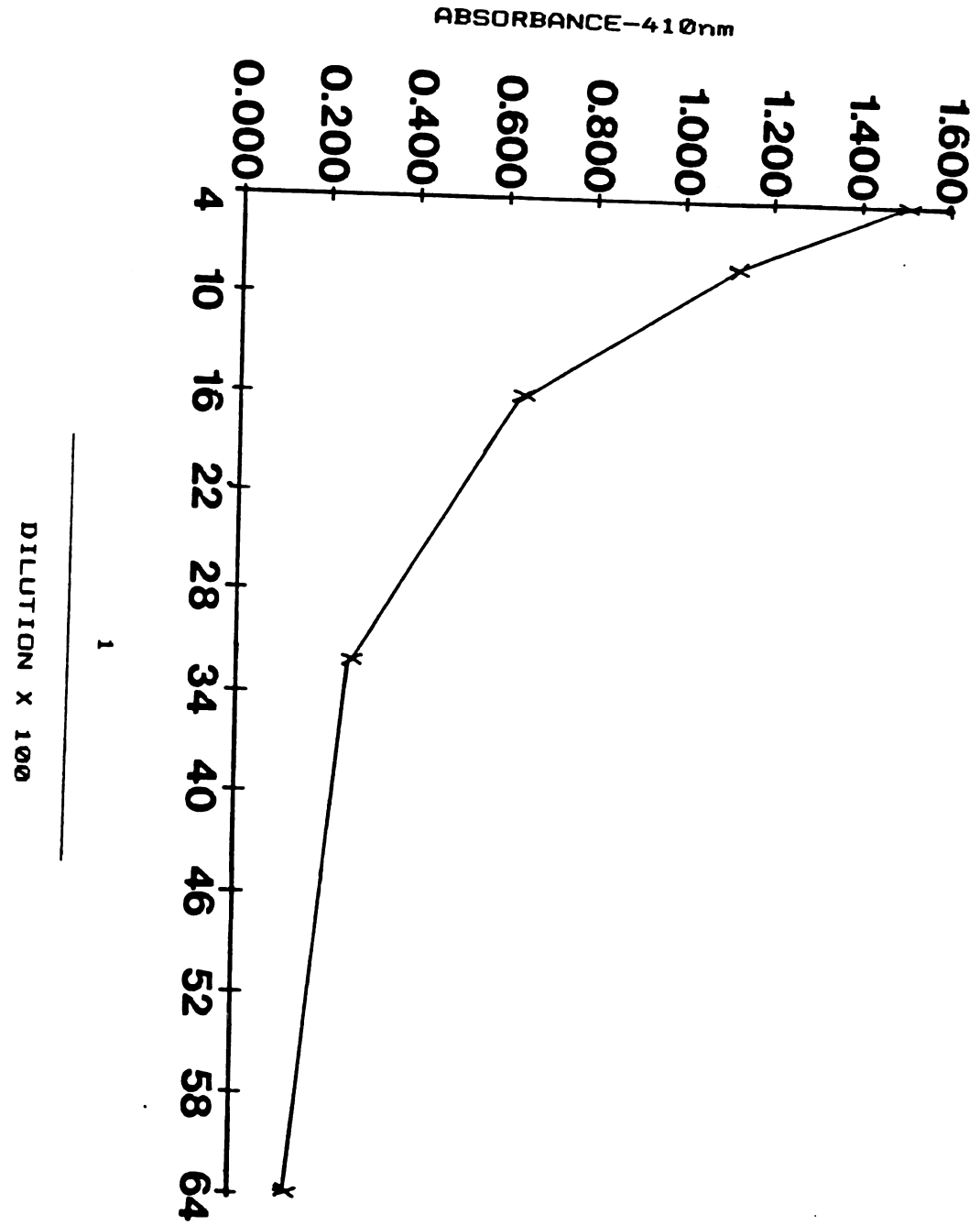
ABSORBANCE-410nm
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
4
10
16
22
28
34
40
46
52
58
64
DILUTION X 100
1

Figure 2. Competitive indirect ELISA standard curve to DP1. Each data point represents triplicate determinations in a single microtiter plate. In this standard curve, absorbance obtained with 0.1ug/ml of free precursor was significantly different (P=0.05 by Student's t-test) than absorbance obtained using precursor diluent only. Absorbance values represent the mean of four replications. Standard deviation was always 0.02 absorbance units or less. The absorbance value when preimmune serum was used was 0.00.

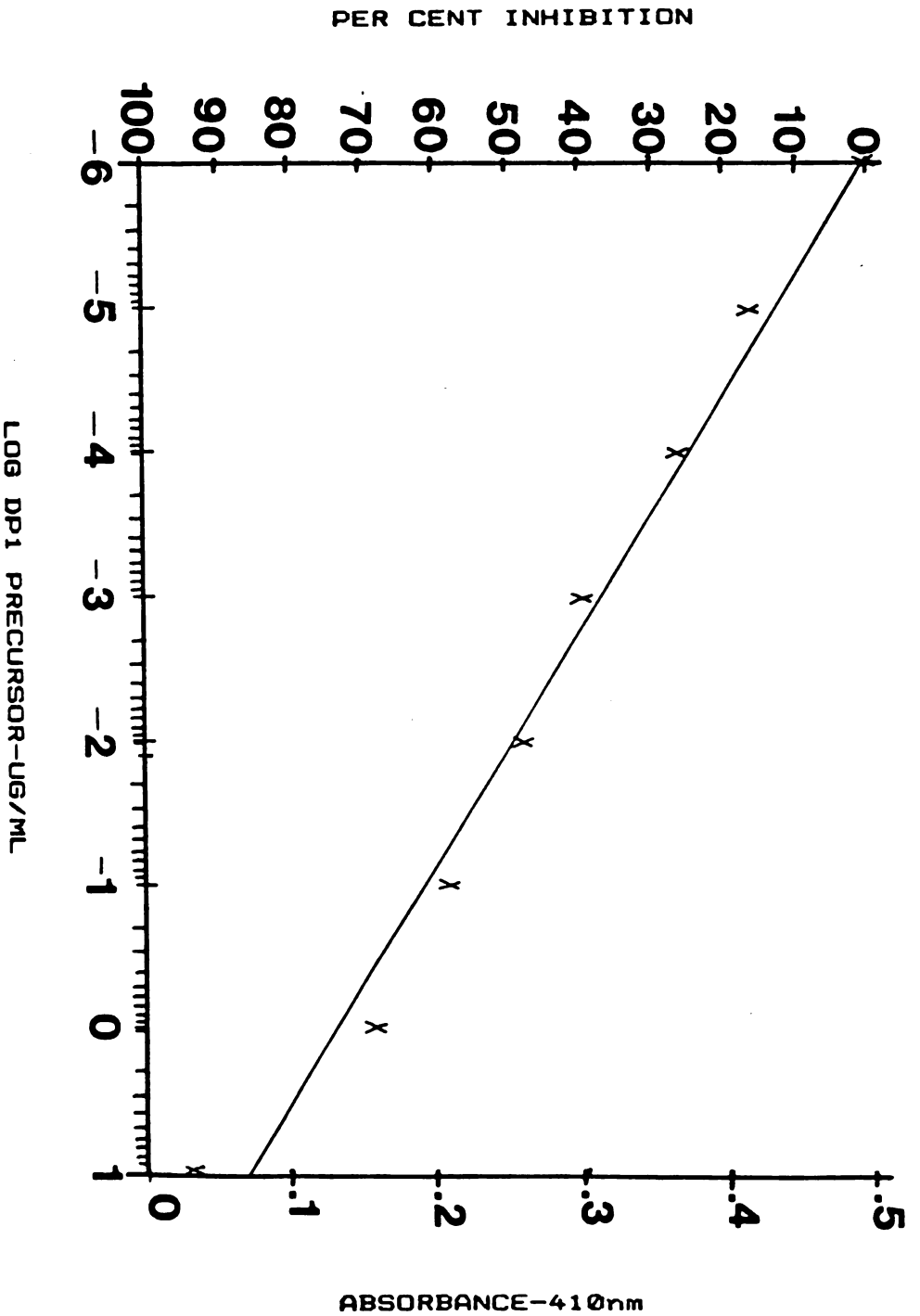
PER CENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
-6
-5
-4
-3
-2
-1
0
1
0
.1
.2
.3
.4
.5
LOG DP1 PRECURSOR-UG/ML
ABSORBANCE-410nm

Table 1. Sensitivity of various extensin precursor antibodies to extensin precursor antigens in indirect competitive ELISA (CEIA).[a]

Extensin precursor bound to immuno-plate (solid phase)	Free extensin precursor added in CEIA	50% inhibition[b]
P1	P1	0.03
P1	P2	0.07
P1	DP1	0.03
P1	DP2	0.16
P2	P1	0.41
P2	P2	0.17
P2	DP1	14.0[d]
P2	DP2	21.0[d]
DP1	P1	*[c]
DP1	P2	*
DP1	DP1	0.01
DP1	DP2	0.07
DP2	P1	*
DP2	P2	*
DP2	DP1	0.06
DP2	DP2	0.32

[a]All values are in ug/ml of extensin precursor in 0.1M PBS, pH 7.5. Antisera homologous to the bound precursor was added with free precursor, incubated 1 hour, then washed. Amount of bound antibody was determined with anti-rabbit peroxidase followed by peroxidase substrate. There were four replications per immunoplate for each treatment. Each experiment was performed at least two times with comparable results.

[b]ug/ml extensin precursor required to inhibit binding of antibody by 50% to the precursor solid phase. Inhibition was determined by regression analysis. The correlation coefficient (r) for all curves except the P2-DP1 combination were greater than 0.93. The r value for the P2-DP1 curve was 0.77.

[c]*=no competition demonstrated at 10 ug/ml free extensin precursor.

[d]values were extrapolated based on experimental values lower than the value indicated.

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PART II

LOCALIZATION OF EXTENSIN BY IMMUNOFLUORESCENCE MICROSCOPY

ABSTRACT

We have investigated the cellular distribution of insoluble extensin in hypocotyls of cucumber (Cucumis sativus L.) seedlings subjected to a disease resistance inducing heat shock followed by inoculation with the fungal pathogen Cladosporium cucumerinum Ell and Arth., using antibodies to the soluble precursor forms of extensin. Antisera to glycosylated precursor (P1 and P2) and deglycosylated precursor (DP1 and DP2) forms of extensin were produced. Plant tissue cross-sections were treated with anti-precursor antibodies followed by fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin. Fluorescence microscopy revealed that anti-precursor antibodies bound only to the upper and lower epidermal cell walls of sections from inoculated and non-inoculated heat-shocked plants. Anti-precursor antibodies did not bind to sections from non heat-shocked plants. Antibodies to all four forms of precursor bound to the epidermis and sub-epidermal cell walls, indicating that proteins that cross-react with these antibodies are present in heat shocked, inoculated seedlings. The significance of these findings in relation to disease resistance is discussed.

INTRODUCTION

The role that hydroxyproline-rich glycoproteins (HRGPs), such as extensin, play in disease resistance is not known. Hammerschmidt, et al. (11) have shown that extensin increases in the cell walls of cucumber seedlings infected with the fungus Cladosporium cucumerinum Ell. and Arth. the cause of cucumber scab. This increase in extensin correlates with resistance to C. cucumerinum. Further evidence for the possible role of extensin in the defense mechanism of plants was demonstrated by Leach et al. (14,15) who showed that the bacterial agglutinin from potato was similar to soluble extensin. Recent work by Stermer and Hammerschmidt (19) has shown that a brief heat shock enhances the accumulation of extensin in the cell walls of cucumber seedlings. Inoculations of heat shocked seedlings with C. cucumerinum 24h after the shock resulted in further enhancement of extensin. The increase in cell wall hydroxyproline content observed in aged carrot discs has also been postulated to occur as part of a response to wounding in excised tissue (2). In each instance described above soluble extensin becomes insolubilized in plant cell walls with time. Other workers have suggested that the increase in extensin biosynthesis may be part of the plant's defense mechanism against invading pathogens (3,5).

The soluble extensins produced in wounded carrot root tissue and from tomato cell suspension cultures have been well characterized biochemically (1,18,22). A common amino acid sequence is present in both carrot and tomato extensin (1,18). Because a similar amino acid

composition is present in both these kinds of tissue we believed that cucumber extensin may also contain a similar amino acid sequence.

The purpose of this study was to determine the distribution of extensin in cucumber seedlings subjected to heat shock and infected with the fungus C. cucumerinum. Recently developed antibodies to glycosylated (P1 and P2) and deglycosylated (DP1 and DP2) forms of tomato extensin (4) were used to visualize immunocytochemically these glycoproteins in cucumber seedlings.

MATERIALS AND METHODS

Materials. All inorganic chemicals were reagent grade or better. Bovine serum albumin (BSA) (fatty acid free and fraction V), polyoxyethylenesorbitan monolaurate (Tween 20), and 2,2-azino-di (3-ethylbenzthiozaline) sulfonic acid (ABTS) were obtained from Sigma Chemical Co., St. Louis, Mo; Freund's complete and incomplete adjuvants, sheep normal IgGs, and fluorescein conjugated sheep anti-rabbit IgG (FITC-IgG) from Cooper Biomedical, Malvern, PA; immunoassay microtiter plates (immunoplates) from Nunc Intermed, Roskilde, Denmark; and Nalgene Sterilization filter units, from Nalge Company, Rochester, New York. Purified glycosylated and deglycosylated forms of extensin precursors were generously provided by J.J. Smith (18).

Antigen production. Glycosylated precusors, (P1 and P2), and deglycosylated precursors, (DP1 and DP2), were prepared by direct elution of the cell surface of intact tomato cell suspension cultures according to the method of Smith, et al. (18).

Immunization protocol. This protocol is described in previous work (4). Four rabbits were injected repeatedly for more than one year. They were bled weekly and the titer was checked by the indirect ELISA method described in previous work (4).

Plant and fungal material. Cucumber (Cucumis sativus L., cv. Marketer; Burpee Seed Co., Warminster, PA) susceptible to C. cucumerinum were grown in rolled germination paper and placed in darkness for 5 days at 22 C (10). C. cucumerinum cultures were grown on potato

dextrose agar at 18 C (21).

Heat shock and inoculation of seedlings. Seedlings were treated by dipping the cotyleons and hypocotyls in a 50 C water bath for 40 seconds (19). The shocked seedlings were then rolled on moistened germination paper and incubated in the dark at 22 C. Twenty-four hours after heat shock one-half of the seedlings were inoculated by spraying with a spore suspension of 3 X 10^6 spores per ml. The etiolated seedlings were rolled again in germination paper and incubated at 22 C.

Sectioning and staining procedures. Individual plants were selected for sectioning 6, 12, 18, 24, 48, 72, and 96 h after heat shock. The apical 2 cm. of tissue were excised below the hook region and sectioned immediately. Cross sections were made using a Hooker microtome (Lab-Line Instruments, Inc., Melrose Park, IL). Control sections were prepared from non-heat shocked or non-heat shocked, inoculated plants. Sections were stained with fluorescent antibody by using the indirect method, modified from Leach, et al. (15) which is described below and summarized in the form of a flow diagram in Appendix B. Each treatment was performed using at least 3 plants.

Labelling of the tissue sections was performed at room temperature. The sections were first placed on the upper portions of a Nalgene sterilization filter unit, Type S (115 ml, 0.45 micron pore) and rinsed carefully with 5ml of 0.15M saline (PBS, pH 7.5) in a plastic wash bottle. They were then immersed in 1ml of sheep normal IgG (A_{280nm} = 1.0) in PBS, incubated for 20 min and rinsed several times with PBS by applying a vacuum below the filter membrane for 3-4min to draw the fluid off the sections. The step involving sheep normal IgG was done to prevent nonspecific binding of anti-precursor antibodies. Purified

anti-precursor antibodies (A_{280nm} = 0.1) in PBS or normal rabbit IgGs (A_{280nm} = 1.0) were added to the sections. After a 20 min. incubation, the sections were rinsed with PBS and suctioned again. FITC-IgG, diluted forty-fold in PBS was added to the sections which were then incubated in the dark for 20 min. Following a final rinse, the sections were carefully washed off the filter unit onto glass slides and blotted dry. A drop of mounting buffer (9 parts glycerol to 1 part PBS, pH 7.6) was added to the sections which were then covered with a No. 1 coverslip. Sections were viewed X 250 in a Leitz Laborlux 12 microscope equipped with a high-pressure mercury lamp as an ultraviolet light source and with epi-illumination. The spectrum of incident ultraviolet light was controlled with exciter filters BP 450-490; a barrier filter (LP 515) was used for protection of the eyes. Photographs were taken with a Wild Heerbrug 35 mm camera, professional Kodak Ektachrome (ASA 100) film and 15s or 30s exposures.

RESULTS

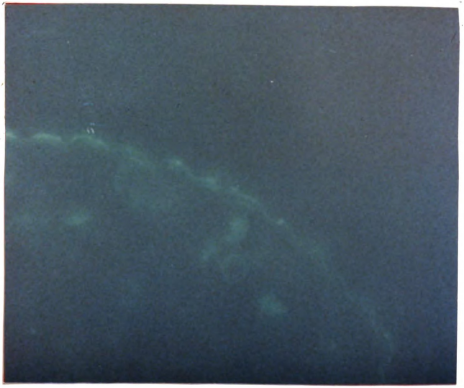
Staining of extensin precursors in cucumber hypocotyls. To localize the precursors, plant tissue sections were first treated with sheep normal immunoglobulins to minimize nonspecific binding of antibodies to the tissues. The staining procedures for immunofluorescence were done on tissue placed on a Nalgene filter unit which allowed gentle, thorough, rapid rinses.

In sections of hypocotyl, antibodies against the glycosylated precursors (P1 and P2) bound only to the upper and lower epidermal cell walls of heat shocked and heat shocked, inoculated seedlings, as indicated by the apple-green color of the fluorescent dye (Fig. 1 and 2). Fluorescence was also visible in the epidermis 12h after heat shock of uninoculated seedlings and immediately after inoculation of heat shocked seedlings. Fluorescence immediately after fungal inoculation was probably due to the prior heat shock and not the presence of the fungus. No difference in the intensity of fluorescence was visible between heat shocked or heat shocked and inoculated sections treated with antibodies made against P1 or P2. Control sections treated with pre-immune rabbit serum did not bind the FITC-labelled goat anti-rabbit immunoglobulins (Fig. 3 and 4). Fluorescence (other than autofluorescence) was also seen on the xylem vessels and collenchyma (Fig 5). Membranes apparently were not stained.

Antibodies specific for the protein portion of the glycosylated precursors were also produced in an attempt to eliminate cross-

Figure 1. View of cross section of cucumber hypocotyl indirectly stained with P1 antibody followed by anti-rabbit-FITC 72 h after heat shock (40 seconds at 50 C).

Figure 2. View of cross section of cucumber hypocotyl indirectly stained with P1 antibody followed by anti-rabbit-FITC 72 h after heat shock and 48 h after inoculation with spores of Cladosporium cucumerinum (3×10^6 spores per ml).



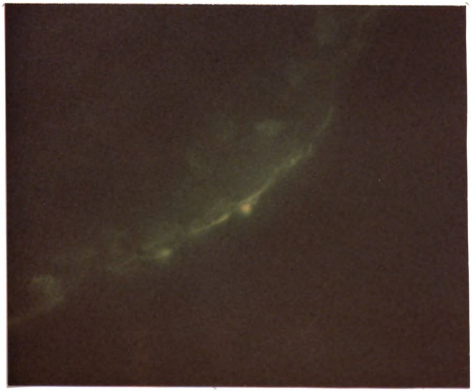
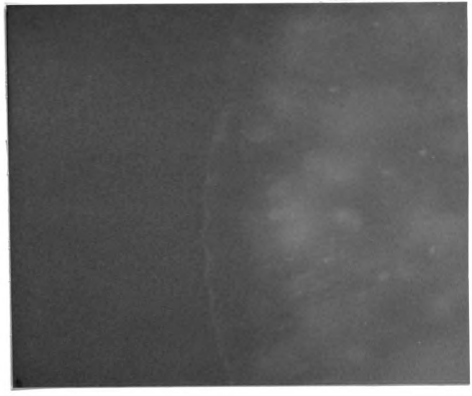


Figure 3. View of cross section of cucumber hypocotyl indirectly stained with rabbit normal serum followed by anti-rabbit-FITC 72 h after heat shock.

Figure 4. View of cross section of unshocked, uninoculated cucumber hypocotyls indirectly stained with P1 antibody followed by anti-rabbit-FITC.

Figure 6. View of cross section of unshocked cucumber hypocotyls indirectly stained with P1 antibody followed by anti-rabbit-FITC.



reactivity between glycosylated and deglycosylated forms of precursor and to eliminate cross-reactivity between the carbohydrate moiety of the precursors and polysaccharides present in the cell wall. The carbohydrate component was removed from glycosylated molecules by hydrolysis with hydrogen fluoride (18). Immunofluorescence of hypocotyl sections treated with antibodies made against both forms of deglycosylated precursors (DP1 and DP2) indicated that the protein portion of extensin precursors were specifically located in the upper and lower epidermal cell walls of heat shocked and heat shocked, inoculated tissue sections.

DISCUSSION

The immunofluorescence procedures described above indicate that forms of extensin precursor or precursor-like glycoprotein which cross-react with all four types of anti-precursor antibodies accumulate on or in the upper and lower epidermal cell walls of cucumbers subjected to a brief heat shock. This demonstration of the accumulation of high extensin precursor levels in response to heat shock is consistent with previous studies demonstrating the accumulation of cell wall HRGPs in cumcumber tissue subjected to heat shock (19). Additionally, the accumulation of cell wall HRGPs has been demonstrated in plant tissues infected with fungi (6.7,11). Esquerre-Tugaye et al. (8) have concluded that the accumulation of this glycoprotein acts as a defense mechanism which becomes efficient if started early in the host. Stermer and Hammerschmidt (19) have demonstrated that the extensin content of cucumber cell walls increases before the onset of resistance in heat shocked seedlings. They suggest that the cross-linking of extensin by an increase in peroxidase activity is crucial for disease resistance. The presence of extensin in the epidermal cell wall of heat shocked cucumber tissue is consistent with the theory that the greater cross-linking of extensin could be responsible for the resistance of heat shocked seedling cell walls to digestion by C. cucumerinum enzymes (19). Extensin may function in defense by directly forming a structural barrier to fungal invasion or it may indirectly provide sites for lignin deposition.

It was expected that antibodies prepared against soluble extensin from tomato bound to the epidermis and cell walls of a non-solanaceous plant. Soluble carrot and tomato extensin have now been well characterized by biochemical and molecular biological approaches (1,18,22). The amino acid composition of the HRGPs is similar for carrot (5) and tomato (18) cell walls, and tobacco (16) and potato agglutinins (14,17). Thus, antibodies prepared against the protein portion of soluble extensin of tomato appear able to bind to common sequences of the glycoprotein found in cucumber. This observation supports the theory that similar forms of extensin are present in all dicotyledonous plants.

Results from enzyme-linked immunosorbent assays discussed in Part I of this thesis show that anti-P1 antibody strongly cross-reacts with all other precursor forms. Anti-P2 antibody appears to bind primarily to the carbohydrate moiety of the native glycosylated protein, whereas, anti-DP1 and anti-DP2 antibodies do not react with anti-P1 or anti-P2 presumably because the protein portion of the glycosylated molecule is unavailable for antibody binding. Since anti-P1 and anti-P2 glycosylated forms of extensin precursor were able to absorb and fluoresce in the epidermal cell walls of heat shocked tissue, such precursor forms are presumably present in such tissue. More specifically, since anti-DP1 and anti-DP2 forms were able to absorb and fluoresce, the protein portion of the extensin molecule is located in cucumber cell walls subjected to heat shock. Since it is unlikely that the deglycosylated forms of precursor are present in the cell wall (R. Hammerschmidt, personal communication), the glycosylated forms must be configured in the cell wall in such a way as to allow antibody binding.

Previous studies (9,12,13,15) have described the production of highly specific fluorescent antibody preparations to hydroxyproline-rich glycoproteins (HRGPs) in plants. We have described methods for the production of anti-extensin precursor antibody in rabbits and for the use of these antibodies for the localization of extensin precursor in heat shocked plant tissue. Our results support the views of Stermer and Hammerschmidt (11,19) who consider the enhancement of extensin to be a factor in disease resistance. This system could provide a useful method to study the interactions of extensin in lignin synthesis and cell wall bound phenols in normal and abnormal cell wall metabolism (11,23,24).

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APPENDIX A

FLOW CHARTS FOR INDIRECT ELISA

All quantities are per well of microtiter plate. Dilutions for reagents vary from batch to batch (e.g. antisera, enzyme conjugates) will not be given.

Indirect ELISA- see Part I for reagent sources

200ul 10mg/ml precursor in 50mM carbonate-bicarbonate, pH 9.6

↓ incubate 4°C, overnight;

↓ wash 1 min. in H_2O using microtiter plate washer; air dry

200ul 1.0% BSA in PBS

↓ incubate 37°C 30 min; wash 1 min.

25ul 1.0% BSA in PBS/0.01% Tween-20

+

25ul purified antisera, diluted in PBS

↓ incubate 37°C 1h; wash 1 min.

50ul goat-antirabbit peroxidase diluted in 1% BSA/PBS/Tween

↓ incubate 37°C 30 min; wash 2 min.

100 ul ABTS/H_2O_2 solution

↓ incubate 37°C 30 min., rooom temperature

100ul HF/EDTA stopping solution

Read A_{410}

Indirect ELISA for quantitation of deglycosylated forms of precursor-see Part I for reagent sources.

200ul of DP1 or DP2 in 50mM carbonate-bicarbonate, pH 9.6

↓ incubate 4°C overnight;

↓ wash 1min with H_2O using immunoplate

↓ washer

200ul 1.0% BSA in PBS

↓ incubate 37°C 30min; wash 1 min

25ul 1.0% BSA/PBS/0.01% Tween 20

+

25ul diluted DP1 or DP2

+

25ul diluted anti-DP1 or anti-DP2

↓ incubate 37°C 1h; wash 1min

75ul goat anti-rabbit peroxidase conjugate diluted in 1.0% BSA/PBS/Tween

↓ incubate 37°C 30min; wash 2min

100ul ABTS/H_2O_2 solution

↓ incubate 30min, room temperature

100ul HF/EDTA stopping solution

Read A_{410}

Indirect ELISA for quantitation of glycosylated forms of precursor-see Part I for reagent sources.

200ul P1 or P2 in 50mM carbonate-bicarbonate, pH 9.6

↓ incubate 4°C overnight;

↓ wash 1min with H_2O using

↓ immunoplate washer

200ul 1.0% BSA/PBS

↓ incubate 37°C 30 min; wash 1min

25ul 1.0% BSA/PBS/0.01% Tween-20

+

50ul mixture containing diluted P1 or P2 and anti-P1 or anti-P2

(pre-incubated in glass test tubes for 2h, room temperature)

↓ incubate 37°C 1h; wash 1min

100ul goat anti-rabbit peroxidase conjugate diluted in 1.0% BSA/PBS/Tween

↓ incubate 37°C 30 min; wash 2 min

100ul ABTS/H_2O_2 solution

↓ incubate 30 min; room temperature

100ul HF/EDTA stopping solution

Read A_{410}

APPENDIX B

FLOW CHART FOR INDIRECT FLUORESCENT ANTIBODY PROCEDURE

Dilutions for reagents that vary from batch to batch (e.g. antisera, FITC-labelled enzyme conjugate) will not be given.

Indirect fluorescent antibody procedure for localization of precursor in plant tissue sections-see Part II for reagent sources

Tissue sectioned with Hooker microtome

↓ sections collected on Nalgene filter unit (0.45um pore); rinsed 3x PBS, pH 7.5

sections flooded with sheep normal IgG in PBS

↓ incubate 20min room temperature; rinse 3x PBS, pH 7.5

Purified anti-precursor antibodies in PBS or normal rabbit IgGs in PBS added to sections

↓ incubate 20min room temperature; rinse 3x PBS, pH 7.5

FITC-conjugated sheep anti-rabbit IgGs added to sections

↓ incubate 20min in dark, room temperature; rinse 3x PBS, pH 7.5

sections washed off filters onto glass slides

↓ blot dry

drop of mounting buffer added to sections

sections covered with No. 1 coverslip; view sections under epifluorescence