Isolation and characterization of barley stripe mosaic virus protein
by David John Gumpf

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:

Dialysis of barley stripe mosaic virus (BSMV) against 1.0 M calcium chloride resulted in the degradation of the virus to its component protein and nucleic acid. The protein, free of nucleic acid, obtained by this method was used for characterization. Lowering the pH of a BSMV-protein suspension to below 6.3 resulted in the polymerization of this protein into virus-like rods having a "stacked-disk" appearance as observed by electron microscopy. Sedimentation values, as observed by density gradient centrifugation, and electrophoretic mobilities of the polymerized protein, at pH 6.0 were indistinguishable from those of the intact virus. Non-polymerized protein at a pH above 6.3-6.4 had a relatively low sedimentation value, and an electrophoretic mobility 2/3 that of BSMV. Immunodiffusion patterns of BSMV-protein showed three antigen bands: a slowly diffusing major antigen showing partial identity with BSMV, and two faster diffusing minor antigens. These latter two antigens were identical to those formed on the degradation of BSMV or BSMV-protein with Leonil SA, an anionic detergent, and to virus related protein antigens present in crude sap from BSMV infected barley.

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VIRUS PROTEIN

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ABSTRACT

Dialysis of barley stripe mosaic virus (BSMV) against 1.0 M calcium chloride resulted in the degradation of the virus to its component protein and nucleic acid. The protein, free of nucleic acid, obtained by this method was used for characterization. Lowering the pH of a BSMV-protein suspension to below 6.3 resulted in the polymerization of this protein into virus-like rods having a "stacked-disk" appearance as observed by electron microscopy. Sedimentation values, as observed by density gradient centrifugation, and electrophoretic mobilities of the polymerized protein, at pH 6.0 were indistinguishable from those of the intact virus. Non-polymerized protein at a pH above 6.3-6.4 had a relatively low sedimentation value, and an electrophoretic mobility $2/3$ that of BSMV. Immunodiffusion patterns of BSMV-protein showed three antigen bands: a slowly diffusing major antigen showing partial identity with BSMV, and two faster diffusing minor antigens. These latter two antigens were identical to those formed on the degradation of BSMV or BSMV-protein with Leonil SA, an anionic detergent, and to virus related protein antigens present in crude sap from BSMV infected barley.

INTRODUCTION

A great deal of information on the structure and properties of a virus may be obtained by degradation of the virus followed by a study of its isolated components. Virus particles may be degraded by a variety of methods, including treatment with an alkaline buffer (Schramm et al., 1955), amino alcohol (Newmark and Myers, 1957), cold 67% acetic acid (Fraenkel-Conrat, 1957) and cold calcium chloride (Yamazaki and Kaesberg, 1963).

The majority of available information on the structure and properties of rod-shaped viruses, pertains to tobacco mosaic virus (TMV). Schramm et al. (1955) noted that TMV protein isolated by alkaline degradation, which they called A-protein, was polymerized to non-infectious rod-shaped particles resembling TMV by lowering the pH of the protein solution to under 6.0. X-protein, the virus protein not combined with TMV-RNA, isolated from TMV-infected leaves, was also observed to polymerize to form rod-shaped particles at pH 5.0 in 0.1 M ammonium acetate (Takahashi and Ishii, 1952, 1953). X-ray diffraction patterns of the polymerized protein showed the same helical arrangement of subunits, as the intact TMV (Franklin, 1955; Rich et al., 1955), even in the absence of virus nucleic acid. TMV-protein may also polymerize in a loose less orderly fashion, having a "stacked-disk" appearance (Franklin and Commoner, 1955).

According to Klug and Franklin (1957), A-protein is an aggregation of about six protein subunits in disks 70 Å long and having a molecular weight of about 100,000. Harrington and Schachman (1956) reported that non-polymerized A-protein has an S_{20}^W of 4. Lowering the pH will promote further polymerization of the disk-shaped structures to the rod forms by causing the disk-shaped structures to stack up end to end and then the disks will

gradually come into correct position. This polymerization may be reversed by a readjustment of the pH to 7.0 or by dialysis against distilled water, resulting in the protein having a disk shape (Takahashi and Ishii, 1952).

At pH values between 3.2 and 6.0, when the protein is polymerized into virus-like particles, its electrophoretic mobility and isoelectric point become similar to those of the intact virus (Kramer and Wittman, 1958). The protein at a pH around 7.0, however, has a slower electrophoretic mobility than TMV (Schramm et al., 1955; Rich et al., 1955).

The surface potential of TMV is determined only by the protein surface of the virus particle (Kramer and Wittman, 1958). The surface of the protein fragments obtained by degradation of TMV by alkali is not uniformly charged. The part of the surface that originally formed the outside wall of the virus is more negatively charged than the inside wall. When the pH is lowered to 6.0 the less charged parts of the surface become isoelectric while the more charged parts remain charged. Polymerization or aggregation resulted in the joining and covering of the isoelectric parts of different A-protein particles, thereby removing these parts from contact with the surrounding medium and leaving only the charged parts on the outside wall of the polymerized aggregates. The aggregates would therefore have the same shape and surface potential as the original virus. In reality, the difference in electrophoretic mobility at pH 7.0 between the virus and A-protein would result solely from the fact that in the virus the protein is polymerized whereas the A-protein is not. The preceding observations of Kramer and Wittman (1958) agree with those of Kleczkowski (1959), who

showed that the nucleic acid of TMV makes no appreciable contribution to the surface potential and that the greater the aggregation of the protein the closer the electrophoretic mobility approached that of the virus. It may be presumed that when the polymerized protein is in solution the spaces normally taken up by the RNA in the intact virus are replaced by water and anions to make up for the missing phosphate groups of the RNA (Caspar, 1964).

Serological studies furnish additional information regarding TMV and its related proteins. X-protein reacted serologically with X-protein antiserum as well as with TMV antiserum. The potency of the X-protein antigen, however, is greatly increased when it is reacted at pH 5.3 (Takahashi and Gold, 1960). TMV likewise cross-reacted with both sera (Takahashi and Ishii, 1953). Commoner and Rodenberg (1955) also reported that virus proteins which occur consistently in TMV-infected plants exhibit specific immunochemical cross-reactions with TMV antisera. A-protein reacted serologically with TMV antiserum. The higher the degree of polymerization of either A-protein (Rappaport, 1965) or X-protein (Takahashi and Gold, 1960) the more closely, serologically, they resembled the intact virus.

Studies of barley stripe mosaic virus (BSMV) were undertaken in order to provide information on some aspects of its structure and properties. A survey of the literature indicated that BSMV is structurally similar to TMV. BSMV is a virus of helical symmetry with an average length of 128 mu (Harrison et al., 1965) and a diameter of about 20 mu (Brandes, 1959; Gibbs et al., 1963). These values compared with a length of 300 mu and a diameter of 15 mu for TMV (Hall, 1964). A central canal was visible by

electron microscopy of negatively stained preparations of both TMV (Hall, 1964) and BSMV (Harrison et al., 1965), indicating that both viruses are essentially hollow cylinders. The x-ray diffraction pattern of BSMV is layer lines with near-meridional reflections occurring on every fifth layer line corresponding to a repeating length of 130 Å (Finch, 1965). The pitch of the helix is 26.1 Å ($\pm$ 0.2 Å) (Finch, 1965). TMV also gives a pattern of layer lines on x-ray diffraction; however, its near-meridional reflections occur on every third layer line, corresponding to a repeating length of 69 Å, and its helix pitch is 23 Å (Caspar, 1964).

Immunodiffusion of purified BSMV resulted in the formation of a precipitation band at the border of the antigen depot composed of two precipitation lines (Ball, 1961). A front precipitation band of non-sedimentable antigens, analogous to X-protein found in sap of TMV-infected leaves, was observed in the immunodiffusion of sap from BSMV-infected barley (Hamilton and Ball, 1966).

Hamilton (1964) reported the presence of a prominent front precipitin line upon immunodiffusion of sap from BSMV-infected barley in the presence of 0.5 and 1.0% Leonil SA, an anionic detergent. This same front precipitation band was also observed by immunodiffusion of purified BSMV in gel containing the detergent (Hamilton and Ball, 1966). An increase in prominence of the front precipitation band upon immunodiffusion was paralleled by a decrease in the prominence of the rear precipitation band (Hamilton, 1964). According to Hamilton and Ball (1966) BSMV was degraded by Leonil SA into faster diffusing virus-related antigens.

The purpose of this report is to describe some of the properties of BSMV-protein by the use of techniques used in the study of TMV. Specifically the problem was threefold: 1) isolation of BSMV-protein free of nucleic acid by means of the calcium chloride degradation method of Yamazaki and Kaesberg (1963); 2) a study and partial characterization of this protein and 3) polymerization of the isolated protein in vitro to rod-shaped particles.

MATERIALS AND METHODS

A. Preparation of Virus

A Montana isolate of BSMV derived from a single local lesion on Chenopodium amaranticolor L. after isolation from Hordeum vulgare L. var. Vantage, was used throughout this investigation. The virus previously shown to sediment at the same rate, by ultracentrifugation, as the type strain of BSMV (AC 69 obtained from The American Type Culture Collection) and to react with antiserum to the same strain. BSMV was grown on barley (Hordeum vulgare L., var. Blackhulless), in a greenhouse maintained at a temperature of about 20°C. Young plants at the three leaf stage were inoculated by rubbing the leaves with steam-sterilized cheesecloth pads soaked with an extract from BSMV-infected leaves and Celite.¹ Infected leaves of the inoculated plants were harvested two weeks following inoculation. The leaves were macerated in a food chopper and were stored at -10°C if not immediately used.

The macerated tissue was mixed with $\frac{1}{2}$ weight volume 0.02 M K_2HPO_4 and strained through several thicknesses of cheesecloth. This extract was then heated at 40°C for 1 hr in a water bath, and then clarified by a low speed centrifugation of 3,000 x g for 20 min. The supernatant fluid was then centrifuged for 2 hr at 79,400 x g. The pellets from this high speed centrifugation were overlayed and resuspended with 0.5 ml of 0.02 M tris (hydroxymethyl) aminomethane (Tris) -0.0064 M citrate buffer at pH 6.8. This suspension was then clarified by a low speed centrifugation as before and the pellets were discarded. The virus was further purified by density gradient centrifugation. Density gradient columns were prepared by layering

¹Diatomaceous silica manufactured by Johns-Manville Company.

4, 4, 4, and 4 ml of 0.02 M Tris-0.0064 M citrate-0.1% Igepon T-73, pH 6.8 - buffered sucrose solutions containing 600, 500, 400, and 300 mg sucrose per ml, respectively, in 1 x 3-inch cellulose nitrate tubes (Brakke, 1959). The columns were stored for 12 hr at 4°C. Three ml of the virus preparation containing 10-15 mg/ml, as determined spectrophotometrically², were floated on top of the columns, followed by 8.0 ml of the 0.02 M Tris-0.0064 M citrate-0.1% Igepon buffer on top of the virus preparation. The columns were centrifuged for 5 hr at 23,000 rpm, 90,000 x g. After centrifugation the virus zones were removed from the columns with a bent hypodermic needle attached to a 5 cc syringe. Virus was removed from the buffered sucrose-Igepon liquid by centrifugation at 144,880 x g for 1½ hr. The virus pellets were resuspended in 0.5 ml 0.02 M Tris-0.0064M citrate buffer, pH 6.8.

B. Isolation of Protein

BSMV-protein free of nucleic acid was obtained by a modified method of Yamazaki and Kaesberg (1963). Purified virus suspended in 0.02 M Tris-0.0064M citrate pH 6.8 was dialyzed against 1.0 M calcium chloride buffered with 0.02 M Tris-0.0064M citrate, pH 6.3, at 4°C for 12 hr. After dialysis the precipitated virus nucleic acid was removed by centrifugation at 20,000 x g for 30 min. The supernatant fluid, which was almost exclusively protein, was then dialyzed against several changes of 0.02 M Tris-0.0024M HCl buffer at pH 8.5 for 24 hr to remove the calcium and chloride ions. During the early stages of dialysis the protein precipitated, but went back into

² Virus concentration was determined by relating the absorbancy at 260 mu of a range of BSMV concentrations obtained gravimetrically. An absorption of 2.8 at 260 mu in a 1 cm light path is equivalent to a concentration of 1 mg/ml.

solution with continued dialysis. Following removal of the calcium and chloride ions, the protein solution was centrifuged for 1½ hr at 144,880 x g to remove any undegraded virus. The supernatant fluid was used as the purified protein preparation.

Concentrations of protein used in the following experiments were determined spectrophotometrically on the Beckman model DU Spectrophotometer with a 1 cm light path. An absorption reading of 1.7 at 280 mu, is equivalent to 1 mg/ml of BSMV-protein. Protein concentrations were determined using Lowry's (1959), biuret (Gormall et al., 1949) and dry weight determinations.

C. Production of Antisera

Purified virus and protein preparations diluted with 0.01 M potassium phosphate-0.14 M NaCl, pH 7.0, to a concentration of 1 mg/ml of virus and protein respectively, and emulsified with an equal volume of Freund's complete adjuvant, were used for injection into rabbits. Three intramuscular injections of 0.5 mg, 0.5 ml per hind leg, were given at 7 day intervals. Serum was collected on the second and third week following the final injection and stored at -10°C. Serum titers were determined by the micro-precipitin method of van Slogteren (1954) using both BSMV and BSMV-protein at 0.5 mg/ml as the constant antigen concentration. Titers of BSMV antiserum and BSMV-protein antiserum were 1:512 and 1:128 respectively. Immunodiffusion patterns of the BSMV antiserum gave better resolution against both BSMV and BSMV-protein than did the BSMV-protein antiserum.

D. Amino Acid Analysis

BSMV-protein samples were hydrolysed with about 100 times their weight of constant boiling 6N HCl at 110°C in a vacuum for 24 hr. To remove the HCl the samples were lyophilized and then redissolved in small amounts of distilled water and lyophilized again. This process was repeated three times. Two samples of the hydrolysates weighing 0.55 mg and 0.67 mg, respectively, were then dissolved in 1 ml of distilled water and analysed on a Technicon Automatic Amino Acid Analyzer.

E. Polymerization and Sedimentation

BSMV or BSMV-protein (0.5 mg/ml) was dialysed against a variety of buffers at pH 6.0, 7.0 and 8.0 for 2 hr at room temperature. Included in this series of buffers were the following: 0.1M potassium phosphate; 0.01M potassium phosphate; 0.017M borate; 0.125M borate; 0.001M ethylenediamine tetracetic acid (EDTA); and 0.01M EDTA. Following dialysis, the protein preparations in the 0.1M and 0.01M potassium phosphate buffers at pH 6.0 were made 1% with respect to sodium azide. Density gradient columns were prepared at each pH by layering 1.2, 1.2, 1.2, and 0.7 ml of buffered sucrose solutions, containing 400, 300, 200, and 100 mg sucrose per ml, respectively, into ½ x 2-inch cellulose nitrate tubes. The 0.1M and 0.01M potassium phosphate buffers at pH 6.0 were made 1% with respect to sodium azide and the pH readjusted before it was used to make the sucrose solutions for the density gradient columns. The columns were stored for 12 hr at 4°C, before 0.2 ml of the virus and protein suspensions were layered on top. The columns were then centrifuged at 39,000 rpm, 173,000 x g for 1 hr. After centrifugation the columns were examined for

visible zones and also scanned by means of an ISCO Density Gradient Fractionator with attached recording ultraviolet analyser.

F. Electron Microscopy

Purified virus and protein suspensions at concentrations of 1 mg/ml were dialyzed at room temperature against 0.01M potassium phosphate, 0.1M potassium phosphate, and 0.01M EDTA buffers at pH 6.0, 7.0, and 8.0 for 2 hr. After dialysis the pH 6.0 protein preparations in the potassium phosphate buffers were made 1% with respect to sodium azide. All preparations were then combined with equal volumes of 2% phosphotungstic acid, adjusted to the appropriate pH with 2N NaOH, and incubated for 2-5 min with shaking at room temperature. The suspensions were then sprayed on grids coated with a collodion film, and examined and photographed in a Siemens Elmiskop I electron microscope. A magnification of about 20,000 diameters was used for these studies.

G. Serological Studies

Immunoelectrophoretic analyses of BSMV and BSMV-protein were carried out by a modification of the method of Williams and Gräbar (1955). Glass photographic plates (3¼ x 4¼-inch) were coated with a film of Formvar and then filter paper electrodes were attached to the glass with Double Stick tape. A 2mm layer of masking tape was placed around the periphery of the slides to form a spacer for cover slides. Warm 1% agarose was then pipetted between the slides. The cover slides aided in the formation of a bed of uniform thickness. The cover slides were removed, and circular wells, 7mm in diameter, were cut with a cork borer and the wells then filled with the antigens. Buffers used were 0.01M potassium phosphate with 0.02M

potassium chloride, at pH 6.0, 7.0 and 8.0. The buffers used as the agarose solvents were the same as those used for electrophoresis except that the agarose solvents contained 0.02% sodium azide. All antigens were dialysed 2 hr prior to electrophoresis in the electrophoresis buffers at room temperature and the pH 6.0 protein preparations were made 1% with respect to sodium azide.

Electrophoresis was carried out at 4°C with a current of 5 ma per slide, giving a potential of about 7 volts/cm for 4 hr. After electrophoresis had been concluded, a trench was cut in the agarose bed parallel to the direction of current flow and filled with undiluted antiserum. Identification of the transported antigens was facilitated by setting up an immunodiffusion system in the agarose bed on a straight line with and at various distances from the origin. The wells were then charged with the same antigens as those that were subjected to electrophoresis. The plates were incubated at 20°C in a humid environment. Migration distances were determined by measuring the distance between the origin and the arc formed by the immunoprecipitin lines. Migration rates are given as $\text{cm} \times 10^{-3}/\text{min}$.

Serological activity of BSMV, BSMV-protein and BSMV-related antigens was determined by the gel-diffusion method (Ouchterlony, 1958). Quantities of the same 1% agarose gel used for immunoelectrophoresis were placed in Formvar-treated petri dishes. Wells, 7 mm in diameter, were cut with a cork borer. The wells were cut, one in the center, and the others on the circumference of a circle so that all wells are equidistant from each other. The center well was filled with undiluted antiserum, and the peripheral wells with the antigens to be tested. The plates were incubated at 20°C in

a moist environment.

RESULTS

A. Absorption Spectra

The ultraviolet absorption spectra of BSMV and BSMV-protein are shown in Figure 1. BSMV was observed to have an absorption spectrum with a maximum between 260 and 270 mu, a minimum between 250 and 255 mu, a 260/280 ratio of 1.0-1.2, and a max/min ratio of 1.02-1.20. BSMV-protein had an ultraviolet absorption spectrum typical of protein free of nucleic acid with a maximum between 278 and 280 mu, a minimum at 250 mu, a 260/280 ratio of .47-.57, and a max/min ratio of 2.1-3.43.

B. Amino Acid Analysis

The partial amino acid composition of BSMV-protein is presented in Table I. These results are an average of two separate determinations. No analyses were performed to determine the concentrations of tryptophan, which is destroyed; or cystine and cysteine which are oxidized by acid hydrolysis. The values given for serine and threonine were not extrapolated to zero hydrolysis time.

C. Polymerization and Sedimentation

It was with some difficulty, as will be explained further in the discussion, that sedimentation studies of the virus and virus protein were carried out. As may be seen (Table II) BSMV-protein at pH 7.0 and 8.0 sedimented relatively slowly in a density gradient column as compared to the intact virus. A lowering of the pH to 6.0 was accompanied by an increased sedimentation of the protein to a value approaching that of the whole virus. Polymerization and sedimentation results given in Table II, were obtained by using the potassium phosphate buffers with sodium azide treatment at pH 6.0. Previous experiments suggested that the addition of

Figure 1. Ultraviolet absorption curves of BSMV and BSMV-protein
a) BSMV in 0.02M Tris-0.0064M citrate pH 6.8. b) BSMV-protein in
0.02M Tris-0.0024M HCl pH 8.5. Absorbancy is given in arbitrary units.

TABLE I. Amino acid composition of BSMV-protein in
ug of 130.97 ug total recovered amino acids.

Amino acid	ug	Per cent
Aspartic acid	18.11	13.83
Threonine	5.66	4.32
Serine	5.10	3.89
Glutamic acid	17.66	13.48
Proline	6.45	4.92
Glycine	3.57	2.73
Alanine	10.65	8.13
Valine	8.03	6.13
Isoleucine	4.20	3.21
Leucine	13.71	10.47
Tyrosine	5.44	4.15
Phenylalanine	5.12	3.91
Ammonia	2.68	2.05
Lysine	5.85	4.47
Histidine	3.41	2.60
Arginine	15.33	11.71

TABLE II. Visible location of BSMV and BSMV-protein zones following centrifugation in 0.01 M potassium phosphate buffered density gradient columns.

pH	Location of zones below meniscus in cm	
	BSMV	BSMV-protein
6.0	2.7 - 3.4	0.7 - 1.1
	2.9 - 3.2*	1.7 - 2.5
7.0	2.9 - 3.1	0.5 - 1.0
	3.3 - 3.5*	
8.0	2.9 - 3.0	0.5 - 1.0
	3.1 - 3.3*	

*Lower virus zones caused by aggregation of BSMV

sodium azide inhibited the gross aggregation of BSMV-protein at pH 6.0. The other buffer systems, EDTA and borate, proved to be unsatisfactory for polymerization and sedimentation at pH 6.0. In these buffers the protein rather than polymerizing into rod-shaped particles, aggregated in such a gross fashion that it pelleted upon centrifugation. Sedimentation of both BSMV and BSMV-protein in EDTA and borate at pH 7.0 and 8.0 (Tables III and IV) gave results comparable to those obtained using the potassium phosphate buffers.

These preceding results were obtained by a visual location of light scattering zones in the density gradient columns. No results were obtained employing ultraviolet absorption for location of the zones because of the extremely high absorption of ultraviolet light by the sodium azide present in the density gradient columns.

D. Electron Microscopy

Electron micrographs of BSMV and BSMV-protein are shown in Figure 2. From the examination of two preparations of purified BSMV-protein it was evident that at pH 8.0 the protein was in small disk-shaped particles while at pH 6.0 the protein had polymerized into a loose "stacked-disk" rod form.

E. Serological Comparison of BSMV and BSMV-protein

1. Immunodiffusion

BSMV antiserum was used in the analyses of BSMV and BSMV-protein. Photographs and line drawings of the immunodiffusion systems used for the analysis BSMV and its related antigens are shown in Figures 3-6. Fusion of precipitation lines indicates the presence of common antigens. BSMV produced

TABLE III. Visible location of BSMV and BSMV-protein zones following centrifugation in 0.001 M EDTA buffered density gradient columns.

pH	Location of zones below meniscus in cm	
	BSMV	BSMV-protein
6.0	2.8 - 3.0	pelleted
7.0	2.9 - 3.1	0.5 - 0.8
	3.2 - 3.5*	
8.0	2.9 - 3.1	0.5 - 0.7
	3.3 - 3.5*	

*Lower virus zones caused by aggregation of BSMV

TABLE IV. Visible location of BSMV and BSMV-protein zones following centrifugation in 0.125 M borate buffered density gradient columns.

pH	Location of zones below meniscus in cm	
	BSMV	BSMV - protein
6.0	2.9 - 3.2	pelleted
7.0	2.9 - 3.0 3.1 - 3.3*	0.5 - 0.7
8.0	2.9 - 3.1 3.2 - 3.4*	0.45 - 0.8

*Lower virus zones caused by aggregation of BSMV

Figure 2. BSMV and BSMV-protein in negative contrast with 2% phosphotungstic acid. 2a) BSMV at pH 7.0. 2b) BSMV-protein at pH 8.0. 2c) Polymerized BSMV-protein at pH 6.0, note "stacked-disk" appearance. Magnification 100,000 X

two precipitin lines in a major precipitin band close to the antigen depot (Figure 3 and 6). BSMV-protein produced a major precipitin band close to the antigen depot and two minor precipitin bands midway between the antigen and antiserum depots (Figure 3 and 6). Hooking of the major BSMV-protein precipitin band into the BSMV precipitin band indicates a partial identity of antigens displaying similar serological characteristics. The minor BSMV-protein antigens are not found in purified BSMV preparations, but were always present in the protein preparations used in these experiments. Incubation of BSMV or BSMV-protein with 0.5% Leonil SA at room temperature; pH 7.0, resulted in the formation of two precipitin bands (Figure 4). The band formed closest to the antiserum well is composed of two precipitin lines. The two precipitated antigens are identical to the minor antigens of BSMV-protein (Figure 5 and 6). The major antigen from crude sap of BSMV-infected barley leaves were found bordering the antigen depot, and would appear to be the same as the purified BSMV major antigen. Two other antigen bands were visible in this immunodiffusion system of crude sap which were identical to the minor BSMV-protein antigens and the antigens derived by Leonil SA degradation of BSMV (Figure 6).

The curvature of the major precipitin bands bordering the BSMV-protein wells is a function of antigen concentration and pH. In these serological investigations the antigen concentrations were the same for each system. The difference in curvature that can be observed for the major BSMV-protein antigen in Figures 3, 5 and 6 is therefore a function of the pH. The degree of curvature of the precipitin bands toward the antigen depot was greater at pH 6.0 than at pH 7.0. The pH was also

Figure 3. Immunodiffusion patterns and line drawings of BSMV and BSMV-protein at pH 7.0. Well 1) BSMV-protein; Well 2) purified BSMV. Well 3 was filled with undiluted BSMV antiserum. a) BSMV-protein major antigen; b and c) BSMV-protein minor antigen; and d) BSMV major antigen.

Figure 4. Immunodiffusion patterns and line drawings of antigens at pH 7.0 produced by Leonil SA degradation. Well 1) BSMV with 0.5% Leonil SA. Well 2) BSMV-protein with 0.5% Leonil SA. Well 3) undiluted BSMV antiserum. a and b) antigen bands. Note presence of two precipitin lines in band b.

Figure 5. Immunodiffusion patterns and line drawings of BSMV-protein antigens at pH 6.0. Well 1) BSMV-protein with 1.0% sodium azide; well 2) BSMV-protein; well 3) BSMV-protein with 0.5% Leonil SA; wells 4 and 5 not used. The center well was filled with undiluted BSMV antiserum. Note identity of the minor antigens of BSMV-protein and the antigens formed by Leonil SA degradation of BSMV-protein.

Figure 6. Immunodiffusion patterns and line drawings of BSMV and related antigens at pH 7.0. Well 1) purified BSMV; well 2) BSMV-protein; well 3) crude sap from BSMV infected barley; well 4) BSMV with 0.5% Leonil SA; well 5) crude sap from healthy barley. The center well was filled with undiluted BSMV antiserum. Note two precipitin lines in the major BSMV antigen band (well 1).

observed to be affecting the diffusion rate of BSMV-protein. At pH 7.0 (Figure 3 and 6) the protein diffused a measurable distance from the antigen depot, while at pH 6.0 (Figure 5) it remained at the border of the antigen well. This observed increase in curvature and decrease in diffusion rate at pH 6.0 are a result of increased antigen size caused by polymerization of the protein. BSMV and its related antigens displayed identical immunodiffusion patterns at pH 7.0 and 8.0 except for less curvature of the major components of BSMV and BSMV-protein at pH 8.0.

The serological comparison suggests the following classification of the antigens: the major BSMV-protein antigen is partially identical or similar to the major BSMV antigen found in purified BSMV preparations and in crude sap from BSMV-infected plants; two minor BSMV-protein antigens found as components of BSMV-protein preparations derived by calcium chloride degradation of BSMV, are identical to minor antigens found in crude sap from BSMV-infected barley, and minor antigens formed by Leonil SA degradation of virus or virus protein.

Immunodiffusion patterns using BSMV-protein antiserum were identical to those observed with BSMV antiserum. BSMV antiserum was used solely because it allowed for better resolution of the antigen mixtures.

2. Immunelectrophoresis

BSMV antiserum was used in the immunelectrophoretic analyses of BSMV and BSMV-protein. Electrophoretic mobilities of BSMV and its related antigens at pH 6.0, 7.0 and 8.0 are given graphically in Figure 7. Photographs and line drawings of immunelectrophoresis patterns, showing method of antigen identification, are shown in Figure 8 and 9. It may be seen that

at pH 6.0, 7.0 and 8.0, BSMV and the BSMV-protein antigens migrated anodically. BSMV and BSMV-protein migrated at the same rate at pH 6.0 (Figure 8); at pH values above 6.3-6.4 the major BSMV-protein antigen migrated at about $2/3$ that of BSMV (Figure 9). The minor BSMV-protein antigens migrated identically, at a rate slightly less than that of the major BSMV-protein antigen. Incubation of BSMV or BSMV-protein with Leonil SA resulted in the formation of two antigens, identical serologically to the minor antigens of BSMV-protein, which exhibited a high mobility rate independent of pH between 6.0-8.0.

Figure 7. Electrophoretic mobilities of BSMV and its related antigens at pH 6.0, 7.0 and 8.0 in 1.0% agarose gel. a) antigens formed by degradation of BSMV or BSMV-protein with 0.5% Leonil SA. b) BSMV major antigen. c) BSMV-protein major antigen. d) BSMV-protein minor antigens.

Figure 8. Immuno-electrophoresis patterns and line drawings at pH 6.0, of BSMV, BSMV-protein and antigens formed by the degradation of BSMV or BSMV-protein with 0.5% Leonil SA. Well 1) purified BSMV; wells 2 and 2a) BSMV or BSMV-protein with 1.0% sodium azide; wells 3 and 3a) BSMV or BSMV-protein with 0.5% Leonil SA. The trenches were filled with undiluted BSMV antiserum. a) BSMV-protein major antigen. b and c) BSMV-protein minor antigens.

Figure 9. Immuno-electrophoresis patterns and line drawings at pH 7.0 and 8.0, of BSMV, BSMV-protein, and antigens formed by the degradation of BSMV and BSMV-protein with 0.5% Leonil SA. Wells 1 and 1a) purified BSMV; wells 2 and 2a) BSMV-protein; wells 3 and 3a) BSMV or BSMV-protein with 0.5% Leonil SA. The trenches were filled with undiluted BSMV anti-serum. a) BSMV-protein major antigen. b and c) BSMV-protein minor antigens.

DISCUSSION

Calcium chloride degradation of BSMV was used solely in this investigation primarily because it was simple to perform. As it may be seen from the ultraviolet absorption spectrum of BSMV-protein, the protein component isolated by this method seemed to be free of contaminating nucleic acid. The same relative ultraviolet absorption spectrum could be obtained consistently with different samples. The protein derived by CaCl_2 degradation of BSMV, proved satisfactory for most of the experiments of this investigation, the exception being in the polymerization and sedimentation studies. When the pH was lowered to 6.0 the protein rather than polymerizing to virus like rod-shaped particles would "fall out of solution." If sodium azide was added to this protein suspension the protein would then go into a colloidal suspension of rod-shaped particles. According to Markham (1962) if carboxyl groups predominate on a virus protein, as they do in TMV and assuming they do so in BSMV, addition of salts or lowering the pH results in the cancellation of carboxyl group charges. This causes a reduction of repulsive forces between protein particles, allowing hydrophobic groups on adjacent particles to attract each other, repulse the water molecules, and then come out of solution. Calcium was used to degrade the virus and it is known that proteins have a particular affinity for multivalent ions such as calcium (Haurowitz, 1963), thus furnishing another reason for the precipitation of the protein. Divalent calcium may simply form bridges between protein particles causing gross aggregation. By whatever method the calcium is interfering with the protein, it is eliminated presumably by a chelating action of the sodium azide, or by increasing the ionic strength of the buffer solution thus allowing an orderly polymerization

of the protein to the rod-like particles. The addition of a commonly used chelating agent, ethylenediamine tetracetic acid, however, at varying concentrations, was found to be noneffective.

There is no indication that BSMV-protein possesses a particularly unique amino acid composition which would interfere with polymerization. BSMV-protein was found to have a high aspartic and glutamic acid content which undoubtedly confers a negative charge to the protein at pH values 6.0, 7.0 and 8.0. This conclusion is supported by the high anodic mobility of BSMV and BSMV-protein in the electrophoresis experiments.

Electron micrographs of the polymerized protein show a loosely polymerized "stacked-disk" form at pH 6.0. This type of polymerization has been reported for TMV-protein (Takahashi and Ishii, 1952, 1953; Franklin and Commoner, 1955). It should also be mentioned that at a pH above 6.3-6.4 or when polymerization has not occurred the protein is in the form of a disk. This characteristic was true with non-polymerized TMV-protein (Takahashi and Ishii, 1952).

The antigenic components of BSMV and BSMV-protein reacted with both BSMV antiserum and BSMV-protein antiserum. The major BSMV-protein antigen present very close to the antigen depot, had partial identity reactions with the major BSMV antigen present in purified BSMV preparations and in sap from BSMV infected plants. Minor antigens whether in sap from infected plants, formed by calcium chloride-degradation of BSMV, or formed on degradation of BSMV or BSMV-protein with detergent, diffused rapidly and were precipitated as front precipitation bands about half way between the antigen and antiserum depots.

These rapidly diffusing antigens appeared to be the same as the non-sedimentable antigens observed in extracts of BSMV-infected barley (Hamilton and Ball, 1966), which in turn are analogous to the low molecular weight TMV X-protein (Takahashi and Ishii, 1953).

Immunoelectrophoresis of these antigenic components revealed these interesting facts. BSMV-protein, when in the non-polymerized form, had an electrophoretic mobility $2/3$ that of BSMV. Kramer and Wittman (1958) reasoned that the protein fragments obtained on degradation of TMV are not uniformly charged, the part forming the outside wall being more negatively charged than that forming the inside wall. On degradation both sides would be exposed to the medium which indicates that the less negatively charged inside wall would effectively negate some of the outside wall charges, thereby reducing electrophoretic mobility. When BSMV-protein is polymerized, with the aid of sodium azide, the mobilities of the virus and protein are the same. The protein in this state has the same surface potential as the intact virus. From this fact it is evident that the virus nucleic acid plays no role in the determination of surface potential. Minor antigens, derived by calcium chloride degradation of BSMV had lower mobilities than either virus or protein, regardless of pH. Antigens formed by Leonil SA degradation of BSMV or BSMV-protein exhibited the unique characteristic of having the highest mobility of any of the components. It should also be noted that this mobility was the same at all pH values. Putnam (1948) reported that anionic detergents combine with plant virus proteins, cleaving protein molecules and remaining as a complex, which would then tend to increase anodic mobility. This was

the case with BSMV-protein. Leonil SA degraded both BSMV and BSMV-protein into two antigen complexes, serologically identical to the two minor antigens found in sap from BSMV infected barley leaves and as components of BSMV-protein, but with greatly increased electrophoretic mobility. These minor antigens possessed the same electrophoretic mobilities, but their differing diffusion rates made them detectable.

BSMV would seem to be an excellent choice of virus for further studies because of its relative ease to isolate and purify. At the present time this investigator can see no reason why reconstitution of this virus should not be possible. If reconstitution were to be tried, however, it might be advantageous to use acid or alkaline degradation for the isolation of the protein in order to eliminate problems presumable caused by the divalent calcium ion, since the sodium azide treatment is not always completely reliable.

SUMMARY

Degradation of BSMV by calcium chloride yields a nucleic acid-free protein component suitable for characterization. Immunodiffusion patterns of BSMV-protein show three antigens: a slowly diffusing major antigen showing partial identity with BSMV, and two faster diffusing minor antigens identical to those formed on the degradation of BSMV or BSMV-protein with Leonil SA, an anionic detergent. These latter two minor antigens are identical to non-sedimentable virus-related antigens present in the crude sap of BSMV-infected barley. BSMV-protein at a pH above 6.3-6.4 has a relatively low sedimentation value, as observed by density gradient centrifugation, and an electrophoretic mobility $2/3$ that of BSMV. At pH 6.0, with gross aggregation of the protein inhibited, the sedimentation values and electrophoretic mobilities of the protein are indistinguishable from those of the intact virus. This increase in sedimentation and electrophoretic mobility is correlated with a polymerization of the protein into virus-like rods having a "stacked-disk" appearance as observed by electron microscopy.

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