Characterization of Belladonna Mottle Virus Isolates from Kansas and Iowa

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ABSTRACT

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A mechanically transmissible virus with a narrow host range was isolated from *Capsicum frutescens* 'Hybrid Tokyo Bell.' Isometric virus particles approximately 27 nm in diameter sedimented in sucrose density gradients as a noninfectious 53S top component and an infectious 109S bottom component. The single RNA component sedimented at 26S. Through the use of antiserum to the Iowa Physalis mottle strain of belladonna mottle virus, the virus was identified serologically and called belladonna mottle virus, Kansas strain. Both the Kansas and Iowa strains were unusual among plant viruses in that they separated into two cathodic electrophoretic forms between pH 5 and 10. The Kansas and Iowa strains may be differentiated by host range. No seed transmission was found for either strain in *C. frutescens, Physalis alkekengi,* or *Datura stramonium.*

Additional key words: Electrophoretic heterogeneity, pepper virus, seed transmission, tymoviruses.

Belladonna mottle virus (BdMV) was first reported in Germany from *Atropa belladonna* (17) and has been isolated from other hosts in Illinois and Iowa (13, 18). Isolation of a Kansas strain of BdMV from garden-grown pepper (*Capsicum frutescens* L. var. 'Hybrid Tokyo Bell') is reported here. Like the Iowa Physalis mottle strain (BdMV-I) (13), the Kansas strain (BdMV-K) has two centrifugal components, only one of which is infectious and contains RNA. Both strains are unique among plant viruses in that they have two cathodic electrophoretic forms. A preliminary report was published (11).

MATERIALS AND METHODS

Virus culture and host range. The BdMV-K and BdMV-I (ATCC No. PV 183) strains were maintained in pepper, *C. frutescens* L. 'California Wonder,' in *Nicotiana glutinosa* L., or in *Datura stramonium* L. For host range studies, infected pepper leaves or infected *N. glutinosa* leaves were ground in 2 ml of 0.02 M potassium phosphate pH 7.0 (phosphate buffer) per gram fresh weight, and the crude homogenate was mechanically inoculated onto carborundum-dusted leaves of test plants. After 3–4 wk, back inoculations were made to pepper to check for symptomless hosts.

In vitro properties. Infected pepper leaves were ground as described above. The thermal inactivation point (TIP) was determined by heating 1.0-ml samples of the homogenate for 10 min in a water bath, cooling them on ice, and assaying them for infectivity on pepper. The dilution end point (DEP) was determined in tenfold serial dilutions. Longevity in vitro (LIV) tests were made with homogenate stored at 4 C and at room temperature.

Purification. Virus was purified from infected pepper leaves by the method of Moline and Fries (13), except that 0.2% 2-mercaptoethanol was added to the extraction buffer and the freezing step was omitted. Plants for virus purification of both strains of BdMV were grown concurrently but well separated in the same greenhouse. Virus concentration was estimated with an extinction coefficient of $E_{260}^{0.1\%} = 8.0$. For serology, the virus was further purified by sucrose density gradient centrifugation (3). For amino acid analysis, the virus was further purified by sucrose density gradient centrifugation followed by equilibrium centrifugation in cesium chloride (7). Samples were collected, dialyzed extensively against distilled water, concentrated by ultracentrifugation, and resuspended in 0.02 M phosphate buffer or distilled water for serology or amino acid analysis, respectively. For serology, only the bottom (B) component was collected, whereas both the top (T) and B components were collected for amino acid analysis.

Density gradient centrifugation. Sedimentation coefficients of whole virus and of extracted viral RNA were estimated by sucrose density centrifugation on log linear columns as described by Brakke and Van Pelt (3). Cowpea mosaic virus (CPMV), bean pod mottle virus, tobacco mosaic virus, and Panicum mosaic virus were used as standards. Extracted RNAs of CPMV and brome mosaic virus (BMV) served as RNA standards. Particle density was estimated by equilibrium centrifugation in cesium chloride (7). The volumes were modified for the IEC 460 fixed-angle rotor; 2.5 ml of cesium chloride in 0.05 M phosphate buffer (1.2 density) containing the virus was layered onto 2.5 ml of cesium chloride solution (1.5 density). The cesium chloride was then overlayed with mineral oil to fill the tube. After centrifugation at 225,000 *g* at 4 C for 36 hr, the gradients were fractionated by carefully lowering a 20-gauge needle attached to a 1.6-mm ID tubing to the bottom of the centrifuge tube. Fractions of three drops (about 80 μl) were collected by means of a peristaltic pump at room temperature. A 10μl sample was taken immediately for determination of the refractive index from which the density of the fraction was determined using the method of Ifft et al (5). Another 50μl sample of the fraction was taken, diluted with 2 ml of phosphate buffer, and the A_{260} determined. Infectivity was determined on alternate fractions after they had been diluted in 2 ml of phosphate buffer.

Serology. Antiserum to BdMV-K was prepared by injecting two rabbits intramuscularly three times at weekly intervals with 1 mg of virus in 1 ml 0.02 M phosphate buffer. Antiserum also was

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prepared against BdMV-I in a similar manner. Agar double diffusion and intragel-specific adsorption tests were made in plates containing 0.75% Ionagar No. 2 and 0.02% NaN_3 (1).

Electron microscopy. Purified preparations of BdMV-K were placed on carbon-coated Formvar grids and negatively stained with either 2% phosphotungstic acid or with a uranyl formate stain prepared by mixing 5 mg of uranyl formate in 1 ml of water, adding 50 μl of concentrated formic acid, and centrifuging at 10,000 *g* for 5 min. The clear amber supernatant was used. The grids were observed on a Phillips 201 electron microscope.

Electrophoresis. The electrophoretic forms of BdMV-K were separated on cellulose polyacetate, as described by Niblett and Semancik (15), with Sepraphore III and either High Resolution buffer (Gelman Instrument Company, Ann Arbor, MI) diluted to 0.02 M, or a series of buffers that were 0.03 in veronol, sodium phosphate, or sodium acetate (depending on the pH desired), which also contained 0.02 M NaCl. Samples (5–10 μl containing 50–100 μg of virus) were applied to the center of the Sepraphore III strip and a constant potential of 250 V was applied for 25 min at room temperature. The strips were cut in half longitudinally, and one half of each strip was fixed and stained in 7% acetic acid containing 0.05% nigrosin. The stained strip was then matched to the unstained half and the areas corresponding to virus bands were cut out and eluted in 2 ml of 0.02 M phosphate buffer. Eluates were assayed for infectivity on pepper and checked serologically for reaction with BdMV antiserum.

RNA extraction. Viral RNA was extracted as described by Brakke and Van Pelt (2) with 0.2 M ammonium carbonate buffer, pH 9.0, containing 0.2% sodium diethyldithiocarbamate, 0.002 M ethylenediamine tetra acetate (EDTA), 100 μg/ml of crude bentonite, and water-saturated phenol.

Amino acid analysis. Virus samples (1.0 mg) were mixed with an equal volume of 12 N HCl and hydrolyzed in sealed, nitrogen-filled tubes for 20, 48, and 72 hr at 110 C. The acid was removed in a rotary evaporator, and the residue was dissolved in 0.2 M sodium citrate buffer, pH 2.2, and analyzed on a Beckman model 120 C amino acid analyzer equipped with an automatic integrator.

Base ratios. Base composition was determined by acid hydrolysis and paper chromatography as described by Knight (8) and by the Randerath procedure (14,20). RNA analyzed by the Randerath procedure previously had been collected from sucrose density gradients (3) and extensively dialyzed in deoinized water. BMV RNA was the control.

Seed transmission. Seeds from Chinese lantern plants, *Physalis alkekengi* L., *D. stramonium,* and pepper plants that were infected with either BdMV-K or BdMV-I were planted. The seedlings were checked visually at the four-leaf stage for virus symptoms, then ground at a ratio of 1 g of tissue per 2 ml of 0.02 M phosphate buffer, and the homogenate then was checked serologically in agar double diffusion tests for the virus.

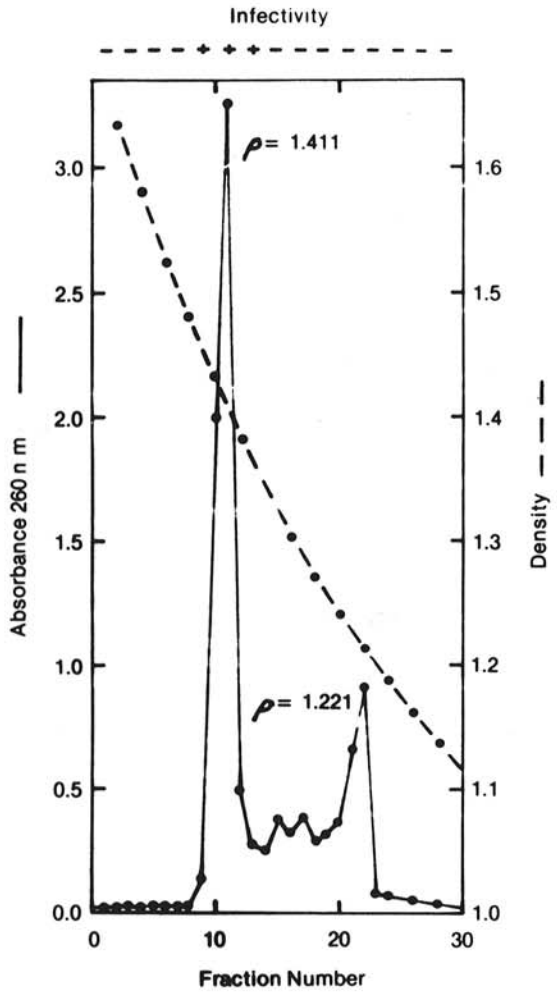

Fig. 1. Absorbance and infectivity of fractions of the Kansas strain of the belladonna mottle virus (BdMV-K) collected after cesium chloride equilibrium centrifugation.

RESULTS

Host range. At 5–7 days after inoculation, BdMV-K produced large indistinct lesions resembling a yellow mottle in *D. stramonium; Physalis floridiana* Rybd.; *P. alkekengi; Cyanopsis tetragonolobax* (L.) Taub.; *C. frutescens* 'California Wonder,' 'Hybrid Tokyo Bell,' 'Tabasco,' and 'Yolo Wonder;' and *Vinca rosea* L. At 17–21 days after inoculation, *N. glutinosa* showed similar symptoms which were expressed 10–14 days later than in the above hosts. *Centaurea cyanus* L. was a symptomless host. The following plants were not infected by BdMV-K: *Cucurbita maxima* Dcne. 'Acorn,' 'Butternut,' and 'Zucchini;' *Citrullus vulgaris* Schrad. 'Charleston Gray;' *Cucumis sativus* L. 'National Pickling;' *C. melo* L. 'Hood's Heart of Gold' and 'Rockford;' *Phaseolus vulgaris* L. 'Bountiful,' 'Roger's Cherokee Wax,' 'Cherokee Wax,' 'Henderson Lima,' 'Black Valentine,' 'Kentucky Wonder Pole,' 'Michigan Navy,' and 'Pinto;' *Glycine max* (L.) Merr. 'Wayne' and 'Clark 63;' *Pisum sativum* L. 'Dwarf Gray Sugar,' 'Landreth's Green Pod,' and 'Laxton's Progress;' *Lycopersicon esculentum* Mill. 'Rutgers;' *N. tabacum* L. 'Hicks,' *Xanthi* NC,' and *N. rustica* L.; *N. clevelandii; Brassica campestris* var. *napobrassica* (L.) DC.; *Beta vulgaris* L.; *Pastinaca sativa* L.; *Proboscidea louisiana* (Mill.) Thell.; *Allium cepa* L.; *Vigna unguiculata* (L.) Walp. 'Early Ramshorn;' *Chenopodium quinoa* L.; *C. amaranticolor* Coste and Reyn.; and *C. murale* L.

Several differential hosts for BdMV-K and BdMV-I were found (Table 1). BdMV-I infected *N. clevelandii, N. rustica, N. tabacum* L. 'Xanthi NC' and *L. esculentum*; but BdMV-K did not. *V. rosea* was a host for BdMV-K but not for BdMV-I. Although comparisons were limited, BdMV-K does not infect *N. clevelandii, N. tabacum* L., 'Xanthi NC,' or *L. esculentum,* but BdMV-type does (17).

TABLE 1. Differential hosts for strains of belladonna mottle virus (BdMV)

	BdMV strain		
	Kansas[a]	Iowa[a]	Type[b]
Capsicum frutescens	+	+	+
Datura stramonium	+	+	+
Lycopersicon esculentum	−	+	+
Nicotiana clevelandii	−	+	+
Physalis floridiana	+	+	ND[c]
Nicotiana glutinosa	+	+	+
Nicotiana rustica	−	+	ND
Nicotiana tabacum L., Xanthi NC	−	+	+
Vinca rosea	+	−	ND

[a] Determined concurrently under identical conditions.
[b] Reported by Paul et al (17).
[c] Not determined.

The host ranges of BdMV-K and BdMV-I were determined by using infected *N. glutinosa* leaves and infected pepper leaves to see if the virus inhibitor reported from pepper leaves (12) had any effect on the host range of either of the BdMV strains. The host range was the same whether infected pepper or *N. glutinosa* leaves were used as the source of inoculum.

In vitro properties. The TIP for BdMV-K is between 75 and 80 C. The DEP is 10^{-7}. The LIV at room temperature is between 14 and 21 days; at 4 C, infectivity was lost between 4 and 5 mo. Our values are similar to those reported for BdMV-type (17) and BdMV-I (13). The BdMV-K strain attains high concentrations in plant tissues; it yields more than 200 mg of purified virus from 100 g of virus-infected pepper leaves 21 days after inoculation.

Density gradient centrifugation. In linear-log sucrose gradients, BdMV-K sediments as two components. Sedimentation coefficients were estimated at 53S for the T component and at 109S for the B component. Only the B component was infectious. The $A_{260/280}$ (not corrected for light scattering) ratios of T and B were 0.89–0.91 and 1.72–1.75, respectively. The $A_{260/280}$ ratio of unseparated BdMV-K was 1.52–1.54. The absorption maximum for BdMV-K was 259 nm; the minimum was 245 nm. RNA was obtained only from B component. It sedimented at 26S on linear-log sucrose gradients and had maximum and minimum absorption at 259 and 235 nm, respectively. The extracted nucleic acid was confirmed as RNA by its susceptibility to RNase, resistance to DNase, and by hydrolysis in 0.3 N KOH at 37 C for 18 hr. The densities of the T and B components of BdMV-K were 1.221 and 1.411, respectively, as determined in cesium chloride equilibrium gradients. Infectivity was associated only with the B component (Fig. 1) and its RNA. Similar properties were reported for BdMV-I (13).

Serology. In injected rabbits, BdMV-K elicited a strong immune response. Titers reached 1/320 in agar double diffusion tests at an antigen concentration of 500 μg/ml. Both T and B components were serologically active although antiserum was produced only against B component. In agar double diffusion tests, when either BdMV-K or BdMV-I antiserum was placed in the center well and BdMV-K and BdMV-I antigens were placed in the surrounding wells, one homologous precipitin band formed with no spur formation. There was no serological reaction with healthy plant extracts and no reaction of BdMV with normal serum collected from the rabbits before injection of the antigen. The intragel-specific adsorption performed with BdMV-K and BdMV-I antiserum and antigens in all possible combinations indicated that the two virus strains were the same serologically. The BdMV-I antiserum (ATCC PVAS 82) has been reported to be distantly related to BdMV-type strain (13).

No reaction occurred with BdMV-K or BdMV-I tested against the following antisera: turnip yellow mosaic, southern bean mosaic, elm mosaic, cucumber mosaic, cowpea mosaic, tobacco ringspot, Panicum mosaic, brome mocaic, tobacco mosaic, and wheat streak mosaic.

Electron microscopy. Measurements of negatively stained purified virus preparations gave an average diameter of 27 nm for the isometric virus particles of BdMV-K.

Electrophoresis. When analyzed on Sepraphore III strips, both BdMV-K and BdMV-I separated into electrophoretic forms that migrated toward the cathode between pH 5.0 and 10.0 (Fig. 2). Two forms were found in Kansas and Iowa isolates of BdMV isolated from *Datura,* pepper, *N. glutinosa,* and *Physalis.* CPMV, which was run simultaneously, also had two electrophoretic forms, but they carried a net negative charge (as do most plant viruses) and migrated toward the anode during electrophoresis. Both

TABLE 2. Base compositions of RNA from the Kansas strain of belladonna mottle virus (BdMV-K) and brome mosaic virus (BMV)

RNA	Base Compositions[a]			
	Gua	Ade	Cyt	Urd
BdMV-K[b]	19.6	19.6	38.5	22.2
BdMV-K[c]	13.7	20.9	37.3	28.1
BdMV-type[d]	17.5	22.8	32.8	26.9
BMV[e]	27.0	26.4	19.8	26.7
BMV[f]	24.6	25.7	22.2	27.5
BMV[g]	28.0	27.0	21.0	24.0

[a] Molar percentage.
[b] Acid hydrolysis (8); results are the average of seven different hydrolyses on RNA extracted from three different viral purifications. The coefficients of variation are 2.73, 2.56, 2.69, and 4.63% for Gua, Ade, Cyt, and Urd, respectively.
[c] Enzyme hydrolysis (19); results are the average of two different hydrolyses on RNA extracted from one viral purification. The coefficients of variation are 6.53, 8.01, 3.50, and 5.03% for Gua, Ade, Cyt, and Urd, respectively.
[d] Reported for type strain (15).
[e] Acid hydrolysis (8); results are the average of three different hydrolyses on RNA extracted from two different viral purifications. The coefficients of variation are 2.38, 2.57, 2.98, and 2.54% for Gua, Ade, Cyt, and Urd, respectively.
[f] Enzyme hydrolysis (19); results are the average of two different hydrolyses on RNA extracted from one viral purification. The coefficients of variation are 2.40, 1.23, 1.42, and 2.29% for Gua, Ade, Cyt, and Urd, respectively.
[g] Reported for type strain (10).

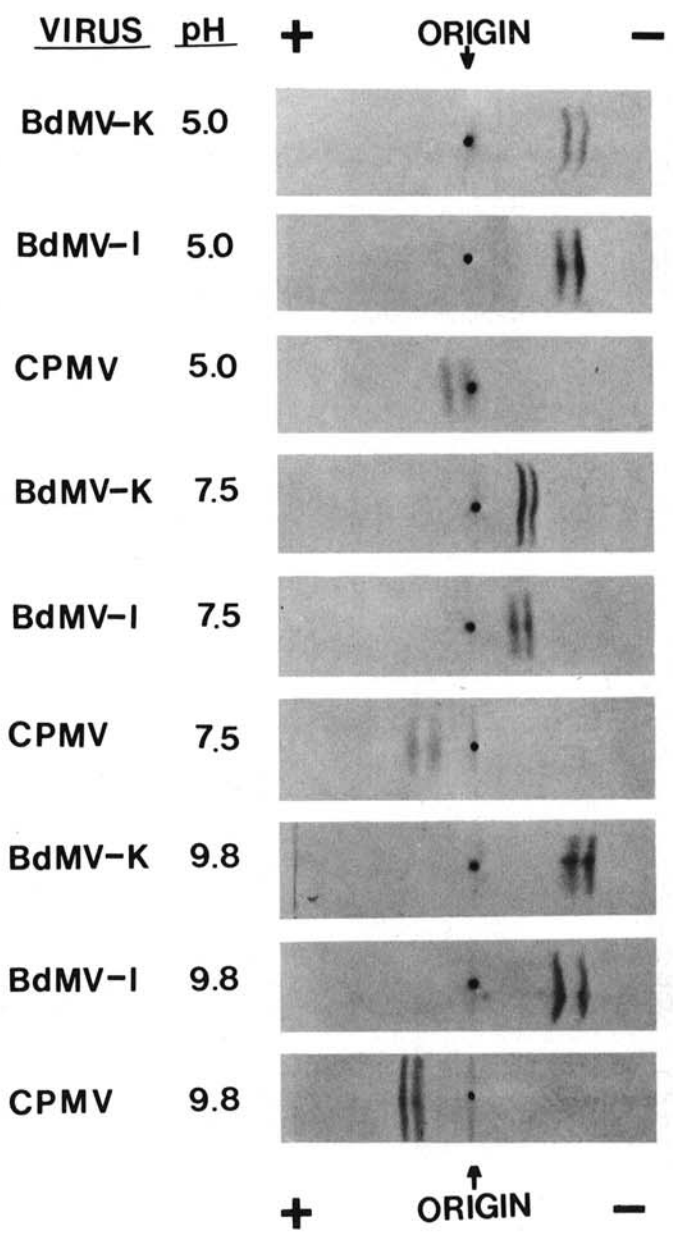

Fig. 2. Electrophoresis of two strains of belladonna mottle virus (BdMV-K, BdMV-I), and cowpea mosaic virus (CPMV) on Sepraphore III cellulose polyacetate strips at various pH levels.

electrophoretic forms of BdMV-K were eluted from the Sepraphore III strips, both were infectious, and both reacted with BdMV-K antisera.

Base ratios. Table 2 shows the base composition of the RNA from BdMV-K. The high cytosine content and low guanine content of the RNA from BdMV-K is characteristic of viruses in the tymovirus group (4). Analysis of BdMV-K RNA by the Randerath procedure gave results similar to those obtained by acid hydrolysis but detected 0.2% of a minor component with chromatography characteristics identical to those of dihydrouridine. Base ratios of the BMV RNA, as determined by both procedures, agreed well with those reported (10).

Amino acid analysis. The results of the amino acid analysis of BdMV-K and BdMV-I are given in Table 3. Hydrolysis was performed for 20, 48, and 72 hr with three replications run for each hydrolysis time. Arginine, aspartic acid, histidine, leucine, lysine, methionine, phenylalanine, serine, and tyrosine recovery increased with hydrolysis time. Values for 72-hr hydrolyses were used. Alanine, glycine, isoleucine, proline, threonine, and valine recovery decreased with hydrolysis time. Values for these amino acids were obtained by extrapolating back to zero time hydrolysis by regression analysis (19). Cystine and tryptophan were not determined. The amino acid compositions of BdMV-K and BdMV-I are similar, and the relative amounts of each amino acid are similar to the values reported for BdMV-type strain (16). The greatest difference in amino acid compositions of BdMV-K and BdMV-I occurs in isoleucine, but smaller differences also occur in arginine, glycine, serine, and threonine. Apparently, these differences are too small to allow the two strains to be differentiated serologically.

Seed transmission. No symptoms were seen and no virus was detected serologically on 203, 110, and 35 seedlings grown from seed of BdMV-K infected Chinese lantern, pepper, and *Datura* plants, respectively, or on 185 and 27 seedlings grown from seed of BdMV-I infected Chinese lantern and *Datura* plants, respectively.

DISCUSSION

The Kansas and Iowa strains of BdMV are unusual among plant viruses in that they have cathodic electrophoretic heterogeneity. Both BdMV-I (13) and BdMV-K also have two centrifugal components with sedimentation coefficients of 54S and 114S, and 53S and 109S, respectively. Several other tymoviruses are cathodic (eggplant mosaic, Andean potato latent, eggplant mosaic-Abelia latent strain, BdMV-type strain, dulcamara mottle, Scophularia mottle, wild cucumber mosaic, and okra mosaic viruses), but all have a single electrophoretic form (9). The cathodic electrophoretic heterogeneity of BdMV-K and BdMV-I was also confirmed by polyacrylamide gel electrophoresis (R. F. Lee, *unpublished*). The nature of the cathodic electrophoretic heterogeneity was not determined. The 53S component of BdMV-K is presumed to be the empty virus capsid, based on its low $A_{260/280}$ ratio (0.90), its density (1.221), its lack of infectivity, and its lack of extractable RNA. The 109S component of BdMV-K must represent the complete virion; based on centrifugal analysis, it contains a single 26S RNA component.

The BdMV-K possesses many of the properties characterisitic of other BdMV isolates (13,16,18) and of other tymoviruses (4,9). The TIP, LIV, and DEP are comparable to those of other strains of BdMV (6,13,17,18). The high cytosine and low guanine content of the RNA (4,6,18) and the two centrifugal components (4,9) are characteristic of these viruses. The BdMV-K strain appears serologically identical to the BdMV-I strain that is distantly related serologically to BdMV-type strain (13). The amino acid compositions of BdMV-K and BdMV-I are similar and resemble that reported for BdMV-type strain (16).

However, BdMV-K may be differentiated from other strains of BdMV by its host range (Table 1). The BdMV-K strain does not infect *N. clevelandii, N. tabacum* 'Xanthi NC,' or *L. esculentum,* although other strains of BdMV do. The BdMV-K strain infects *V. rosea,* but the BdMV-I strain does not. The BdMV-I strain infects *N. rustica,* and the BdMV-K strain does not.

The BdMV-K strain is prevalent in Kansas. Since its isolation in 1974, it has been isolated from Chinese lantern and pepper 10 times within a 40-km radius of Manhattan, KS. One isolation was from a pepper plant in a commercial nursery. All isolates from Kansas were identified as BdMV-K by their reaction with BdMV-K antiserum and their ability to infect *N. glutinosa* but not *N. rustica.*

The mechanism by which BdMV-K is spread is not known. We found no evidence of seed transmission in the hosts tested, nor was seed transmission found for the type strain (16). The virus has the potential for becoming widespread, as *Physalis* is a perennial weed throughout the Midwest. No information is available on vectors in Kansas, but the flea beetle, *Epithrix atropae* is a vector in Europe (16).

TABLE 3. Amino acid composition of the Kansas (BdMV-K), Iowa (BdMV-I), and type (BdMV-type) strains of belladonna mottle virus

Amino acid	Mole % Amino Acid		
	BdMV-K	BdMV-I	BdMV-T[a]
Ala[b]	11.56	11.14	8.50
Arg[c]	3.81	4.42	2.51
Asp[c]	7.66	7.27	5.62
Glu[b]	8.33	8.55	8.79
Gly[b]	5.64	6.30	6.86
His[c]	1.40	1.46	0.00
I leu[b]	8.85	7.29	9.10
Leu[c]	8.23	8.19	9.46
Lys[c]	5.26	5.05	4.90
Met[c]	2.25	2.43	0.96
Phe[c]	2.33	2.33	2.63
Pro[b]	8.95	9.11	8.26
Ser[c]	8.51	8.05	12.54
Thr[b]	7.65	8.32	8.38
Tyr[c]	2.22	2.38	2.07
Val[b]	7.35	7.71	7.67
Cys	...[d]	...[d]	1.14
Try	...[d]	...[d]	0.59

[a] Reported values for BdMV-type strain (6).
[b] Values extrapolated to time zero.
[c] Values for 72-hr hydrolysis.
[d] Not determined.

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