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

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by

GARY ALLEN SECOR

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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 poly-

acrylamide 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°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°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-HC1 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-HC1 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.

Enzymatic digestion. -- 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 HC1 (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.

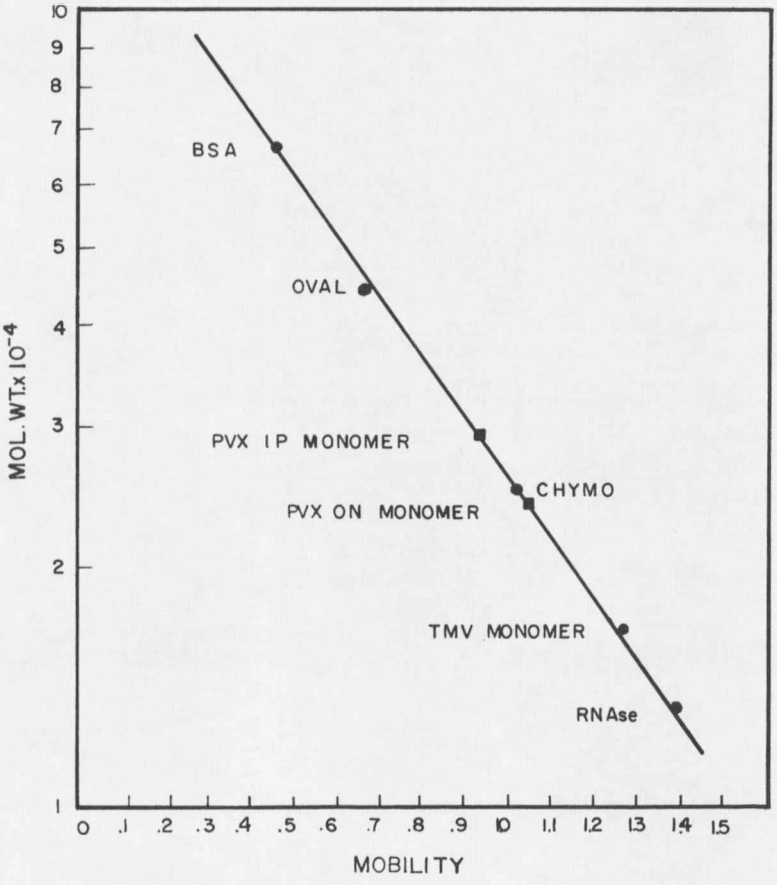

Figure 1. Estimation of molecular weights utilizing the SDS split gel electrophoresis system. Mobility equals the ratio of the distance the protein migrates to the distance chymotrypsinogen migrates. Both graphs plotted by method of least squares.