Communication

Complete Amino Acid Sequence of a Polypeptide from Zea mays Similar to the Pathogenesis-Related-1 Family

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ABSTRACT

A polypeptide serologically related to the tobacco pathogenesis-related-1 family of proteins has been purified from the root tissue of maize (Zea mays L.), and the complete amino acid sequence has been determined. The mature protein has a calculated molecular weight of 14,970 and isoelectric point of 4.2. The maize protein shows 86 to 88% amino acid identity with the tobacco pathogenesis-related-1 family and 55% identity with the tomato p14 protein.

The infection of plants with certain viral, viroid, fungal, and bacterial pathogens results in the accumulation of a group of polypeptides collectively referred to as the PR1 proteins (1, 16). These proteins have also been shown to accumulate during environmental and chemical stress (2). In addition to their induction by pathogens, PR proteins are also produced in healthy tissue (3, 7, 12). Characterization of PR proteins has been described in monocot and dicot families, although the majority of the molecular characterizations of PR proteins has been accomplished using tobacco as a model system (1, 10, 11, 17).

Recent work has provided information regarding possible in vivo activity of certain subsets of the PR proteins. These include chitinase activity (PR-P and PR-Q) (6), β-1,3-glucanase activity (PR-2, PR-N, and PR-O) (4), and a putative proteinase/α-amylase inhibitory activity (PR-S) based on sequence homology with a maize proteinase/α-amylase inhibitor (14). A subset of PR proteins for which no function has yet been described is the PR-1 family. The PR-1 family in tobacco consists of three polypeptides (PR-1a, b, and c) that show 90% homology with each other (13). The presence of PR-1 related proteins has also been described in other plant species (1, 17).

In this communication, we report the complete amino acid sequence of a polypeptide isolated from the root tissue of maize (Zea mays). Examination of the amino acid sequence reveals a striking similarity to the PR-1 family of proteins from tobacco and clearly establishes the existence of a homologous counterpart in maize (MPR-1) to the PR-1 family of proteins.

MATERIALS AND METHODS

Plant Material

Three-week-old seedlings of maize (Zea mays var Pioneer B37 × H84) were obtained from French Agricultural Research Inc. (Lamberton, MN). Seedlings had been infected with second instar larvae of the western corn rootworm (Diabrotica virgifera). Roots of seedling were also infected with an unidentified fungus. After removal of Diabrotica larvae, roots were separated and washed extensively with water.

SDS-PAGE and Western Blot Analysis

Denaturing gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (5), and silver staining was done according to the methods employed by Morrissey (9). A corn polypeptide serologically related to tobacco PR-1 was assayed by Western blot analysis. Samples to be analyzed were microdialyzed against several changes of water subsequent to concentration by lyophilization. Concentrated samples were analyzed by SDS-PAGE. Gels were blotted onto nitrocellulose (MSI Laboratories) and probed with antiserum to tobacco PR-1, 1:1000 dilution (15).

Protein Purification

Twenty-five grams of root tissue was homogenized in extraction buffer (25 mM KH2PO4 [pH 7.5] and 10 mM Na2S2O3) by two 60-s pulses with a Tekmar Tissuemizer at room temperature. Buffer was added at a ratio of 10 mL/g of tissue (wet weight). The homogenate was filtered through three layers of cheesecloth and the filtrate centrifuged (12,000g) at 4°C for 15 min. The supernatant fluid was applied to a DEAE-Cellulose column (2.5 x 20 cm; Sigma Chemical Co.) equilibrated with 25 mM KH2PO4 (pH 7.5). Bound material was eluted with a linear salt gradient of 0 to 700 mM KCl in equilibration buffer. Immunopositive fractions were pooled and precipitated by addition of ammonium sulfate to 90% saturation. The suspension was centrifuged (12,000g) at 4°C for 15 min. The pellet was resuspended in 5 mL of water and applied to a Sephacryl S-200 HR column (2.5 × 120 cm;

1 Supported by Ohio Board of Regents.
2 Present address: Glaxo Research Laboratories, 5 Moore Drive, Research Triangle Park, NC 27709.
3 Abbreviation: PR, pathogenesis-related.
4 Protein identification resource accession No. A33155.
Pharmacia Fine Chemicals) equilibrated in water. Immunopositive fractions were pooled and lyophilized to dryness. The material was taken up in 100 μL distilled water and purified by reverse phase HPLC using a Polypore Phenyl column (2.1 × 30 mm; Brownlee). Protein was eluted at 30°C with a linear gradient of 18 to 90% acetonitrile in 0.1% TFA over 60 min.

**Chemical Sequencing**

Reduction and alkylation were performed by incubating the reverse phase purified material in 6 M guanidine-HCl, 1 M Tris-HCl (pH 8.6), 10 mM EDTA, and 20 mM dithiothreitol (Calbiochem) for 1 h at 37°C under nitrogen. At this point, 4-vinylpyridine (Sigma) was added to 50 mM, and the incubation was continued for an additional 30 min at room temperature. The modified protein was desalted by HPLC as described above.

Digestion with sequence grade trypsin (Boehringer Mannheim) was carried out in 0.1 M Tris-HCl (pH 8.5), for 5 h at room temperature with an enzyme:substrate ratio of 1:100. Digestion with sequence grade endoproteinase Asp-N (Boehringer Mannheim) was performed in 0.1 M Tris-HCl (pH 8.5), for 4 h at room temperature with an enzyme:substrate ratio of 1:100. Endoproteinase Glu-C (Boehringer Mannheim) digestions were performed in 50 mM ammonium carbonate (pH 7.5), for 16 h at room temperature with an enzyme:substrate ratio of 1:100.

Peptides from the first tryptic digest and the Asp-N digest were isolated by HPLC with an Aquapore OD300 column (1 × 250 mm; Brownlee) and eluted with a linear gradient of 0 to 54% acetonitrile in 0.1% TFA over 60 min. A second tryptic digest was performed in order to recover a peptide which was lost on the OD300 column. This peptide was purified by using a Polypore RP (2.1 × 30 mm; Brownlee) column at 80°C employing a linear gradient of 0 to 54% acetonitrile in 0.1% TFA over 60 min. Peptides generated by the Glu-C digestion were isolated by using the Polypore RP column under the same conditions, except with a linear gradient of 0 to 72% acetonitrile over 30 min. All peptides were sequenced twice to resolve any ambiguities. Automated Edman degradations were performed using the Applied Biosystems 477A liquid-pulse sequencer and phenylthiohydantoin amino acids were identified on an Applied Biosystems 120A PTH analyzer.

The C-terminal peptide and the N-terminal peptide from the tryptic digest were isolated and submitted for analysis by mass spectrometry. Tandem quadruple Fourier transform mass spectrometry was performed on the C-terminal peptide and fast-atom bombardment mass spectrometry was performed on the N-terminal peptide.

**RESULTS**

Our laboratory is involved in studying the interactions between maize and the Western corn rootworm. One aspect of this research involves examining corn root polypeptides that may play a role in this plant/pest interaction. In our efforts to characterize the composition of root proteins we observed the presence of a polypeptide serologically related to the PR-1 family of proteins from tobacco (data not shown).
We monitored for the presence of this polypeptide in subsequent fractionations of crude corn root extracts by Western analysis.

Figure 1A shows the elution profile of corn root homogenates fractionated on DEAE-cellulose. Western analysis of individual fractions showed that the polypeptide eluted between 0.25 and 0.35 M KCl. The pooled DEAE material was applied to a Sephacryl S-200 HR column. Figure 1B shows a typical elution profile in which the immunopositive polypeptide elutes between fractions 53 and 57. These fractions were then applied to reverse-phase HPLC (Fig. 1C) which represents the final purification step. The major peak eluting with a retention time of 15 min showed similar electrophoretic mobility to the immunopositive polypeptide in crude root extracts (data not shown). Western blot analysis of extracts from Diabrotica larvae, fungi, and pathogen-free root tissue show no detectable levels of the polypeptide. We are examining whether such plant-pest interactions are responsible for the accumulation of this protein.

The complete amino acid sequence of MPR-1 was determined by aligning overlapping peptides generated by endoproteinase Glu-C, endoproteinase Asp-N, and trypsin digestions (Fig. 2). The N-terminal of the protein was confirmed by sequencing the first nine amino acid residues of the intact polypeptide. Direct sequencing of the intact polypeptide and the N-terminal Glu-C peptide (G1) resulted in a signal of about 5% of that expected. Mass spectrometry data revealed that the majority of the protein contains pyroglutamate instead of glutamine at the N-terminal position which accounts for the reduced signal. The C-terminal residue of the trypic fragment (T5) was confirmed to be tyrosine by mass spectrometry confirming that the sequence obtained by Edman degradation represents the entire length of the fragment (data not shown).

Figure 3 shows the sequence comparison of MPR-1 with the deduced amino acid sequences of the tobacco PR-1 family (13) and the tomato p14 polypeptide (8). There is 67% amino acid identity between the corn protein and tobacco PR-1a, 66% with PR-1b, and 68% with PR-1c. The tomato PR protein, p14, showed the least identity, 55%, with MPR-1. Comparison of the corn protein with all three tobacco variants and tomato show that 61 residues can be regarded as highly conserved (invariant) between the three genera. These invariant positions include all six cysteine residues.

**DISCUSSION**

The mature form of MPR-1 is a 140 amino acid polypeptide with a calculated $Mr$, 14,970. The estimated $pI$ was calculated to be 4.2 based on the amino acid sequence. The N-terminus of MPR-1 was found to be blocked by pyroglutamate, which is consistent with PR proteins p14 and PR-1a (8). Comparison of the corn sequence with the tobacco PR-1 (13) family and tomato p14 (8) reveals a high degree of amino acid identity shared between these proteins. The homology between the corn, tobacco, and tomato proteins suggests a close evolutionary relationship.

The use of immunodetection techniques has shown the presence of polypeptides serologically related to the tobacco PR-1 family in a variety of plant families including the Graminae (17). Recently, a protein serologically related to tobacco PR-1b has been purified from maize (PRm 2) (11), but from the information presented in that report, we cannot conclusively state that we are examining the same polypeptide. Although no physiological function has been ascribed to this subclass of PR proteins, the determination of the primary structure of PR-1 related proteins from a variety of different plant species will provide additional information that may be useful in determining the evolutionary history and possibly provide insight into structure/function relationships of this class of proteins.
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LITERATURE CITED