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DOE/ER/13717--4

DE93 011613

SUMMARY OF RESULTS OF STUDIES COMPLETED ON

DOE GRANT No. DE-FG02-87ER 13717

Section 1.- Role of Proteolytic Enzymes in Degradation of Plant Tissues.

Most bacterial plant pathogens that degrade plant tissues have the ability to produce extracellular proteases. For many years, primary research emphasis has been placed on the nature and function of pectolytic enzymes and, to a lesser degre, of cellulaes, in efforts to understand the mechanisms by which plant tissue is macerated. Recent studies on the importance of hydroxyproline-rich glycoproteins (HPRG's) in the nature and function of plant cell walls have led to the question as to whether proteolytic enzymes are also involved in tissue maceration and act in concert with other cell wall degrading enzymes in the process. The primary objective of this section of the research was to determine whether proteolytic enzymes, in combination with other enzymes, are involved in the degradation of plant cell walls and thus may be essential for pathogenesis by certain soft rot bacteria.

The proteolytic enzymes of Ecc.-

The proteolytic enzymes of strain SR394 of Erwinia carotovora subsp.carotovora (Ecc) grown on various media were examined by isoelectrofocusing in polyacrylamide gels over a pH range of 3-10. In addition to the main protease present in culture filtrates, low concentrations of several other proteases were present in extracts from potato tubers infected by Ecc. Proteases from all these sources were similar and had the following properties: pH optimum near 8.0, calcium dependent, insensitive to serine proteinase and SH-proteinase inhibitors, inhibited by EDTA, and highly thermostable. These enzymes degraded gelatin, soluble collagen, and Hide Powder Azure, and showed weak activity on casein, but did not degrade insoluble collagen or elastin.

The main proteases of Ecc have isoelectric points at pH near 4.2 and 5.1. However, when bacteria were grown in media supplemented with cell walls, a very faint band of alkaline protease was detected. This protease, produced in media containing plant cell walls, was thought to be involved in tissue degradation since free hydroxyproline was detected in this medium after incubation with the bacterium. However, Ecc proteases did not degrade potato lectins, which are hydroxyproline-rich glycoproteins. Similarly, hydroxyproline-rich proteins isolated from carrot roots, and ionically-bound proteins extracted from potato cell walls, were not degraded by Ecc proteases. It was concluded that free hydroxyproline, which can be detected in filtrates of media containing cell walls, was not derived from soluble hydroxyproline-rich proteins, but, rather, from insoluble cell wall proteins. In general, bacterial proteases did not degrade proteins present in potato tissue extracts.

Effect of Ecc proteases on plant cell walls.-

Potato cell walls free of ionically-bound proteins released hydroxyproline-containing compounds when incubated with filtrates of various culture media containing Ecc proteases. However, free hydroxyproline was detected only after hydrolysis of these compounds with 5N HCl. Complete inhibition of proteolytic activity by alpha-macroglobulin did not have any influence on pectin degradation or on the associated release of hydroxyproline-containing substances from cell walls. Also, complete inactivation of pectin lyase by heating stopped cell wall degradation even though proteases in the reaction mixture still retained about 60% of their activity.

Removal of a large portion of the cell wall polysaccharides, by boiling at pH 1.0, did not facilitate the liberation of free hydroxyproline by the Ecc protease. Since Ecc creates reducing conditions either when grown in sythetic media or in potato tubers, it was postulated that hydroxyproline may be released only under reducing conditions due to activation of some proteases and increased susceptibility of some proteins to degradation when in the reduced state. However, the rate of cell wall degradation remained the same with and without 5 mM sulfite, as determined by electrophoretic patterns of degradation products.

When Ecc was grown in a medium containing potato cell walls, hydroproline was released into the medium. However, when cell walls were treated with culture filtrates containing proteases, hydroxyproline was not detected in the medium. This indicated the possibility that some undetected specific protease could have been adsorbed to cell walls and was discarded with cell wall debris when filtrates were prepared. However, no proteolytic activity was desorbed from cell wall debris by rinsing this material with IN KCl and subsequently with 0.1% SDS.

Plant cell walls did not adsorb and did not inactivate proteolytic activity of crude filtrates; in contrast, pectin lyase was strongly bound to cell walls and caused immediate and rapid change in the physical properties of the cell wall (shrinkage, agglutination). Preincubating cell wall suspensions with proteolytic enzymes did not affect binding by pectin lyase and the subsequent degradation of pectic compounds.

Several factors, such as: a) insolubility of polypeptide cell wall material treated with Ecc proteases, b) the failure of degraded cell walls to release amino acids and the requirement for subsequent acid hydrolysis for this release to occur, and c) the liberation of large amounts of other than polypeptide components by bacterial enzymes suggested that evidence for degradation of cell wall proteins could be obtained more readily by labelling the polypeptides *in muro* and detecting the products released by simple colorimetric methods. Thus, potato cell walls were stained with the amino group reagent, trinitrobenzene sulphonic acid (TNBS) and then subjected to enzymatic degradation by Ecc enzymes or extracted with 5 M ammonia. TNBS-stained material was liberated from cell walls only with the 5 M ammonia treatment.

The ammonia-dissolved material from cell walls: 1) had immunolcical properties similar to those of potato lectins, 2) agglutinated red blood cells, 3) contained hydroxyproline, and 4) reacted with protein reagents. After SDS electrophoresis, two protein bands were detected; one of these bands disappeared after tratment with crude Ecc culture filtrates. Increased solubilization of cell wall proteins was observed following incubation of cell walls with culture filtrates at pH 5,8 and subsequent ammonia extraction.

SUMMARY

Free hydroxyproline was not released from potato cell wall after treatment with partically purified proteolytic enzymes of Erwinia carotovora susp. carotovora (Ecc) obtained from various media and from infected rotting tubers. These proteases did not degrade potato lectins or hydroxyproline-rich proteins isolated from carrots, even when these proteins were denatured by boiling or treated with reducing compounds.

The Ecc proteases did not degrade other proteins present in potato tuber extracts or proteins ionically bound to cell walls. Furthermore, deglycosylation of cell walls by boiling them at pH 1.0 did not promote solubilization of cell wall proteins by protease-containing filtrates of Ecc. Treatment with Ecc proteases did not increase electrolyte release from potato tuber slices and did not affect vital staining with neutral red. "Neither cell wall degradation nor electrolyte leakage by pectin lyase was accelerated in the presence of proteases. However, lectin-like, hydroxyproline-containing proteins extracted from cell walls with 5 M ammonia were degraded when incubated with Ecc proteases. Cell walls pretreated with proteinases of Ecc and subsequently extracted with 5 M ammonia also gave a higher yield of cell wall soluble compounds.

To sum up, Ecc proteases appear capable of degrading at least one type of cell wall protein in vitro, but we were unable to obtain evidence that these proteases can attack cell wall proteins in muro. The results indicate that some glycosidic alkali-labile bonds have to be broken before Ecc proteases can degrade cell wall proteins. Thus, these proteases may play a role in cell wall degradation only when acting in concert with other enzymes that split glycosidic bonds.

Publications

Lewosz, J., Kelman, A, and Sequeira, L. 1992. The protease of Erwinia carotovora subsp. carotovora and its relation to degradation of plant cell walls. Phytopathology (to be submitted)

Lewosz, J., Kelman, A., and Sequeira, L. 1898. Release of hydroxyproline-rich glycoproteins (HPRG's) from potato cell walls by enzymes from Erwinia carotovora subsp. carotovora. Phytopathology 79:1181.

Lewosz, J., Kelman, A., and Sequeira, L. 1989. Demonstration of activity of pectate lyase, polygalacturonase, and protease on SDS-polyacrylamide gels. Proc. 5th. Intl. Fallen Leaf Conf., South Tahoe, CA., p. 30.

Section 2. Induction of Polygalacturonases Important in Plant Pathogenicity of Pseudomonas solanacearum.

Plant cell wall degradation is known to play a role in many plant diseases, including bacterial wilt caused by Pseudomonas solanacearum. Although most well-known bacterial plant pathogens (such as Erwinia carotovora subsp. carotovora) rely primarily on pectate lyase to macerate host tissue, P. solanacearum produces no detectable pectate lyase activity. Rather, it produces two types of extracellular plant cell wall-degrading enzymes: cellulase and polygalacturonase (PG). Recently, it has been shown genetically that the major extracellular endo-PG produced in culture by P. solanacearum is only a minor component of bacterial wilt disease. However, we had observed that exo-PG activity increased substantially when bacteria grew in the presence of plant cells, while endo-PG activity remained constant. The primary objective of this section of the research was to characterize the role of all the PGs in bacterial wilt disease development.

Polygalacturonase isozymes produced. P. solanacearum wild-type strain K60 produces at least three extracellular PGs, PehA, PehB, and PehC, with isoelectric focusing points of 9.15, 7.5, and 5.5, respectively. PehA is an endo-PG; the other two enzymes have only exo-PG activity as determined by viscometry and reducing sugar assays.

Regulation. Direct enzyme activity assays and reporter gene expression studies indicate that the PehA endo-PG is produced at similar levels in planta and in various culture media, while production of the exo-PG isozymes is induced more than 10-fold in the presence of plant tissue and actively repressed in the presence of rich culture media, specifically glutamine. Inactivation of a regulatory locus called pehR results in a roughly 10-fold reduction in the levels of all PG activities. Trans-merodiploid strains carrying pehR on a multicopy plasmid produce about 8X the normal level of PG activity. These data suggest that pehR is a trans-acting positive regulator of PG production. Northern blot results indicate that pehR affects pehA expression at the transcriptional level; enzymes assays suggest that while pehR is absolutely required for PehA expression, some exo-PG activity is still present in pehR mutant strains.

Possible mode of action of pehR. The DNA sequence of the pehR locus is still incomplete, but preliminary results suggest that pehR belongs to a family of prokaryotic environmentally-responsive positive regulatory elements. In particular, the first ORF of the locus contains a region with high amino acid homology to the conserved histidine protein kinase encoded by, among others, the Agrobacterium tumefaciens virA gene. This result suggests that pehR may encode a protein that transmits a signal, possibly plant-produced, resulting in transcriptional activation of the PG structural genes when bacteria are growing in plant tissue.

Relationship between pehR expression and phosphorylation of a cytoplasmic membrane protein. P. solanacearum has an 85-Kd membrane protein that is phosphorylated on a tyrosine residue in wild type bacteria. This is particularly noteworthy because phosphorylation on a tyrosine residue is rare or unknown in prokaryotes. Interestingly, there is a substantial reduction in phosphorylation of this protein in pehR mutant strains. Preliminary results

suggest that the reduced level of phosphorylation in pehR strains is due to a soluble inhibitory factor present in these mutants. Bacterial signal transduction pathways often make use of phosphate transfer to transmit information.

Role in pathogenicity. Although marker-exchange mutants in the pehA locus are only slightly reduced in virulence on eggplants relative to wildtype strains, transposon mutants unable to produce either PehB or PehC are substantially reduced in virulence. Double pehA/pehB and pehA/pehC mutants are even less virulent. In addition, pehR regulatory mutants that produce only about 10% of wildtype levels of exo-PG isozymes and virtually no endo-PG are similar to the double structural gene mutants in pathogenicity. In short, although the exo-PGs appear to play an important role in bacterial wilt virulence, eliminating one or reducing levels of both does not render a strain completely nonpathogenic. We have not yet generated a mutant that produces no PG whatsoever, so no final conclusion is possible. In light of these results and those of other researchers studying the role of EPS in bacterial wilt disease development, it seems unlikely that the disease will be reduced to a single genetic or biochemical factor. It appears that a complex group of traits are involved in wilt pathogenesis, including many that allow the bacterium to live in plant tissue.

Publications

Allen, C., and L. Sequeira. Pseudomonas solanacearum exo-polygalacturonase contributes to virulence and is induced in planta. (In preparation.)

Atkinson, M., C. Allen, and L. Sequeira. 1992. Tyrosine phosphorylation of a cytoplasmic membrane protein from Pseudomonas solanacearum. J. Bacteriology 174:4356-4360.

Allen, C., Y. Huang, and L. Sequeira. 1991. Cloning of genes affecting polygalacturonase production in Pseudomonas solanacearum. Mol. Plant-Microbe Interactions 4: 147-154.

Allen, C., M. Atkinson, and L. Sequeira. 1991. Role of polygalacturonase in virulence of Pseudomonas solanacearum. Presented at the Third International Congress of the International Society for Plant Molecula Biology, Tucson, Arizona October 6-11, 1991.

Lewosz, J., C. Allen, and L. Sequeira. 1990. Factors influencing the production of pectinolytic enzymes by Pseudomonas solanacearum. Phytopathology 80:982.

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5/14/93