A serological comparison of potato virus X protein prior and subsequent to partial enzymatic hydrolysis
by Gary Allen Secor

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Botany
Montana State University

Abstract:

Potato virus X protein in the native and depolymerized states were serologically compared prior and subsequent to partial enzymatic hydrolysis. SDS gel electrophoresis and Sephadex chromatography were used to detect changes in molecular weight of the protein. Reciprocal cross-absorption and Ouchterlony double diffusion tests were conducted to detect changes in antigenic specificity of the protein. It was concluded that the PVX protein molecules prior and subsequent to hydrolysis are serologically identical, although a 15% difference in molecular weight exists. The region(s) of the polypeptide chain lost must constitute an immunosilent portion(s) of the molecule. Degraded viral protein is more susceptible to hydrolysis by trypsin and chymotrypsin than is native protein.

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Date December 2, 1971

A SEROLOGICAL COMPARISON OF POTATO VIRUS X PROTEIN PRIOR AND SUBSEQUENT TO PARTIAL ENZYMATIC HYDROLYSIS

by

GARY ALLEN SECOR

A thesis submitted to the Graduate Faculty in partial
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Botany

Approved:

William G. Walter
Head, Major Department

James F. Shepard
Chairman, Examining Committee

K Goering
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

Potato virus X protein in the native and depolymerized states were serologically compared prior and subsequent to partial enzymatic hydrolysis. SDS gel electrophoresis and Sephadex chromatography were used to detect changes in molecular weight of the protein. Reciprocal cross-absorption and Ouchterlony double diffusion tests were conducted to detect changes in antigenic specificity of the protein. It was concluded that the PVX protein molecules prior and subsequent to hydrolysis are serologically identical, although a 15% difference in molecular weight exists. The region(s) of the polypeptide chain lost must constitute an immunosilent portion(s) of the molecule. Degraded viral protein is more susceptible to hydrolysis by trypsin and chymotrypsin than is native protein.

INTRODUCTION

The importance of maintaining native virus particles following purification is obvious, for to study these infectious agents, they must be chemically and structurally complete, as well as biologically functional. Otherwise experimentation may result in erratic results. For example, errors in identification, composition, biological function and relationships with other viruses may result if a portion of the structural molecule is changed or lost upon purification. It has been demonstrated with some plant viruses that purification may result in the production of incomplete virions. Koenig et al (12) recently reported that the molecular weight of the potato virus X (PVX) protein subunit may be affected by the method in which the virus is purified from tobacco. When infective homogenates are incubated overnight at room temperature, the authors suggested the presence of proteolytic enzymes in tobacco sap which are capable of converting the normal protein subunit with a molecular weight of 29,800 to one with a molecular weight of 24,000. Studies with tobacco mosaic virus (TMV) by Rees and Short (20) have shown that purification of this virus from beans results in particles which are serologically distinct from those purified from tobacco, presumably due to the action of carboxypeptidases on virus particles.

Other studies with viruses have shown that artificial enzymatic digestion may markedly affect certain viral properties. Koenig et al

(12) demonstrated that trypsin treatment of purified PVX duplicates the subunit molecular weight loss that occurs upon incubation of infective homogenates. In addition, the electrophoretic mobility of the trypsin treated PVX was distinct from that of the untreated PVX. However, detailed serological studies were not conducted. Bawden and Pirie (1) have shown that pepsin digestion of PVX results in a loss of infectivity and serological activity. Studies with TMV have been most enlightening for correlating protein alteration in particles and changes in antigenic specificity. Harris and Knight (7) have shown that treatment of TMV with carboxypeptidase results in the removal of the three C-terminal amino acids. Resultant TMV particles are serologically distinct from undigested virions. Surprisingly, the removal of twelve additional amino acids from the C-terminal end of the subunits had no further effect on antigenic specificity (10). Sengbusch and Wittmann (23) have also demonstrated the susceptibility of TMV to serological change following a different form of alteration in primary structure. A single amino acid substitution in the TMV polypeptide chain may result in a molecule serologically distinct from wild type protein, provided that the substitution occurs at the proper location.

Niblett and Semancik (15) demonstrated that the electrophoretic components of cowpea mosaic virus and of bean pod mottle virus would migrate at a different rate following digestion by certain enzymes.

Chidlow and Tremaine (3) reported the action of various enzymes on cowpea chlorotic mottle virus and concluded that trypsin digestion caused disassembly of the virus and a subunit molecular weight loss of 20%. Reassembled trypsin digested virus was serologically compared to intact virus and found to be non-identical. By contrast, carboxypeptidase and chymotrypsin removed 0.5% and 10% of the protein respectively, caused no dissociation of the virus, and the serological properties of the virus were unaffected.

Pettersson (18) studied enzymatic digestion of adenovirus hexons and the effect this digestion had on antigenic specificity of the hexons. He found that trypsin digested 5-10% of the hexon but no change in serological properties accompanied the loss. However, when subtilisin, papain or chymotrypsin were used, a great deal of the hexon was digested, leaving only the hexon "core", which was serologically distinct from intact hexons.

SDS polyacrylamide gel electrophoresis has become a reliable technique for the study of protein molecules, especially as a tool for determination of molecular weights. Origination of acrylamide gel electrophoresis by Ornstein and Davis (4, 16) was followed by the use of SDS in the system by Shapiro et al (25). Weber and

Osborn (32) confirmed accurate molecular weight determination using SDS gel electrophoresis. The action of SDS as a denaturant of proteins was explained by Reynolds and Tanford (21) and Pitt-Rivers and Ambesi Impiombato (19), who reported that as SDS denatures a protein, it attaches to the protein and the resultant amalgams vary directly in their molecular radius with molecular weight. Consequently, an accurate relationship between Stoke's radius and molecular weight exists which can be used for molecular weight determination via SDS polyacrylamide gel electrophoresis. Several papers have confirmed the accuracy of molecular weight determination using SDS gel electrophoresis (13, 24, 34). Dunker and Rueckert (5) introduced split gel electrophoresis which allowed the test molecule to be electrophoresed in the same gel as a known molecule, further increasing the reliability of molecular weight determination with SDS gel electrophoresis.

Likewise, SDS column chromatography has been used for the study of proteins (9). Fish et al (6) have elucidated the action of SDS as a denaturant of proteins and their use in gel chromatography, and found that a static relationship exists between Stoke's radius and molecular weight. Page' and Godin (17) and Shalla and Shepard (24) have accurately determined molecular weights of protein molecules using Sephadex G-200.

It is the purpose of this paper to demonstrate the enzymatic digestion of PVX and of its degraded protein by both natural and artificial methods and to report a serological comparison of the two resultant forms of PVX and of PVX degraded protein. In his review on some molecular aspects of antigenicity, Sela (22) points to the role of conformation in antigenic specificity. He cites several cases in which native and denatured proteins are not cross-reactive with each others' antibody populations due to the role of secondary and tertiary structure in determining immunopotency. Such a case in point is PVX. Studies by Shepard and Shalla (24, 28) have shown that as PVX is depolymerized, the released protein subunits undergo unfolding and enter into a new conformational state. This change in conformation of the protein is accompanied by a change in the antigenic specificity of the protein so drastic that PVX and the degraded protein are only slightly related as antigens. It is therefore necessary to compare the PVX forms separately from the PVX degraded protein forms since the two are antigenically distinct.

Because PVX is a good model virus for study, since natural enzymatic digestion does occur, it was of interest to study the effect on the immunological properties of the viral protein that partial polypeptide chain loss would have. SDS split gel polyacrylamide electrophoresis and SDS Sephadex G-200 column chromatography were used as an assay system for primary structural changes.

Ouchterlony double diffusion and reciprocal cross-absorption studies were used for detection of changes in antigenic specificity.

MATERIALS AND METHODS

Virus purification. -- The isolate of potato virus X (PVX) was the same as that used in previous studies in this laboratory (29). Nicotiana tabacum L. var. 'White Burley' was the systemic increase host. Purification of the virus followed the procedures of Koenig and Bercks (11) and Wetter (32). In general, this included homogenization of fresh systemically 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 several cycles of differential ultracentrifugation.

Three alternative schemes were used in the basic purification procedure. Immediately purified PVX (PVX-IP) was prepared by emulsifying infective homogenates from freshly harvested leaves with chloroform within three minutes after homogenization (12). Alternatively, PVX was purified by filtering infective homogenates through cheesecloth and allowing this crude juice to incubate overnight at room temperature before further purification. These preparations were designated as PVX-ON (12). A third method of preparing PVX was also used. These preparations, (PVX-M), consisted of crude homogenates which had been allowed to remain at room temperature or at 4°C from fifteen minutes to several hours before chloroform clarification and further purification, as described.

Purity of the viral preparations was ascertained by (i) clarity of the final high speed pellet (ii) reactivity of viral preparations in radial immunodiffusion plates containing antiserum prepared against healthy host material (28) (iii) homogeneity of degradation products in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis systems, and (iiii) reactivity of healthy host material with antiserum provoked by the viral preparations.

Concentrations of viral and degraded viral protein preparations were determined spectrophotometrically as per Shepard and Secor (29).

Preparation of degraded PVX protein. -- Degraded PVX protein was obtained by depolymerization of the subunits with either pyridine or SDS. Pyridine was added to viral preparations at a final concentration of 30% and then removed by dialysis overnight against a large volume of 0.005 M sodium citrate pH 8.0. For SDS degradation, SDS and 2-mercaptoethanol were each added to the virus solution at a final concentration of 1%, incubated at 37°C for two hours. The resultant viral protein was used for either serological studies or for application on electrophoretic gels.

Acrylamide gel electrophoresis. -- SDS split gel polyacrylamide electrophoresis was used for determination of molecular weight and as an assay of homogeneity (5). PVX protein was prepared for electrophoresis by SDS incubation of virus (24) and electrophoresed on SDS split gels having a final concentration of 10% acrylamide plus 1% SDS (24). Gels were stained with 0.025% Coomassie brilliant blue (Colab Chicago Heights, Ill.) and destained with 7.5% acetic acid plus 5.0% methanol (2), or specifically stained with antibody (24). Molecular weights of degraded viral protein were estimated by comparing their mobilities to the mobilities of known proteins prepared in the same manner. The following standard proteins were used: bovine serum albumin (BSA MW=67,000), ovalbumin (oval MW=45,000), chymotrypsinogen (chymo MW=25,000), tobacco mosaic virus U-1 strain monomers (TMV monomer MW=17,300) and ribonuclease (RNAse MW=13,700). TMV (from Dr. T. A. Shalla, University of California, Davis) monomers were prepared by incubation of TMV at 37°C for two hours in 1% SDS plus 1% mercaptoethanol.

Preparative SDS gel electrophoresis was also conducted in a similar manner, but in larger glass tubes (12cm X 20mm). Three to five mg of protein in 400 μl of buffer were electrophoresed per tube at 40-50 milliamps for 12-16 hours. The PVX degraded protein bands were located with antibody, according to the method described by Shalla and Shepard (24).

Sephadex chromatography. -- Sephadex G-200 equilibrated with 0.05 M Tris-HCl pH 7.2 containing 1% SDS was used for confirmation of molecular weight determinations of degraded viral protein. Molecular weights were estimated by comparing the mobilities of known proteins, similar to the method of Shalla and Shepard (24). The protein standards; BSA, oval, chymo and cytochrome C (cyto C MW=13,700), were prepared by incubation of 1% SDS plus 1% mercaptoethanol for two hours at $37^{o}C$. The fractions, usually 2.2 ml, were collected with an automatic fraction collector and monitored at 280nm with a Beckman DB spectrophotometer.

Antiserum production. -- Antisera to whole PVX and to PVX-M degraded protein antisera were prepared by emulsifying 2 mg of appropriate antigen with an equal volume of Freund's incomplete adjuvant and injecting a rabbit intramuscularly every seven days over a four-week period.

Antisera against PVX-IP and PVX-ON degraded protein were provoked by injecting degraded PVX protein purified by the preparative SDS gel system. Four weekly injections of 2-3 mg of degraded viral protein were administered over a one month period.

Bleedings were conducted 2-6 weeks following the initial injection by cutting the marginal ear vein of the rabbit and collecting 40-50 ml of blood. The antiserum was allowed to separate from the clotted red cells, collected, centrifuged at 10,000 g for 10 minutes and stored frozen at $0^{o}C$.

Serology. -- Antisera were titered by either tube precipitin or double diffusion methods. The tube precipitin procedure was used to evaluate whole virus antisera activity. Antiserum was serially diluted with 0.05 M Tris-HCl pH 7.0 containing 0.85% saline. An equal volume of virus at 0.2 mg/ml was added and the mixture was incubated overnight at 4°C. Double diffusion tests were carried out in 0.9% ionagar dissolved in 0.05 M Tris-HCl pH 7.0 containing 0.85% saline. For antiserum titer determination to degraded protein, two fold dilutions of serum were made, placed in the central antiserum well and degraded protein at 0.1-0.2 mg/ml placed in the peripheral antigen wells. Reactions were allowed to develop overnight at room temperature before evaluation. For establishing serological relationships, degraded protein concentrations ranged from 0.1-0.2 mg/ml.

<u>Enzymatic digestion</u>. -- Trypsin (3X crystallized Worthington Freehold, N.J.) and chymotrypsin (3X crystallized Sigma St. Louis, Mo.) were used for digestion experiments. Unless otherwise specified digestion with trypsin and chymotrypsin was carried out a ratio of 1 µg enzyme per 1 mg of substrate overnight at room temperature in 0.05 M Na-citrate buffer pH 8.0. Following incubation of either whole virus or degraded protein with chymotrypsin, the enzyme was inhibited with L-1-tosylamide-2-phenylethyl chloromethyl ketone HCl (TPCK Sigma St. Louis, Mo.) using a modified procedure of Shaw et al (27). TPCK was dissolved in absolute methanol and added to

the virus-enzyme mixture at a ratio of three parts TPCK to one part enzyme and incubated for two hours at room temperature. Aqueous N-α-P-tosyl-L-lysine chloromethyl ketone (TLCK Sigma St. Louis, Mo.) was used to inhibit trypsin after incubation and was also used at a ratio of three to one (27, 28).

The time required for complete inhibition of trypsin with TLCK and chymotrypsin with TPCK was determined. Hide powder azure (Calbiochem Los Angeles, Calif.), an insoluble chromogenic subtrate, was used to assay the effectiveness of inhibition of enzymatic activity. Five mg of hide powder azure were mixed with 2 ml of 0.005 M Na-citrate buffer pH 8.0 in several tubes. One hundred µg of trypsin plus 300 µg of TLCK were mixed and 100 µg of chymotrypsin plus 300 µg of TPCK were mixed, and these mixtures were added to the tubes containing the hide powder azure at hourly intervals. Solubilization of dye due to enzymatic digestion was spectrophotometrically analyzed at 595 nm with a Beckman DB spectrophotometer after allowing the hide powder azure plus enzyme-inhibitor mixtures to incubate for various times at 37°C.

RESULTS

Effect of purification on the PVX subunit. -- Koenig et al (12) recently reported the effect that method of purification may have upon the molecular weight of the PVX subunit. The report stated that PVX subunit could possess a molecular weight of either 24,000 or 29,800. In the present study, experiments were conducted to determine whether similar results could be produced using the strain of PVX routinely used here and under our conditions. Preliminary examination of PVX was conducted with preparations which varied in time between homogenization and chloroform emulsification from fifteen minutes to several hours (PVX-M). PVX-M protein was prepared by incubating virus with sodium dodecyl sulfate (SDS). Molecular weights of the subunits were estimated from an SDS split gel electrophoresis molecular weight curve (Fig. 1) contructed from data with known protein standards (Fig. 2).

Initial experiments indicated that virus particles in PVX-M preparations were composed of mixtures of protein subunits, i.e., both intact and partially degraded polypeptide chains. These preparations frequently contained subunits of both molecular weights in approximately equal ratios, as determined by intensity and size of the stained portions of the SDS split gels (Fig. 3). The molecular weights of the two sizes of subunits was calculated to be 29,000 for the larger and 24,500 for the smaller.

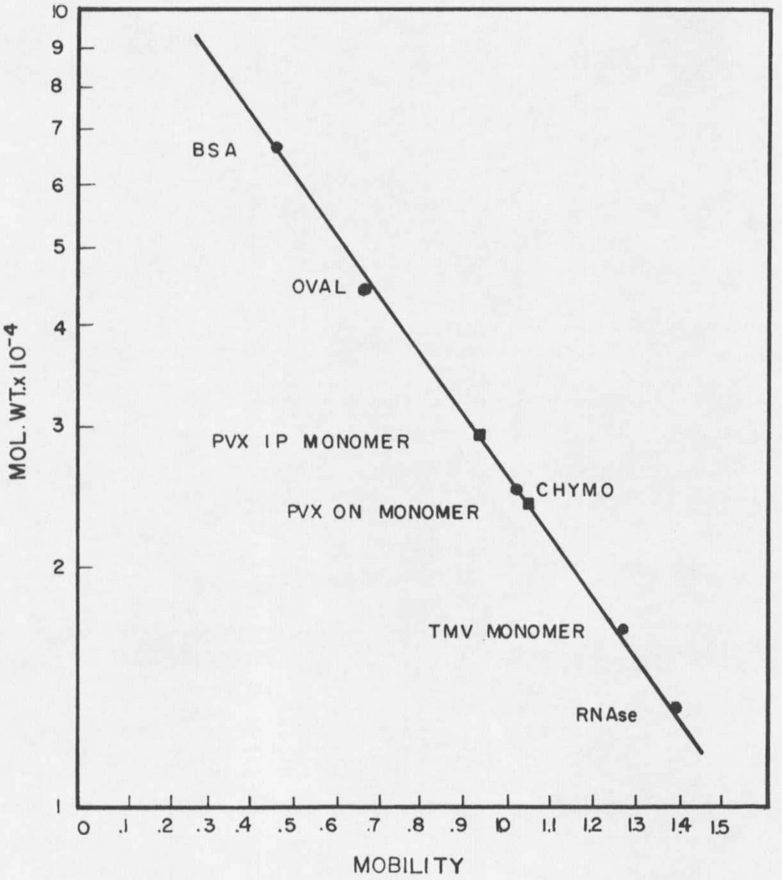

Figure 1. Estimation of molecular weights utilizing the SDS split gel electrophoresis system. Mobility equals the ratio of the distance the protein migrates to the distance chymotrypsinogen migrates. Both graphs plotted by method of least squares.

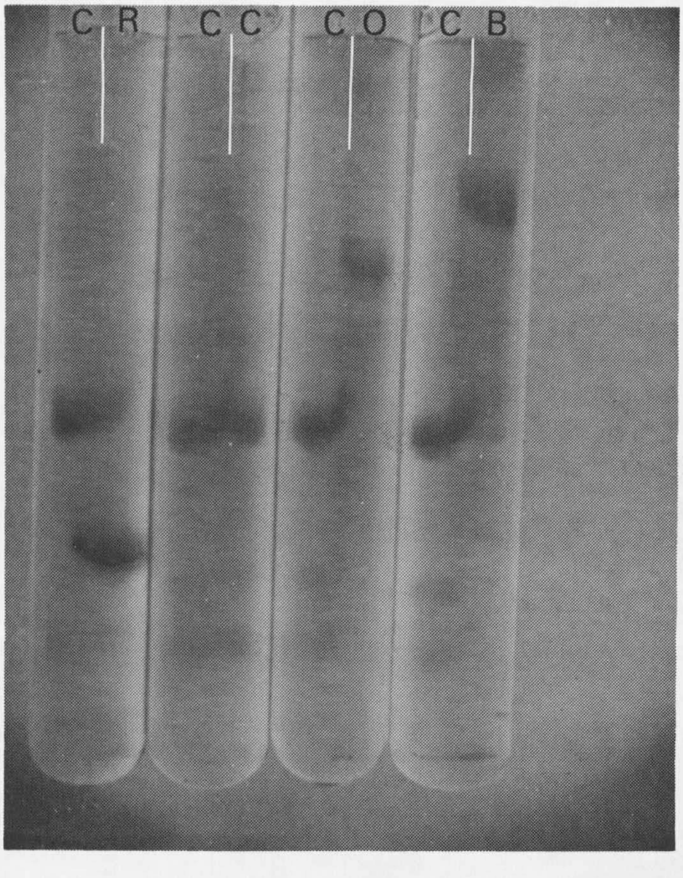

Figure 2. SDS split gels of four of the standards used to construct the molecular weight estimation curve shown in figure 1. C=chymotrypsinogen, R=ribonuclease, O=ovalbumin, B=bovine serum albumin.

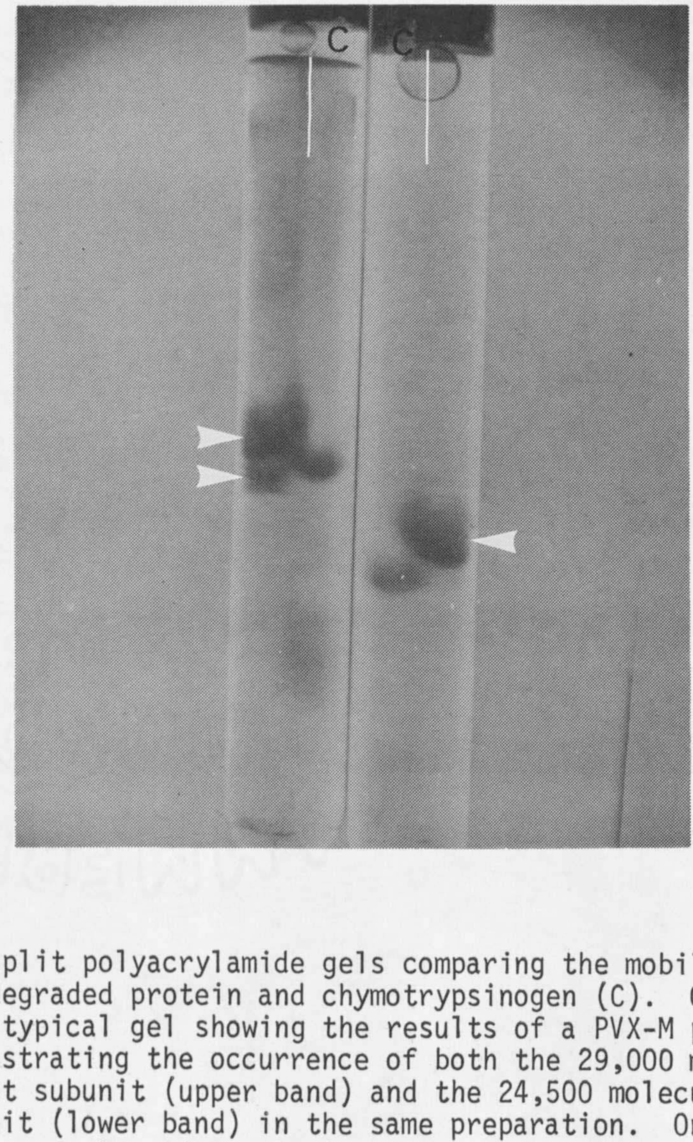

Figure 3. SDS split polyacrylamide gels comparing the mobilities of PVX degraded protein and chymotrypsinogen (C). On the left is a typical gel showing the results of a PVX-M preparation demonstrating the occurrence of both the 29,000 molecular weight subunit (upper band) and the 24,500 molecular weight subunit (lower band) in the same preparation. On the right is electrophoretic analysis of the same preparation after incubation of the virus with trypsin, demonstrating complete conversion to the 24,500 molecular weight subunit.

Crude homogenate manipulation was tested as a means of producing viral preparations composed of subunits of either, but not both, molecular weights. Crude homogenates were emulsified with chloroform immediately after homogenization and, after further purification, PVX-IP was analyzed for subunit molecular weight with SDS split gel electrophoresis. Assays of PVX-IP preparations suggested a homogeneous subunit molecular weight of 29,000±300 (Fig. 1).

Some PVX solutions were purified after allowing the crude homogenate to incubate overnight at room temperature and subsequently purified as described for PVX-ON. These virus suspensions were also analyzed for subunit molecular weight by acrylamide gel electrophoresis, and yielded primarily subunits having a molecular weight of 24,500±500 (Fig. 1). Figure 4 illustrates typical patterns in SDS split gels of PVX-IP and PVX-ON degraded protein compared with the chymotrypsinogen standard.

The consistency of subunit molecular weight was investigated in viral preparations obtained by PVX-IP and PVX-ON purification. Results from twenty experiments indicated that PVX-IP purification consistently produced virus particles composed of the 29,000 molecular weight subunits exclusively, since only these structure units were detectable in electrophoretic gels.

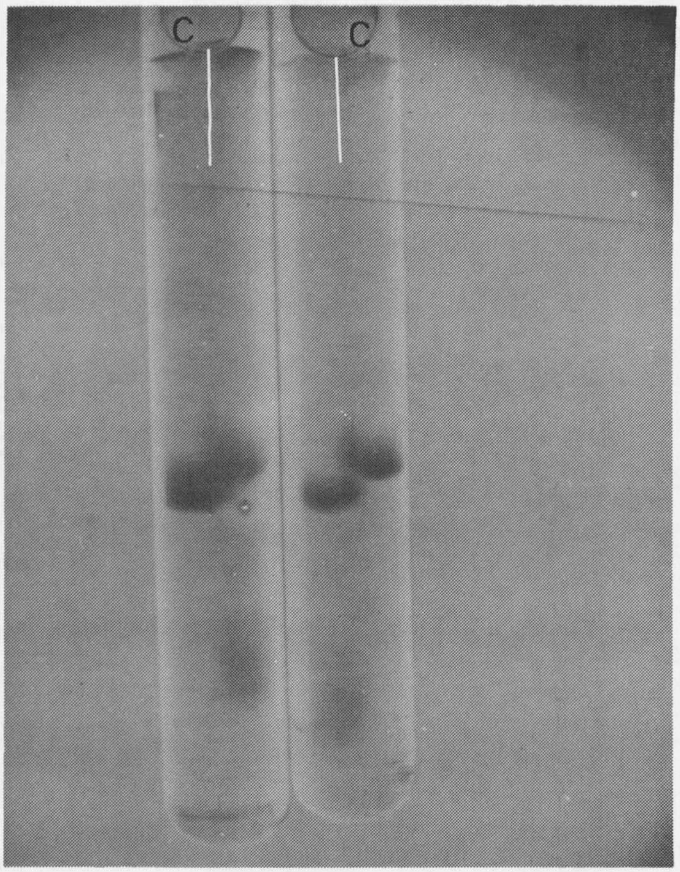

Figure 4. Typical SDS split gels showing patterns obtained after electrophoretic analysis of PVX-IP and PVX-ON protein using chymotrypsinogen as the comparative marker protein (C). On the left is the gel of PVX-IP degraded protein and on the right is the gel of PVX-ON degraded protein. Degraded protein obtained by SDS incubation.

The minimum amount of protein detectable in SDS split gels was determined. Sensitivity of the assay procedure for detecting the subunits in a virus preparation depended upon the minimum amount of protein visible after Coomassie brilliant blue staining. SDS degraded PVX protein was serially diluted in the standard electrophoretic buffer, applied at the top of the gels, and electrophoresed. The minimum detectable concentration of protein after staining was found to be 30 μg/ml in the applied preparation.

However, PVX-ON preparations which contained only the 24,500 molecular weights subunits were more difficult to obtain. Purified PVX-ON suspensions were inconsistent, i.e., sometimes resulting in homogeneous solutions of the 24,500 subunits, but more frequently resulting in solutions containing both the 29,000 and the 24,500 subunits.

Preparations of PVX-IP and PVX-ON were also serologically assayed for homogeneity. PVX was degraded with SDS and the degraded protein electrophoresed on standard sized SDS acrylamide gels. Following electrophoresis for three hours, the gels were surrounded with PVX-M degraded protein antiserum impregnated in agar according to Shalla and Shepard (24). Freshly prepared PVX-IP and PVX-ON assayed in this manner displayed a single component reactive with antibody on the circumference of the gel. The sensitivity of the serological assay method for minimum detectable antigen concentration was ascertained. PVX degraded protein was serially diluted 10 fold from 10 mg/ml to 0.0001 mg/ml, electrophoresed in acrylamide gels, and stained with a constant level of antibody. The results indicated that the antibody assay method was sensitive to 30 µg/ml.

Sephadex gel chromatography of reduced PVX degraded protein in the presence of SDS was tested as an additional method of determining degraded viral protein molecular weights and of confirming those calculated from SDS split gel electrophoresis data. SDS degraded PVX-IP and PVX-ON preparations with a starting volume of 2-3 ml and a concentration of 10-15 mg/ml were chromatographed on Sephadex G-200 equilibrated with 0.05 M Tris buffer pH 7.0 containing 1.0% SDS. Elution profiles compared with similarly treated standards indicated molecular weights for PVX-IP and PVX-ON structure units of 28,500 and 24,000 respectively (Fig. 5).

The values obtained for the molecular weights of PVX-IP and PVX-ON subunits of 29,000 and 24,500 by SDS split gel electrophoresis and 28,500 and 24,000 by SDS Sephadex chromatography agree closely with Koenig's findings of 29,800 and 24,000, and confirms the variability in the PVX protein molecular weight with mode of viral purification.

<u>Preparation of purified PVX-IP and PVX-ON</u>. -- Degradation of

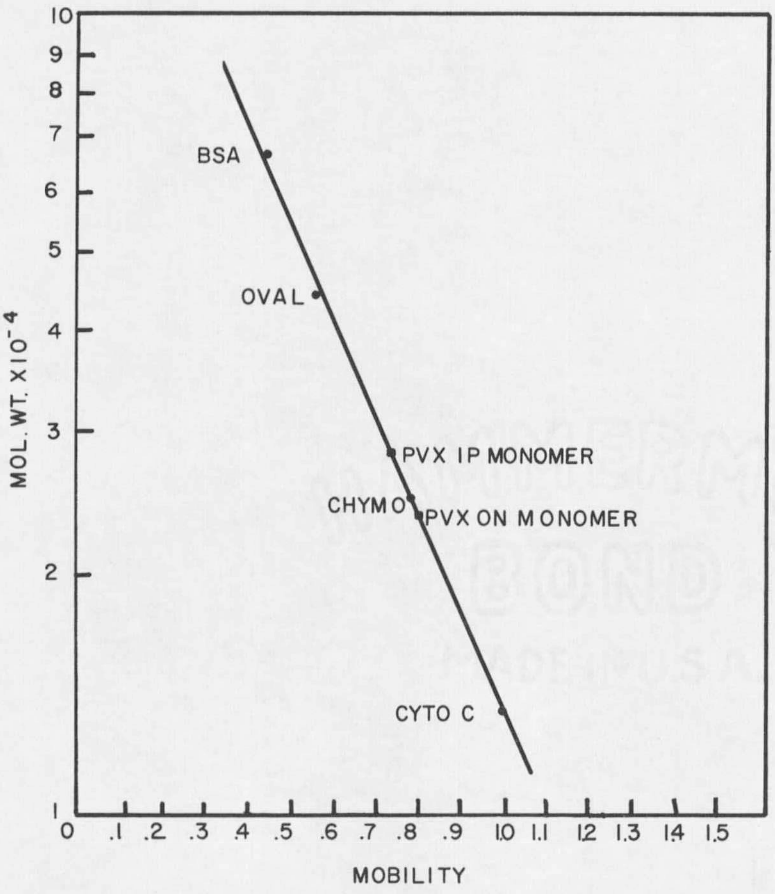

Figure 5. Graph constructed for estimation of molecular weights by SDS Sephadex G-200 column chromatography. Mobility equals the ratio of the elution volume (ml) of the protein to the elution volume of cytochrome C.

PVX-IP resulted in subunit solutions homogeneous in the acrylamide gel assay system. Hence, PVX-IP was prepared in the manner described routinely. However, since production of PVX-ON by crude homogenate incubation was inconsistent, a more reliable method of obtaining virus preparations composed of only the lower molecular weight subunits was investigated. Since Koenig et al (12) reported that incubation of PVX with trypsin duplicated the molecular weight conversion that occurred with crude homogenate incubation, enzymatic digestion was investigated as an alternative means of preparing PVX-ON. Incubation of PVX with trypsin at a substrate to enzyme ratio of 1000:1 for approximately twelve hours resulted in the consistent conversion to virus composed of only detectable subunits with a molecular weight of 24,500 (Fig. 3). Dissociation of the virus subsequent to trypsin digestion as reported for cowpea mottle virus by Chidlow and Tremaine (3) was not observed, as determined by retention of stream birefringence. Trypsin digested PVX was therefore used to prepare PVX-ON except as noted.

Serological relationship between PVX-IP and PVX-ON. -- After it had been confirmed that two molecular weights of PVX protein could be prepared homogeneously, the serological relatedness of the two forms was determined. PVX-IP and PVX-ON were purified and antisera to the two viral forms were prepared. After six weeks, the homologous titer of PVX-ON antiserum reached 1:8192, and the homologous titer of PVX-IP antiserum was 1:4096. Reciprocal cross-absorption tests were conducted to determine if any serological differences existed between these two forms of PVX. The results showed no residual activity to homologous antigen after removal of the immune complex formed by overnight absorption with heterologous antigen at 4 mg virus per ml of antiserum. Control tests conducted by absorption with homologous antigen and subsequent titering to homologous antigen demonstrated that absorption was complete at the levels used. Portions of the antigens used in the cross-absorption studies had been determined to be homogenous with respect to subunit molecular weight prior to use in the reciprocal cross-absorption studies. It was concluded from these studies that PVX-IP and PVX-ON were serologically identical despite the fact they were composed of protein subunits of distinct molecular weights.

Preparation and serological relatedness of PVX-IP and PVX-ON degraded protein. -- Experimentation had shown that the native protein of PVX-IP and PVX-ON were antigenically identical. However, since depolymerization of PVX subunits resulting in a change in antigenic specificity from the native form of the subunit (24, 30) and the efficacy of using denatured protein to determine serological relatedness (31) have been demonstrated, it was necessary to serologically compare the denatured protein subunits of PVX. PVX-IP and PVX-ON were purified and the degraded protein of each species prepared by SDS incubation. As a further precaution against contaminants not detectable with either the chemical or the serological homogeneity assay following electrophoretic analysis, an additional purification step was employed. Acrylamide gel electrophoresis of SDS degraded protein of PVX-IP or PVX-ON was conducted on a preparative scale to further purify the protein to be used for immunization. Antigen bands were identified with antibody and, as shown in figure 6, this method was capable of separating the 29,000 from the 24,500 molecular weight structural proteins. When used to prepare either PVX-IP or PVX-ON degraded protein for immunization, a single band in the gel was observed. SDS split gels were electrophoresed for each preparation to further confirm the homogeneity and molecular weight of the protein. Following preparative gel electrophoresis, that portion of the acrylamide gel containing the degraded viral protein was sliced from the gel, smashed into a slurry with 2 ml of 0.005 M Na-Citrate pH 8.0 buffer and dialyzed 6-8 hours against the same buffer to remove unbound SDS. Antisera to PVX-IP and PVX-ON degraded protein were prepared by injecting this resultant slurry. Antisera to PVX-IP and PVX-ON degraded protein both reached a titer of 1:8 to SDS prepared homologous antigen six weeks after

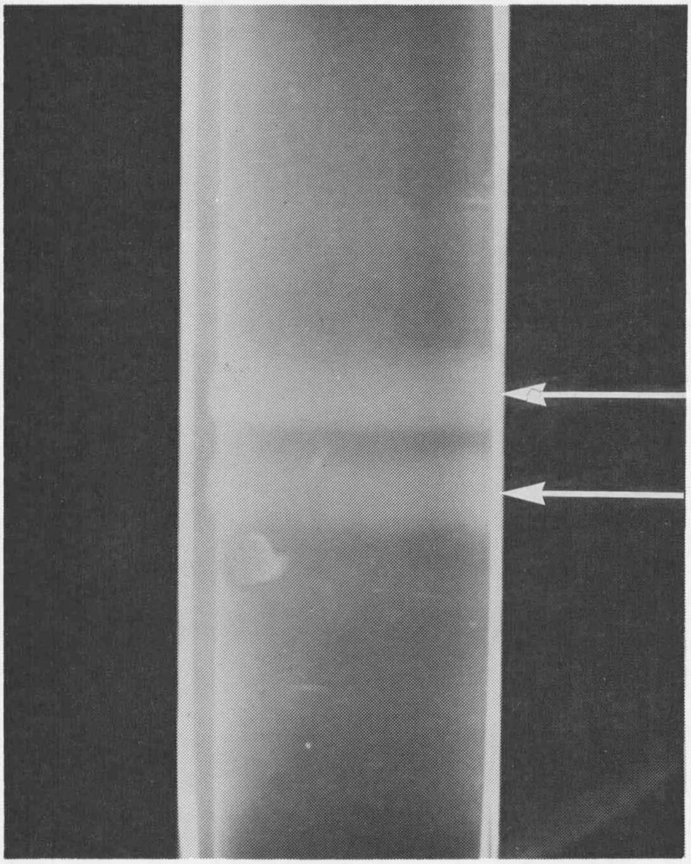

Figure 6. SDS preparative electrophoretic gel of PVX-IP and PVX-ON degraded protein after identification of the protein bands with antibody. The gel illustrates separation possible between the two species of degraded protein, and the usefulness of this method for use in preparing degraded PVX protein for immunization. The upper band represents location of PVX-IP degraded protein and the lower band represents location of the PVX-ON degraded protein.

the initial injection. Ouchterlony double diffusion tests were conducted to detect if any serological differences existed between these two forms of subunits. PVX degraded protein was prepared for serological testing by depolymerization with either 30% pyridine or 1% SDS. Protein prepared with SDS was analyzed for homogeneity before use in double diffusion. Pyridine has been previously shown to yield mainly dimers when used to depolymerize PVX (24). Double diffusion tests conducted with SDS prepared antigen indicated serological identity between the two forms of PVX subunits when tested by the standard method for detecting differences in antigenic specificity via double diffusion (Fig. 7), using antisera to either PVX-IP or PVX-ON degraded protein. Double diffusion assays were similarly conducted using PVX-IP and PVX-ON degraded protein prepared electrophoretically. The degraded viral protein was electrophoresed on standard size SDA acrylamide gels and the protein in the gels identified through the use of antibody. That portion of the gel containing the viral subunits was excised, these discs organized into the desired serological pattern, and surrounded with standard double diffusion agar. A central well was then removed and charged with either PVX-IP or PVX-ON degraded protein antiserum and the reactions allowed to develop. These results also indicated that the two forms of PVX structural protein are se-

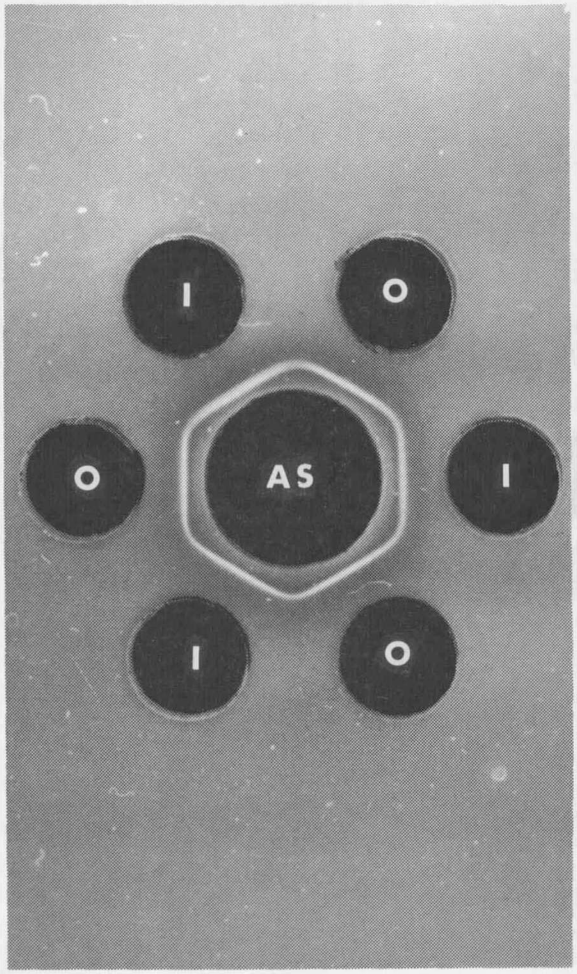

Figure 7. Ouchterlony double diffusion test comparing PVX-IP and PVX-ON degraded protein reacted against PVX-IP degraded protein antiserum. I=PVX-IP degraded protein and O=PVX-ON degraded protein. Notice the continuity of the precipitin lines and the absence of spurs, indicating serological identity of the antigens. Concentrations of both antigen solutions equals approximately 0.2 mg/ml.

rologically identical.

Because it was possible that SDS breakdown of PVX resulted in the production of monomeric solutions of subunits which would not show a single determinant difference between the PVX-IP and PVX-ON degraded protein in double diffusion tests, pyridine degraded PVX-IP and PVX-ON dimers were compared for serological relatedness. Pyridine degradation of each species was performed and the two forms compared in double diffusion using both PVX-IP and PVX-ON degraded protein antisera. The results again indicated serological identity between the two forms of viral protein.

Since trypsin prepared PVX-ON was used in the experiment in most cases, it was of interest to compare the antigenic specificities of trypsin prepared PVX-ON and PVX-ON purified following incubation of crude homogenates. PVX-ON was purified by both methods and the degraded protein obtained by SDS incubation. Standard double diffusion tests were conducted to compare the two forms of PVX-ON protein against PVX-ON degraded protein antiserum. The results of these tests demonstrated that the method of preparing PVX-ON had no effect upon the antigenic specificity of PVX-ON degraded protein.

Effect of trypsin on degraded protein. -- PVX dissociated protein has been shown to be converted into a different conformational state than when assembled into whole virus particles (24).

Tests were conducted to determine whether this denatured, but chemically identical subunit would be more sensitive to trypsin digestion than the native or assembled protein, and hence be serologically distinguishable from undigested protein. Trypsin was serially diluted in tenfold increments from an initial concentration of 10 mg/ml to 0.001 mg/ml. To each concentration was added 1 mg of pyridine degraded viral protein and incubated two hours at room temperature. Each mixture was then inhibited by TLCK.

The time required for inhibition of trypsin by TLCK was determined using the hide powder azure system. The absorbance of the trypsin inhibition test preparation decreased from 0.61 optical density units to 0.015 optical density units after one hour incubation. Viral protein-trypsin preparations were inhibited with TLCK for two hours to insure complete inhibition.

Miki & Knight (14) have reported that PVX protein, degraded with guanidine hydrochloride, resulted in 18 peptides when digested with trypsin at a ratio of PVX protein to trypsin of 10:1. The studies conducted in this experiment demonstrated that PVX degraded protein was sufficiently digested at levels of protein to trypsin of 1:10, 1:1 and 10:1 to have no reactivity remaining when tested against PVX-IP or PVX-ON degraded protein antiserum in double diffusion. However, at levels of viral protein to trypsin of

100:1 and 1000:1, the degraded protein would react with degraded viral protein antiserum, but with a reaction of non-identity when compared to undigested viral protein.

Effect of chymotrypsin on PVX and PVX degraded protein. -- The effect of chymotrypsin on PVX and PVX degraded protein was also tested. Koenig et al (12) have reported that chymotrypsin had no effect upon assembled PVX subunits. This was tested in our laboratory by incubating PVX-IP with chymotrypsin and using TPCK to terminate the reaction. Aliquots of PVX were incubated with chymotrypsin at a ratio of 1000:1, as conducted with trypsin digestion experiments of PVX, and after inhibition of the enzyme, the molecular weight of the viral subunits following SDS degradation was determined with the SDS split gel system. The time required for inhibition of chymotrypsin with TPCK was determined. Using the hide powder azure system again, the absorbance of the chymotrypsin inhibition test preparation decreased from 0.55 optical density units to 0.01 optical density units after one hour. As an extra precaution, the TPCK inhibited viral protein-chymotrypsin mixtures were incubated for two hours to insure complete inhibition. The results obtained indicated that chymotrypsin had no effect upon the molecular weight of the subunits, for the acrylamide gel analysis demonstrated only one stainable band, representative of a molecular weight of

29,000. Chymotrypsin incubated PVX-IP was degraded with SDS and serologically compared to SDS degraded untreated PVX-IP in double diffusion. These results indicated that the serological properties of PVX-IP were likewise unaffected by chymotrypsin incubation.

Experiments were conducted to determine the effect that chymotrypsin would have upon PVX degraded protein. Chymotrypsin was serially diluted from 10 mg/ml to 0.001 mg/ml and to each concentration was added 1 mg of PVX degraded protein which had been prepared with pyridine. The mixture was incubated for two hours at room temperature and the enzyme inhibited by adding TPCK and allowing to incubate for two hours. These studies showed that at levels of viral protein to enzyme of 1:10, 1:1, 10:1 and 100:1, the PVX degraded protein reactivity with degraded protein antiserum was completely eliminated in double diffusion tests. But at levels of viral protein to enzyme ratios of 1000:1, the viral protein would react with PVX degraded protein antiserum in double diffusion, but the reaction was one of non-identity.

DISCUSSION

Presumably an enzyme or enzymes in tobacco sap are responsible for the conversion of the molecular weight of the PVX subunit from 29,000 to 24,500 under certain conditions. This molecular weight conversion can be duplicated by incubating PVX with trypsin. These two molecular weight forms of protein, whether existent as assembled or depolymerized protein, were compared serologically for any difference in antigenic specificity which may occur following a loss of nearly 15% of the polypeptide chain. Purified whole PVX particles composed exclusively of subunits of either molecular weight were compared by reciprocal cross absorption studies. PVX degraded protein of both molecular weights was obtained by both pyridine and SDS degradation. In all cases, the two forms of assembled or degraded PVX protein, i.e., protein consisting of either 29,000 or 24,500 molecular weight, were found to be serologically identical.

This lack of antigenic specificity change with PVX protein is in contrast to TMV experiments whereby one amino acid substitution in or the loss of as few as three amino acids from the protein may cause a detectable serological change. However, the results are in accordance with the experiments with cowpea chlorotic mottle virus in which a 10% loss of protein caused no serological change, but a 20% loss was accompanied by a serological change. In addition, a loss of 5-10% of the molecular weight of adenovirus hexons caused no change in antigenic specificity but a greater loss, (exact data not available), caused a change in antigenic specificity. It seems evident from this data that polypeptide chain loss and antigenic specificity change cannot be necessarily correlated or predicted.

The 15% molecular weight loss has no influence upon antigenic specificity of the PVX molecule, and mode of purification apparently does not render serological identification studies invalid. This is important when considering serological detection of PVX. Antigenic specificity seems to reside in 85% or less of the molecule, meaning that variation can occur in the remaining 15% of the molecule without affecting the ability to be detected by serological methods. If the variation in strains of virus resident in the material tested occurs in the portion of the peptide lost upon enzymatic digestion, identification of PVX would not be seriously impaired. Because of the lack of any antigenic disparity between the two forms of PVX protein, the region or regions, (perhaps both termini are digested), of the polypeptide chain lost, i.e., 15% of the molecular weight, must constitute an immunosilent region or regions of the molecule containing none of the three or four antibody combining sites per monomer as reported by Shepard and Shalla (30). However, since broad spectrum or specific proteases may occur in some plant tissues, for example bean carboxypeptidase (20), mode of purification may influence the structure of certain virions purified and hence the antigenic specificity sufficiently that even identification by immunodiffusion could be questionable. The phenomenon of enzymatic digestion of virions during routine purification may be quite unpredictable and could have conceivably occurred with some virus(es) and gone unrecognized. Digestion of viral protein by some cellular enzymes may occur instantly upon rupture of cells and/or their organelles and lead to confusion in interpretation of data.

The results have shown that degraded protein of PVX is more susceptible to digestion by trypsin and chymotrypsin than assembed protein. The greater susceptibility of the degraded PVX protein subunits to enzymatic digestion may be attributable to the hypothesis that the depolymerized subunits have undergone a change in conformation which allows for the increased digestion, similar to the phenomenon found by Hultin and Sjoqvist (8) with rat liver ribosomes. In their report, they proposed that under certain well-defined conditions, the ribosomes will undergo a change in conformation and a "loop" on the ribosome, which was formerly resistant to chymotrypsin digestion, becomes susceptible to chymotrypsin digestion.

The molecular weights of 29,000 and 24,500 for the two forms of the PVX subunits agree closely with the results of Koenig et al (12). The small discrepancy is possibly attributable to electrophoretic procedural differences. The use of split gels utilizing SDS polyacrylamide electrophoresis is potentially the most accurate method currently in use of determining molecular weights of viral protein in this range. The molecular weights reported also agree closely with the report of the molecular weight of PVX subunits reported by Shalla and Shepard (24) of 25,000. Even though their results of antigenic disparity between PVX and its degraded protein were achieved through the use of the lower molecular weight of the two forms of PVX protein reported in this publication, their results can be extrapolated accurately for the complete subunit since the larger and the smaller are antigenically identical.

Finally, the PVX system may be a model system for exactly locating and identifying the antigenic determinants of a molecule. It is clear that 15% of the PVX molecule is not responsible for antigenicity. Through the use of selective cleavage techniques, either enzymatic or non-enzymatic, modification of PVX protein may result in identification of immunogenic regions of the PVX polypeptide chain.

SUMMARY

Potato virus X (PVX) has been shown to lose a portion of its polypeptide chain during purification or by trypsin digestion. By incubating crude infective homogenates overnight before purifying the virus, or by incubating purified virus with trypsin, a subunit molecular weight change from 29,000 to 24,500 will occur. The change in molecular weight was confirmed by sodium dodecyl sulfate (SDS) split gel polyacrylamide electrophoresis and Sephadex G-200 column chromatography.

The effect of the 15% molecular weight loss due to enzymatic hydrolysis upon the antigenic specificity of PVX and of PVX degraded protein was investigated. It was necessary to compare both molecular weights of the viral protein while in either the native (assembled) state or the degraded (depolymerized) state, for previous studies have shown that PVX and its degraded protein are only slightly related as antigens.

Antisera were made to purified PVX composed of either, but not both, structural unit molecular weights. A preparative SDS gel electrophoresis system was used to prepare degraded protein of either molecular weight for use in the production of antisera to PVX degraded protein. Reciprocal cross-absorption tests and Ouchterlony double diffusion tests were employed to detect differences in antigenic specificity which might accompany the polypeptide

chain loss. Experimental results indicated that no change in antigenic specificity was found to accompany the 15% molecular weight loss in PVX structural protein following enzymatic hydrolysis.

Trypsin digestion of PVX degraded protein was more complete than digestion of native virus. Depending upon the concentration of trypsin, PVX degraded protein was either immunologically nonreactive or reacted with an Ouchterlony precipitin line of nonidentity with untreated PVX degraded protein. Chymotrypsin had no marked influence upon either the molecular weight or the antigenic specificity of PVX. However, PVX degraded protein was also susceptible to digestion by this enzyme. It was hypothesized that conformational changes in the depolymerized PVX protein allows for the greater susceptibility to trypsin and chymotrypsin.

It was concluded that trypsin digestion or incubation of infective homogenates of PVX caused a subunit molecular weight change from the normal of 29,000 to 24,500, and that this 15% molecular weight loss had no influence upon the antigenic specificity of either native or degraded PVX protein. Furthermore, the region(s) lost upon hydrolysis constitutes an immunosilent portion of the molecule.

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