The Effects of Enzymatic Digestion on the Molecular Weight and Antigenic Specificity of Potato Virus X Protein

J. F. Shepard and G. A. Secor

Department of Botany and Microbiology, Montana State University, Bozeman 59715.
Supported by National Science Foundation Grant No. GB-26415.
Montana Agricultural Experiment Station Journal Series Paper No. 326
Accepted for publication 15 April 1972.

ABSTRACT

Two forms of potato virus X (PVX) protein were exposed to proteolytic enzymes in tobacco leaf homogenates and to trypsin. Both enzyme preparations reduced the molecular weight of assembled PVX protein ca. 15%, but did not simultaneously influence the antigenic specificity of the assembled or subsequently dissociated subunits. Treatment of chemically degraded PVX protein (D-protein) with either enzyme preparation, however, resulted in a pronounced reduction in subunit molecular weight with polypeptides of less than 12,000 mol wt being produced. A concomitant, progressive change in antigenic specificity occurred when subunit molecular weight decreased from ca. 23,000 to less than 12,000 even though the latter peptides were still precipitated by D-protein antiserum. The effects of the proteolytic enzyme(s) in tobacco sap on D-protein and on assembled PVX protein could be inhibited through the addition of sodium diethyldithiocarbamate (DIECA) to the extraction medium. Trypsin inhibitors, in contrast, were not inhibitory.

The influence of tobacco enzyme(s) on a third form of PVX protein; i.e., the soluble antigen associated with PVX infection (free-protein), was also investigated. Acrylamide gel electrophoresis suggested that even in the presence of DIECA, the majority of polypeptides in free-protein preparations were of a molecular weight less than 20,000, although some with molecular weights up to 29,000 were also detected when DIECA was used during their isolation.

Phytopathology 62:1154-1160.

Additional key word: serology.

The effects of plant-proteolytic enzymes on the protein structure units of several plant viruses is well documented (5, 6, 8). Of the viruses studied, the effect of such enzymes was most striking for potato virus X (PVX), wherein approximately 15% of the protein subunit was susceptible to enzymatic digestion during initial phases of virus purification (5).

The enzymatic removal of but a single amino acid from the C-terminal end of the tobacco mosaic virus (TMV) polypeptide chain is accompanied by an alteration in the antigenic specificity of that molecule (4). This information, in view of recent reports dealing with the antigenic specificity of PVX structural protein (10, 13, 14), is immediately relevant to the findings of Koenig et al. (5). These investigators reported that the enzymatic conversion of the PVX subunit (by either plant extracts or trypsin) from a molecular weight of 29,800 to 24,000 did not abolish the serological reactivity of digested virions. Detailed results concerning whether or not a minor alteration in antigenic specificity (as per TMV) resulted from subunit molecular weight reduction were not reported, however. Even subtle changes in antigenic specificity which are sensitive to method of purification or to the viral increase host used, present a series of confusing problems if strains of a given virus are to be serologically compared (8). Hence, a description of such potential changes in specificity and means to obviate them become increasingly important.

Less attention, however, has been directed toward the effects of plant proteolytic enzymes on dissociated or otherwise unassembled viral protein. For PVX, two forms of unassembled coat protein have been identified. One form is chemically dissociated PVX protein (D-protein) which has been shown to differ from its assembled counterpart both antigenically and structurally (10, 13). The other identified form of PVX protein is the soluble antigen present in extracts from PVX-infected cells (free-protein). It is antigenically similar but not identical to D-protein, and not reactive with PVX-specific antibody (11). Information on the effects, on unassembled PVX protein, of the proteolytic enzyme(s) present in tobacco extracts may also be of value, particularly if such protein is to be isolated and characterized from tobacco tissue.

In the present study, the effects of the tobacco proteolytic enzyme(s) and of trypsin on the molecular weight of both assembled and unassembled PVX protein were assessed. Also investigated were the possible consequences of proteolytic activity on the antigenic specificity of PVX protein molecules, and (through the use of enzyme inhibitors) means to circumvent it.

MATERIALS AND METHODS.—*Virus purification.*—The isolate of potato virus X (PVX) was the one used previously (10, 13), and was increased in *Nicotiana tabacum* L. 'White Burley'. Purification of the virus in certain instances followed the procedures of Koenig et al. (5). Briefly, this included homogenization of fresh, infected tissue in 0.05 M sodium citrate buffer pH 6.0 containing 0.2% sodium sulfite and 0.2% ascorbic acid; chloroform emulsification; and repeated cycles of differential ultracentrifugation. In other experiments, PVX was purified as before (13).

Preparation of degraded PVX protein.—Chemically degraded PVX protein was prepared by treatment of

virus suspensions with either 30% pyridine or 1% sodium dodecyl sulfate (SDS) (13, 14).

Acrylamide gel electrophoresis.—Polyacrylamide gel electrophoresis in SDS as an assay for homogeneity and molecular weight was conducted after the procedure of Weber & Osborn (15), and sometimes included the split-gel modification of Dunker & Rueckert (3). After electrophoresis, gels were stained with either Coomassie brilliant blue (1) or antibody specific for degraded PVX protein (10). Molecular weights of degraded viral protein were estimated by a comparison of their mobilities against those of the known protein standards previously used (10, 14).

Preparative SDS gel electrophoresis was conducted in a similar manner in 12 cm × 20 mm glass tubes. Three to 5 mg of protein in 400 μliters of buffer/tube were separated by electrophoresis at 40-50 milliamps for 12-16 hr. PVX-degraded protein bands were localized with antibody.

Serology.—Antisera to the complete and partially digested PVX- and PVX- degraded protein antigens were prepared by injecting rabbits intramuscularly with 2 mg of the appropriate antigen emulsified in Freund's incomplete antigen. Antigen was administered at 7-day intervals over a 4-week period. Viral degraded protein immunogens were purified by preparative SDS gel electrophoresis before use.

Bleedings were conducted 2-6 weeks after the initial injection. Antisera were titered for PVX reactivity in tube precipitin tests and for degraded protein reactivity in double-diffusion systems (13).

Trypsin digestion.—Trypsin (3X crystallized; Worthington; Freehold, N.J.) was used in digestion experiments. Unless otherwise specified, trypsin digestion was carried out at concentrations of 1 μg enzyme/1 mg of substrate overnight at room temperature in 0.05 M Na-citrate buffer, pH 8.0. Aqueous N-α-*p*-Tosyl-l-Lysine Chloromethyl Ketone (TLCK; Sigma, St. Louis, Mo.) or soybean trypsin inhibitor (Type I-S, Sigma) was used to inhibit the enzyme after digestion experiments at a ratio of 3 mg inhibitor/mg enzyme (12).

RESULTS.—*Effect of purification on the assembled PVX subunit.*—Experiments with PVX purified after a minimum exposure time to plant sap before further purification (PVX-IP) versus virus purified following an overnight incubation in filtered leaf homogenates (PVX-ON) confirmed the findings of Koenig et al. (5). Immediately purified PVX preparations displayed protein subunits with a homogeneous molecular weight of ca. 29,000. PVX-ON preparations either possessed a mixture of 29,000 and

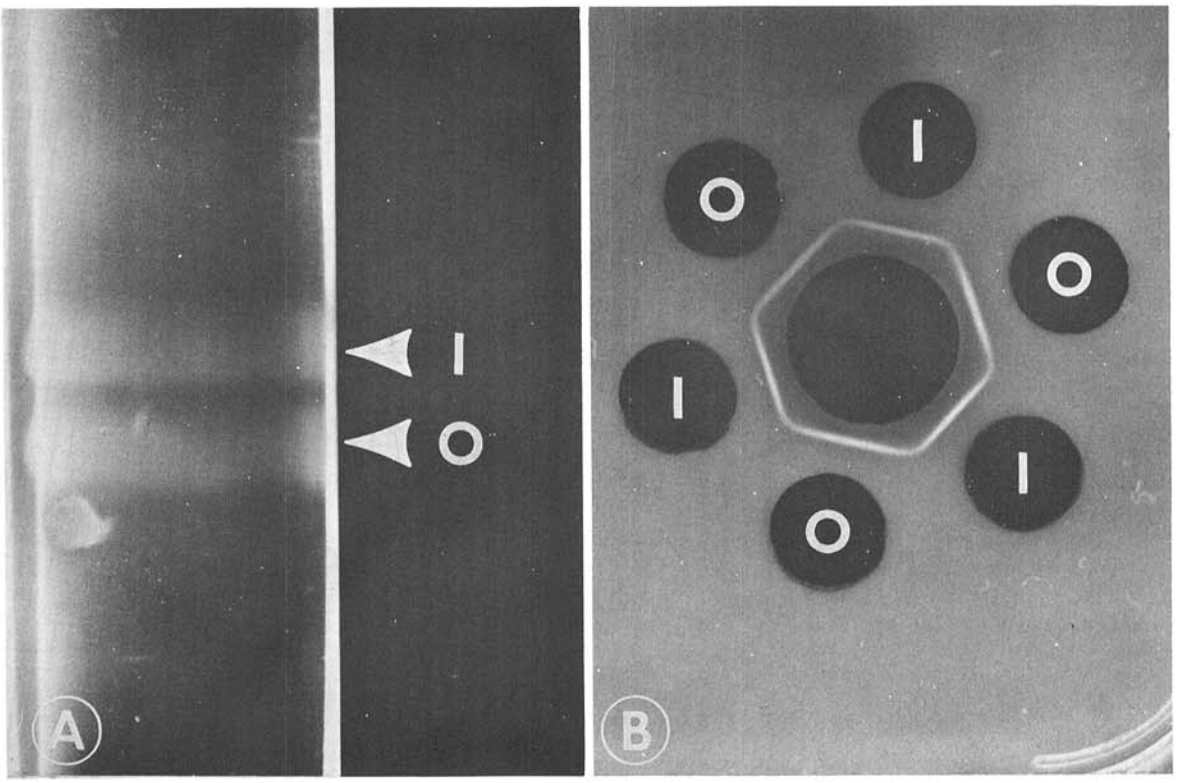

Fig. 1. Electrophoretic separation of, and antigenic relationship between, potato virus X (PVX) protein subunits of 29,000 mol wt (I) and 24,500 mol wt (0). **A)** Preparative acrylamide gel column after electrophoresis and antibody staining. The degraded PVX preparation contained two molecular weight subunits which separated into bands I and 0. **B)** Double-immunodiffusion system containing PVX-degraded protein antiserum in the central depot and PVX protein subunits from excised acrylamide bands I and 0 in peripheral wells. The two molecular weight subunit forms are antigenically identical.

24,500 mol wt subunits or, less often, 24,500 mol wt subunits only. Treatment of either PVX preparation with trypsin produced virus with subunits of a molecular weight of 24,500 only.

Serological relationship between PVX-IP and PVX-ON proteins.—Antisera were prepared against PVX-IP preparations which displayed only 29,000 mol wt subunits and against PVX-ON preparations which had been exposed to a supplementary trypsin digestion to insure the presence of 24,500 mol wt subunits only. After a 6-week immunization period, antiserum titers to homologous antigen were 4,096 and 8,192, respectively, and were the same to the heterologous antigen. Reciprocal cross-absorption tests the PVX-IP antiserum with PVX-ON antigen and of PVX-ON antiserum with PVX-IP preparations revealed that a complete absorption of antibody reactive with heterologous antigen also eliminated serum reactivity toward the homologous antigen. Thus, the two forms of PVX appear to be serologically identical even though they are composed of protein subunits of distinctly different molecular weights.

The chemically degraded proteins of PVX-IP and PVX-ON preparations were also serologically compared. We produced immunogen by degrading both preparations with 1% SDS containing 1% 2-mercaptoethanol followed by preparative gel electrophoresis to reduce the possibility of contamination with heterologous protein. Electrophoresed gels were briefly exposed to degraded protein antiserum to localize the antigen band which was then excised from the gel column, smashed into a slurry with 2 ml of 0.005 M Na-citrate buffer, pH 8.0, dialyzed against the same buffer for 6-8 hr, and finally injected into a rabbit. Resultant antisera to PVX-IP- and PVX-ON-degraded proteins both possessed titers of 8 to homologous antigen 6 weeks after the initial injection. Ouchterlony double-diffusion tests with these antisera against electrophoresed PVX-IP- and PVX-ON-degraded proteins indicated antigenic identity between the two forms (Fig. 1). Higher titered degraded protein antiserum; i.e., with a titer of 32, tested against degraded PVX preparations which contained a mixture of the two molecular weight subunits, also failed to distinguish between the two forms in double-diffusion experiments (Table 1).

Effect of enzymatic digestion on the dissociated PVX subunit.—On the basis of serological and physical data, degraded PVX protein has been suggested to be the denatured form of the protein (10, 14). If so, the dissociated protein subunit may well be even more susceptible to digestion by plant-proteolytic enzymes and trypsin than is the assembled subunit. Preliminary experiments showed that the exposure of PVX-degraded protein (D-protein) to extracts from uninfected tobacco leaves did not result in a detectable change in the antigenic specificity of the D-protein (11), but the possibility remained that an alteration might occur in at least a portion of the exposed antigen.

We prepared PVX D-protein by degrading purified virus with pyridine (13). Uninfected tobacco leaves were homogenized in 1 volume of distilled water/g of tissue; the homogenate was pressed through cheesecloth; and 10 mg of D-protein were added/100 ml of extract. Samples were withdrawn at various time intervals up to 6 hr and compared in Ouchterlony double-diffusion with untreated D-protein at 0.1 mg/ml for detectable changes in antigenic specificity. In no case was such a specificity change observed. However, when the D-protein was reisolated from the plant juice and analyzed by SDS acrylamide gel electrophoresis, a pronounced effect on the molecular weight and antigenic specificity of a large portion (but not all) of the D-protein had indeed occurred. This was ascertained as follows: Ten mg of D-protein were added/100 ml tobacco leaf extract and the mixture was immediately centrifuged at 165,000 *g* in a Spinco 50 Ti rotor for 2 hr. Pellets were discarded, and the supernatant was heated to 65 C for 10-15 min, after which insoluble precipitates were removed by centrifugation at 10,000 *g* for 10 min. Two *g* of ammonium sulfate were added/10 ml supernatant and, following incubation for 1 hr at 4 C, the precipitated D-protein was sedimented at 10,000 *g*/10 min and the pellets were redissolved in a small volume of 0.05 M borate buffer, pH 8.2, containing 1% SDS and 1% mercaptoethanol. Redissolved protein was either dialyzed overnight against the same buffer or incubated for 1 hr at 37 C, and subjected to electrophoresis in acrylamide gel systems along with (in separate tubes) untreated D-protein and molecular weight standards. Molecular weight standards were stained with Coomassie blue and D-protein treatments with specific antibody. The results indicated that even a relatively short exposure of the D-protein to plant extracts resulted in the conversion of a substantial portion of the protein to lower molecular weight forms. A continuous range of molecular weights was found from unmodified subunits with a mol wt of 29,000 to less than 12,000 (the lower limit of technique resolution) (Fig. 2-C). The various regions containing antigen were excised from acrylamide gel columns (as were similar regions from gels containing unmodified D-protein, and those from control experiments of tobacco sap without the addition of D-protein) and compared with the standard D-protein preparation in Ouchterlony double-diffusion. The results showed that antigenic homology existed between 24,500 and 29,000 mol wt D-protein, but molecular weight regions less than ca. 23,000 were antigenically distinguishable from unmodified D-protein. The character and intensity of spur formation changed progressively as the molecular weight decreased (Fig. 3), although a degree of serological reactivity remained even in those fractions of less than 12,000 mol wt (Table 1). Similar results were obtained by incubation of D-protein for various times (10 min - 2 hr) with low concentrations of trypsin (at a ratio of 1×10^{-5} mg trypsin/mg D-protein). Too prolonged an incubation period or too high a concentration of trypsin abolished the serological reactivity of D-protein altogether, and no stainable (with Coomassie blue or antibody) polypeptides could be detected in acrylamide gel systems. Inter-

TABLE 1. Summary of the antigenic specificity relationships between the various forms of potato virus X subunit protein

Compared antigens[a]			
Standard	Test	Antiserum[b]	Antigenic relationship
X-IP[c]	X-ON[d]	X-IP	Identical
X-ON	X-IP	X-ON	Identical
IP-D-protein[e]	ON-D-protein[f]	IP-D-protein	Identical
ON-D-protein	IP-D-protein	ON-D-protein	Identical
D-protein[g]	D-protein(H)[h]	D-protein	Small % of preparation identical, remainder distinct
D-protein	D-protein(HD)[i]	D-protein	Majority of preparation identical, remainder distinct
D-protein	D-protein (T)[j]	D-protein	Not identical
D-protein	Free-protein(W)[k]	D-protein	Distinct
D-protein	Free-protein(WD)[l]	D-protein	Small % of preparation identical, remainder distinct

[a]Results are of whether or not the test antigen possessed all of the antigenic determinants of the standard antigen preparation.

[b]Antiserum for the tests was elicited against denoted antigens.

[c]Potato virus X virions purified without prolonged exposure to plant extracts (X-IP).

[d]Potato virus X virions purified after overnight incubation in leaf homogenates or treated with trypsin so that all subunits displayed a molecular weight of 24,500 (X-ON).

[e]Chemically degraded protein from X-IP virions (IP-D-protein).

[f]Chemically degraded protein from X-ON virions (ON-D-protein).

[g]Chemically degraded protein from a standard potato virus X preparation. Preparation usually contained a mixture of 29,000 and 24,500 mol wt protein subunits (D-protein).

[h]D-protein which had been exposed to tobacco leaf homogenates during the 2-3 hr reisolation procedure (D-protein [H]).

[i]D-protein exposed to leaf homogenates containing 0.01 M sodium diethyldithiocarbamate (DIECA) during reisolation (D-protein[HD]).

[j]D-protein exposed to 10^{-5} mg/trypsin per mg D-protein for 2 hr, pH 8.0 at 37 C. Enzyme then inhibited with soybean trypsin inhibitor.

[k]Soluble antigen associated with PVX infection isolated from leaf homogenates in the presence of distilled water (Free-protein[W]).

[l]Free-protein isolated in the presence of DIECA (Free-protein[WD]).

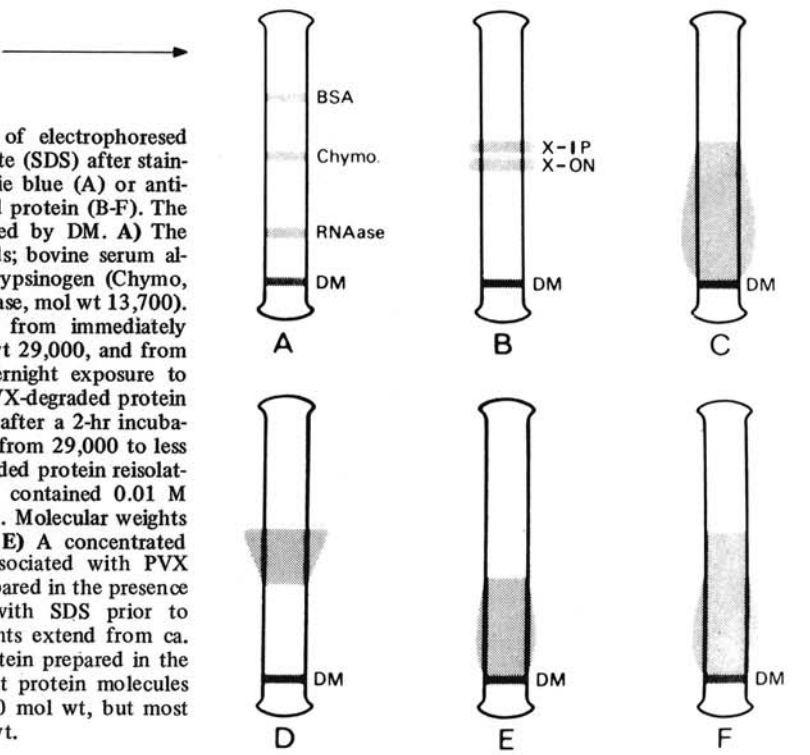

Fig. 2. A diagrammatic illustration of electrophoresed acrylamide gels, in sodium dodecyl sulfate (SDS) after staining (shaded areas) with either Coomassie blue (A) or antibody to potato virus X (PVX) degraded protein (B-F). The Bromphenol blue dye marker is signified by DM. **A)** The relative positions of 3 mol wt standards; bovine serum albumin (BSA, mol wt 67,000), chymotrypsinogen (Chymo, mol wt 25,000), and ribonuclease (RNAase, mol wt 13,700). **B)** The positions of degraded protein from immediately purified (X-IP) PVX preparations mol wt 29,000, and from PVX preparations purified after an overnight exposure to plant sap (X-ON, mol wt 24,500). **C)** PVX-degraded protein reisolated from uninfected tobacco sap after a 2-hr incubation. A broad range of subunit mol wt from 29,000 to less than 12,000 is illustrated. **D)** PVX-degraded protein reisolated from uninfected tobacco sap which contained 0.01 M sodium diethyldithiocarbamate (DIECA). Molecular weights extended from 29,000 to ca. 22,000. **E)** A concentrated preparation of the soluble antigen associated with PVX infection (free-protein). Protein was prepared in the presence of distilled water until incubation with SDS prior to electrophoresis. Protein molecular weights extend from ca. 23,000 to less than 12,000. **F)** Free-protein prepared in the presence of 0.01 M DIECA. Constituent protein molecules ranged from 29,000 to less than 12,000 mol wt, but most polypeptides were less than 20,000 mol wt.

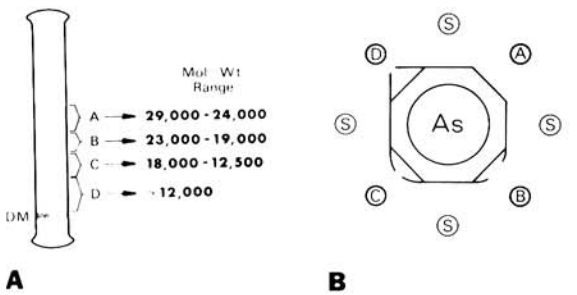

Fig. 3. A) Diagrammatic illustration of the molecular weight classes of PVX-degraded protein (D-protein) in sodium dodecyl sulfate acrylamide gels resulting from the exposure of 1 mg of D-protein to 1×10^{-5} mg trypsin for 2 hr. (DM denotes bromphenol blue dye marker.) **B)** Diagrammatic Ouchterlony immunodiffusion system containing antiserum (As) to PVX D-protein in the central depot and a standard D-protein preparation (S), and D-protein from acrylamide gel regions A, B, C, and D in peripheral depots. Note the increasing length and the changing character of spurs produced over B, C, and D, respectively.

mediate incubation times resulted in a change in the antigenic specificity of the entire preparation (Fig. 4). Thus, the effects of enzymes from plant sap or of trypsin are even more pronounced with PVX D-protein than with PVX virions.

Inhibition of the proteolytic enzyme(s) from tobacco.—To determine whether the effects of the proteolytic enzyme(s) in tobacco extracts on PVX D-protein could be minimized or eliminated, various compounds were added to uninfected tobacco leaf sap prior to, concurrent with, and subsequent to the addition of 10 mg of D-protein/100 ml of extract. Initially, trypsin inhibitors including N-α-*p*-Tosyl-α-Lysine Chloromethyl Ketone (TLCK) and the trypsin inhibitor from soybean were tested at various concentrations, but reisolated D-protein from treated plant extracts invariably displayed the same molecular weight reductions as control experiments without the inhibitor. Chymotrypsin inhibitors were similarly ineffective, as were phosphate, borate, and citrate buffers of varying molarity and pH. In contrast to experiments with the above compounds, the prior or simultaneous addition of 0.01 M diethyldithiocarbamic acid-Na salt (DIECA) to leaf extracts containing D-protein was found to have a decided inhibitory effect on the hydrolysis of PVX D-protein. In acrylamide gel, subunits exhibited a narrower range of molecular weights (29,000-22,000) than with other compounds tested, and a relatively small percentage of the polypeptide chains were of less than 24,000 mol wt (Fig. 2-D).

When DIECA is added to plant sap, it slows the oxidation of phenolics by means of its capacity to chelate iron and other heavy metals. However, experiments with other reducing agents (mercaptoethanol and thioglycolic acid) or with additional chelation compounds (ethylenediamine tetraacetic acid and sodium citrate) were without a comparable inhibitory effect. DIECA also markedly reduced the degree of, but did not totally prevent, digestion of PVX virion subunits in infective plant sap.

Properties of the soluble antigen associated with PVX infection.—A previous report (11) demonstrated that extracts from PVX-infected tobacco contain a soluble antigen which is reactive with PVX D-protein antiserum but not with PVX antiserum. However, the soluble antigen (referred to as free-protein) did not react with identity toward D-protein, indicating some type of structural and/or chemical difference between the two forms of protein. Additional investigation has revealed the presence of a similar soluble antigen associated with the serologically distinct B strain (14) of PVX. In both instances, the concentration of the soluble antigen in infected leaf extracts fluctuates markedly with environmental conditions. When tobacco plants are inoculated with PVX and grown under conditions of 60 C and less than 10-hr illumination, relatively high free-protein concentrations prevail (T.A. Shalla, Univ. of Calif., *personal communication*). In view of the effects of the tobacco proteolytic enzyme(s) on D-protein, their possible influence on the antigenic specificity of free-protein was reexamined.

PVX-infected leaves which displayed relatively high levels of free-protein reactivity in radial immunodiffusion (11) were ground in a mortar with a pestle, and the resulting juice was pressed through cheesecloth. To one-half of the extract was added an equal volume of distilled water, and to the other an equal volume of 0.02 M DIECA in distilled water. The two extracts were then tested for antigenic specificity against one another and against a PVX D-protein

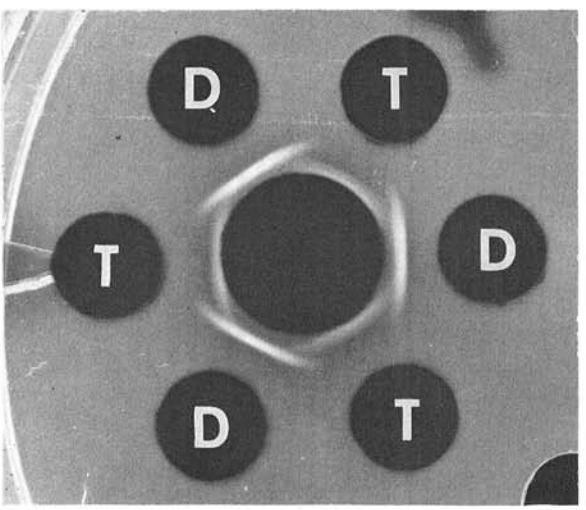

Fig. 4. Double-immunodiffusion plate containing potato virus X (PVX)-degraded protein (D-protein) antiserum in the central well against trypsin-digested (T) D-protein and unmodified D-protein (D). Trypsin digestion was conducted for 3 hr at a ratio of 10^{-3} mg trypsin/mg D-protein, then inhibited with the soybean trypsin inhibitor. The presence of prominent spurs over trypsin digested preparations indicate that most polypeptides were hydrolyzed to a molecular weight of less than 23,000.

preparation in double-diffusion as before (11). The free-protein from the tissue extract in water was serologically different from both the free-protein from the DIECA treated extract and the D-protein preparation. No spurs developed between the DIECA-treated free-protein and the D-protein samples.

To more precisely determine the role of DIECA in preventing spur formation between free- and D-proteins, we isolated free-protein from infected tissue in the absence of and in the presence of DIECA. Infected tissue was vacuum-infiltrated with either distilled water or distilled water containing 0.01 M DIECA (buffers had little effect on the results and therefore were not used), then homogenized in a blender in 1 ml of liquid/g of tissue. Extracts were squeezed through cheesecloth and centrifuged at 165,000 *g* for 2 hr, and the free-protein was isolated by heat clarification and ammonium sulfate precipitation. After incubation in SDS, the preparations were centrifuged at 10,000 rmp/10 min to remove insoluble components and subjected to electrophoresis on acrylamide gels. Gels were stained with antibody to D-protein, and protein molecular weights compared. In the free-protein preparation without DIECA, molecular weights ranged from ca. 23,000 to less than 12,000, with the majority of the protein appearing toward lower molecular weight regions (Fig. 2-E). When prepared in the presence of DIECA, free-protein solutions still were composed largely of low molecular weight; i.e., less than 20,000 polypeptides, although the breadth of the protein molecular weight range was extended to a maximum of 29,000 (Fig. 2-F). These results contrasted sharply with those from experiments with plant sap plus D-protein in which the addition of DIECA resulted in a far narrower molecular weight distribution with the preponderance of polypeptides ranging from 29,000 to 24,000 (Table 1).

The addition of D-protein to sap from PVX-infected leaves inhibited with DIECA followed by the isolation and electrophoresis of the D- and free-proteins resulted in the anticipated appearance of two pronounced protein molecular weight ranges.

DISCUSSION.—The results of this study confirm the finding of Koenig et al. (5) that prolonged exposure of potato virus X (PVX) to tobacco leaf extracts or to trypsin leads to a diminution of the molecular weight of the protein structure unit. However, the hydrolysis was not accompanied by a detectable change in the antigenic specificity of either virions or subsequently dissociated subunits. This is in contrast to reports with tobacco mosaic virus (TMV) (8), but in accordance with those from experiments with cowpea chlorotic mottle virus (2) and adenovirus hexons (7).

The effects of tobacco extracts or of trypsin are far more pronounced on degraded-PVX protein (D-protein) than on assembled PVX protein. In the extreme, exposure of D-protein to trypsin abolished all serological reactivity with anti-D-protein serum and resulted in peptides unresolvable in acrylamide gels. The effects of tobacco proteolytic enzyme(s) on D-protein were of a similar nature of those of trypsin; i.e., drastic reduction in subunit molecular weight, although the reaction was not inhibited by either trypsin or chymotrypsin inhibitors. Sodium diethyldithiocarbamate (DIECA) effectively inhibited the proteolytic activity of tobacco sap to D-protein during a 2-3 hr exposure, and the range of polypeptide molecular weights observed in reisolated D-protein preparations was narrower than that in similar treatments not containing DIECA.

The results of experiments with the soluble antigen associated with PVX infection (free-protein) were less definitive. Whereas the range of polypeptide molecular weights in free-protein preparations was extended up to 29,000 if DIECA was used during isolation, the majority of the polypeptides remained at less than 20,000 mol wt. This extension of the molecular weight distribution made the over-all preparation antigenically identical to untreated D protein even though the majority of the polypeptides in the preparation were antigenically distinct. It appears from this that the antigenic disparity between D-protein and free-protein reported earlier (11) was a consequence of proteolysis of free-protein; but whether all or only a portion of free-protein in vivo was of a 29,000 mol wt remains questionable. If but a small percentage of free-protein was of 29,000 mol wt upon extraction, such a small amount might be rapidly enough digested to produce an over-all antigenic distinctiveness unobtainable in similar experiments with D-protein. Hence, it seems plausible that free-protein polypeptide chains are not of a homogeneous molecular weight in vivo. Rather, they may reflect an even more pronounced intracellular antigen degradation than that reported by Niblett & Semancik (6) for cowpea mosaic virus protein. Alternatively, they may be nascent protein chains released from ribosomes during extraction as described for poliovirus protein (9), or a combination of the two. At any rate, the isolation of polypeptides of this nature from plant extracts for characterization and study may be most difficult.

Of interest is the degree to which PVX D-protein polypeptide chains may be enzymatically digested; i.e., to molecular weights of less than 12,000, and yet combined with the fact that the intensity of spurs remain a precipitant with D-protein antiserum. This, formed by D-protein over polypeptides increased as the size of polypeptides decreased, suggests (i) a stepwise deletion of antigenic structure and (ii) that a major portion of the PVX-degraded protein molecule is directly involved in defining the antigenic specificity of that molecule. If the D-protein is the random coil or loosely ordered equivalent form of PVX protein [all evidence to date (10, 14), including enzyme susceptibility, indicates that it may be], the sequential antigenic determinants must encompass a considerable portion of the D-protein molecule. Since it is generally accepted that an antigen must possess at least two antibody-combining sites to be precipitated by antibody, even 12,000 mol-wt D-protein fragments contain at least two antigenic determinants. It would be enlightening to know what proportion of

the assembled PVX protein structure unit is directly involved in defining antigenic specificity.

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