Purification and Partial Characterization of Maize Dwarf Mosaic Virus Strain A

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ABSTRACT

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Maize dwarf mosaic virus strain A (M-A) was extracted by blending infected leaf tissue in pH 9.0 buffer, precipitating the virus with 4% polyethylene glycol (PEG) in 0.3 M NaCl, and purifying it by centrifugation in the rate-zonal or quasi-equilibrium gradients. Purity was determined by UV absorption spectra and gel electrophoresis. M-A yields from fresh infected tissue were 10–20 μg/g. Virus coat protein migrated as a single component in sodium dodecyl sulfate (SDS) polyacrylamide gels with apparent molecular weights of 30,700 or 35,500 from untreated or carboxymethylated preparations, respectively. A minimum molecular weight of 29,137 was determined by amino acid analysis based on seven histidine residues per subunit. Major differences in number of amino acid residues per subunit between M-A and M-B (maize dwarf mosaic virus strain B), respectively, were glutamic acid 37 and 29, glycine 18 and 34, lysine 22 and 12, serine 15 and 20, and threonine 19 and 25.

Additional key words: protein, amino acids, polyacrylamide gel electrophoresis.

Maize dwarf mosaic virus, a potyvirus, consists of several strains (10) of which A and B are best characterized. Strain A (M-A) (15) infects Johnsongrass (*Sorghum halepense* L.) but strain B (M-B) (11) does not. Both are related serologically to some strains of sugarcane mosaic virus (16). Symptom expressions of the host to infection by these two strains differ enough that infection by M-A is considered by plant pathologists to be more severe than that induced by M-B (5). Only M-B has been chemically analyzed, although yields of purified virus were low (7). Failures with attempts to use the same, or similar purification schemes for all strains, indicate that each strain requires different manipulations (8,17). Polyethylene glycol (PEG) aids purification of "low-yield" viruses (6,16). We were unsuccessful in purifying M-A by using the published methods. Purified M-A was obtained by a modification of the method of Hill et al (7) and a partial biochemical characterization was made. Preliminary reports have appeared (19,20).

MATERIALS AND METHODS

Virus origin, preparation, and purification. The virus isolates were strains A and B of maize dwarf mosaic virus. M-A was obtained from Johnsongrass in Illinois and M-B (isolate ATCC-53) was obtained from the American Type Culture Collection, Rockville, MD.

Sweet corn plants (*Zea mays* L. 'Golden Bantam') grown in steamed composted soil were inoculated mechanically by hand at the two-leaf stage and harvested in batches of 0.5-1.0 kg. The following description adds some detail to the purification procedure given in Fig. 1. Infected leaves were cut into 1.27-cm (1/2-inch) lengths and placed in the buffer (1:4, v/v) in preparation for blending. After the first low-speed centrifugation the pH was adjusted to 7.0 with 1 N HCl or NaOH as necessary, followed by the second low-speed centrifugation, usually within 30 min of the addition of PEG. Whereas the ideal pH for resuspension of the pellet of M-A at this stage is 7.8–8.0, that for M-B is 7.4. Two alternate purification routes can be followed depending upon preference or availability of sucrose and CsCl. Sucrose density gradients (10–40%) were equilibrated for 18 hr after layering. After rate-zonal centrifugation a light-scattering zone 4 cm below the meniscus was removed with a bent needle.

M-B was purified in the same manner as M-A except: the grinding buffer was pH 8.5; 6% PEG was used; and the EDTA-phosphate resuspending buffer was pH 7.4.

After removal of CsCl by dialysis against phosphate buffer, virus concentration was estimated spectrophotometrically using the extinction coefficient ($E^{0.1\%}_{261\ nm} = 2.4\ cm^2\ mg^{-1}$) for tobacco etch virus (7). Infectivity index was calculated by the method of Hill et al (7).

Preparation of viral components. *Protein.* M-A and M-B proteins were prepared by dialyzing purified virus against 0.5 M urea, 50 mM sodium phosphate, pH 7.6, and disrupting the virus by freezing overnight in the presence of 2 M LiCl (3,4). After thawing, the precipitated RNA was removed by centrifuging at 10,000 rpm (12,000 *g*) in a Sorvall SS-34 rotor. The supernatant liquid containing the protein was centrifuged at 40,000 rpm (105,000 *g*) for 1.5 hr in a Beckman Ti 40 rotor to remove undissociated virus. The supernatant containing only protein was analyzed on a GCA-MacPherson series 700 spectrophotometer dialyzed against 50 mM sodium phosphate buffer, pH 7.6, and stored at 4 C. Reduced carboxymethylated protein was prepared as described for M-B (7). Amino acid and tryptic peptide analyses were done only after it was determined the protein was pure according to the system of Hill et al (7).

RNA. RNA was recovered by heating the virus with SDS (sodium dodecyl sulfate) at 50 C for three min, scavenging the SDS with 50% (w/v) sodium perchlorate, and precipitating the RNA with cold ethanol and washing it twice with absolute ethanol (22). Alternatively, both RNA (3) and protein (4) were recovered by using a modification of the LiCl method. The low-speed RNA fraction was resuspended in cold distilled water and the RNA was purified by several precipitations in cold absolute ethanol.

Polyacrylamide gel electrophoresis of virus protein. Coat proteins of M-A and M-B were examined in SDS polyacrylamide gel electrophoresis experiments as described by Weber and Osborn (21). Purified virus was concentrated by centrifugation, then resuspended in 0.01 M sodium phosphate buffer pH 7.8 containing 1% SDS, 1% 2-mercaptoethanol, and 20% glycerol. Samples were heated in a boiling water bath for 1 min to disrupt the virus. Treated virus samples (10–100 μl) were layered on 8 cm cylindrical 7.5% polyacrylamide gels with 0.195% *bis*-acrylamide. Samples were electrophoresed at 2 mA per gel for 20 min, then at 6 mA per gel for 5 hr. Gels were stained overnight in 1% Coomassie Brilliant Blue and 7% acetic acid in 50% ethanol, then destained in 50% ethanol and 7% acetic acid for 24–48 hr. Before measurements, gels were swollen to original size by equilibration in 7% acetic acid. For routine examination to assess the purity of virus preparations, gels were overloaded with 30–50 μg of purified virus in order to detect

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minor host contaminants. For molecular weight estimation, smaller loadings of 5–15 μg in 10–30 μl volumes were used to produce a thin sharp band, suitable for scanning and recording migrating distance. Standard markers were commercially available proteins representing a range of mol wt (bovine serum albumin = 67,000; ovalbumin = 43,000; trypsin, treated with diisopropyl-fluorophosphate (DFP) = 23,300; and ribonuclease-A, = 13,800). In some experiments, marker proteins and maize dwarf mosaic virus protein were reduced and carboxymethylated (2).

RESULTS

Purification. The effect of chloroform in the clarification treatment on final yield of M-A was determined by infectivity dilution curves. Assigning 100% yield to the 5% chloroform treatment, yields by comparison were 95% from 10% chloroform, 76% from 4% chloroform plus 4% butanol, 47% from 5% butanol, and less than 25% from 5 or 10% ethanol treatments.

The pH of infective sap after grinding in the initial pH 9.0 buffer was 7.0 for M-A and 6.5 for M-B, both remaining unchanged after addition of NaCl and PEG.

Contamination of M-A and M-B by host components was markedly affected by varying levels of NaCl (Fig. 2). Contamination of M-A was detected at 0.3 M NaCl but considerably more of it was evident at 0.4 M NaCl; no such contamination of M-B was detected even up to 0.5 M NaCl. Average virus yields in micrograms per gram fresh weight from three experiments for M-A were 4.4 and 4.7 in 0.3 and 0.4 M NaCl, respectively; for M-B they were 5.3, 7.8, 9.8, and 6.8 in 0.2, 0.3, 0.4, and 0.5 M NaCl, respectively.

Yields of M-A and M-B were optimal in 6% PEG treatments. However, some contamination was observed in SDS gels of protein prepared from M-A at PEG concentrations of 6 and 8%, but not at 4%.

The sequence of rate-zonal gradient centrifugation followed by quasi-equilibrium centrifugation in sucrose density gradients yielded relatively pure viral material, although M-A (A260/A280 = 1.25) still contained slightly more absorbing background than did M-B ($A_{260/280}$ = 1.20). Yields were 10–12 (M-A) and 6–10 (M-B) μg/g fr. wt. of infected leaves.

Purification with CsCl gradients was successful only after concentrating the virus through 30% sucrose in the absence of EDTA. Contaminants were easily removed with two or three successive CsCl centrifugations. Virus yields (μg/g fr. wt. of infected sweet corn leaf tissue) were 15–20 for M-A and 12–14 for M-B with values for $A_{260/280}$ of 1.19 and 1.20, respectively.

UV absorption. The absorption spectra for both M-A and M-B

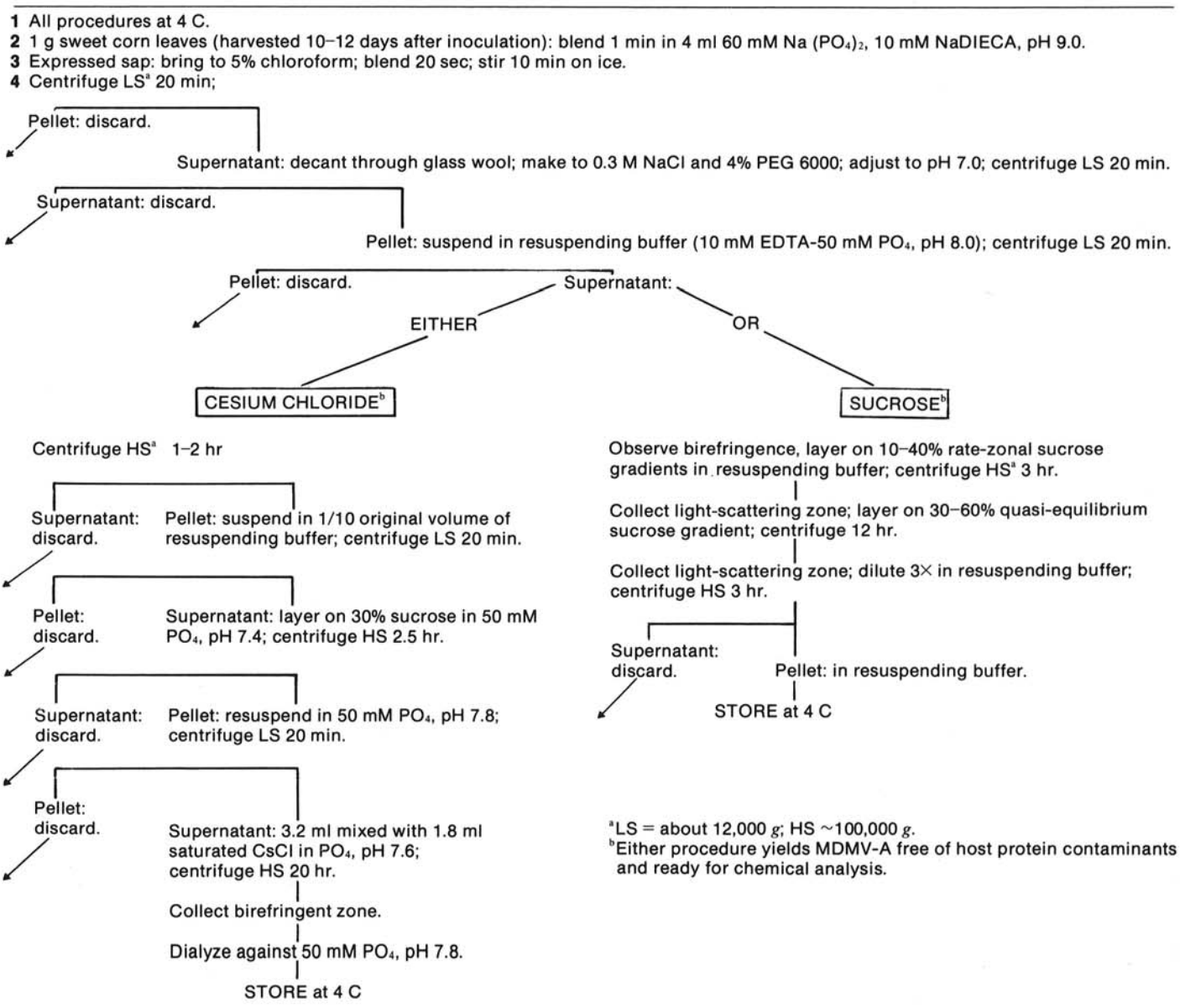

Fig. 1. Flowchart of the procedures for purification of maize dwarf mosaic virus strain A from systemically infected sweet corn leaf tissue.

were typical of the potyviruses: maximum absorption at 262 nm, minimum absorption at 246 nm, and a tryptophan shoulder at 288 nm. The average value for the $A_{260/280}$, corrected for light scattering, indicated an RNA content of slightly over 5%.

In preparation for analyses of the separated viral components we recovered about one-third of the RNA and two-thirds of the protein from the original purified samples by employing several published and widely used methods (Table 1). Yields of RNA and protein were estimated by UV absorbance by using $E^{0.1\%}_{260\ nm} = 25.0$ for RNA and $E^{0.1\%}_{280\ nm} = 1.0$ for protein.

Polyacrylamide gel electorphoresis. The mol wt of M-A protein obtained by following the techniques of Weber and Osborn (21) and Hill et al (7) was estimated to be 30,700; six determinations ranged from 29,500 to 33,000. Since Hill et al (7) used carboxymethylated protein to determine the mol wt of M-B, we also carboxymethylated M-A protein (Fig. 3) and obtained an estimated mol wt of 35,500.

Amino acid composition. We compared the amino acid content of M-A protein with that of M-B and other potyviruses (Table 2). Values calculated for the stable amino acids were averaged from 24 through 72 hr, those for resistant amino acids were skewed toward the 72-hr analysis and those for unstable amino acids were extrapolated to zero time. The best fit for M-A was obtained by using histidine which was present in the lowest concentration, basing this value as a minimum residue of one, then multiplying by integer values until the mol wt approximated that determined from the gel data. Histidine = seven gave the best fit.

A minimum mol wt of 29,131 was determined by calculating the contribution of the various amino acids; this estimated mol wt compares favorably with the mol wt of 30,700 which was determined by the relative migration with known protein markers. The most notable differences between M-A and M-B proteins, respectively, were glutamic acid 37 and 29, glycine 18 and 34, lysine 22 and 12, serine 15 and 20, and threonine 19 and 25.

The tryptic peptide digests (Fig. 4) generally confirmed these differences and compared favorably with that of Hill et al (7) for M-B protein. M-B protein produced 24–25 ninhydrin-positive spots, 8–10 positive for arginine and one for tryptophan. M-A protein produced 30–31 ninhydrin-positive spots, 11–13 positive for arginine and one for tryptophan.

DISCUSSION

The purification procedure used here, although similar to others (1,7,9,12,14,16), incorporates NaCl and alters pH and PEG concentrations to enable purification of both M-A and M-B.

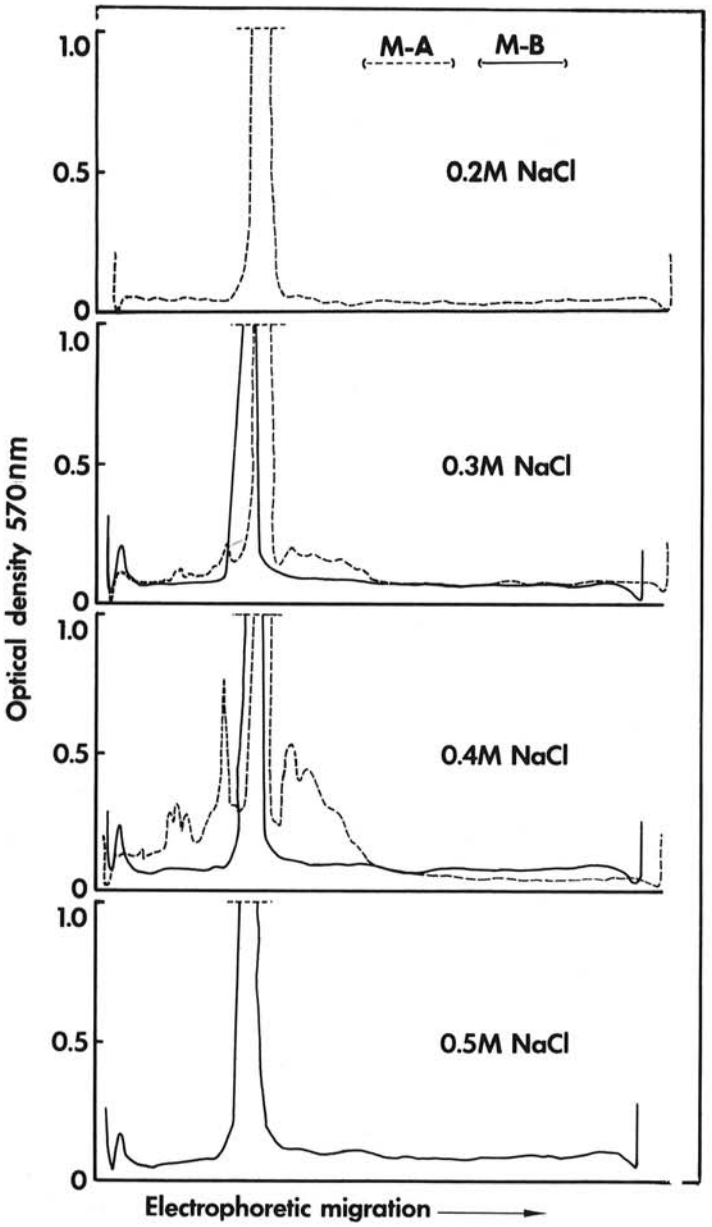

Fig. 2. Electrophoretic migration profiles of maize dwarf mosaic virus strain A (M-A) and strain B (M-B) protein on sodium dodecyl sulfate polyacrylamide gels following NaCl treatments. Equal portions of clarified sap from virus-infected tissue were adjusted with NaCl prior to precipitation with PEG, then purified (M-A at pH 7.0 and M-B at pH 6.5). Gels were scanned in a GCA-MacPherson spectrophotometer with a 0.2-mm slit at 570 nM. Gels were overloaded (~30–50 μg) to detect minor contaminants and read on a 2 OD scale. The primary peak represents M-A or M-B protein and the minor peaks, contaminating host protein.

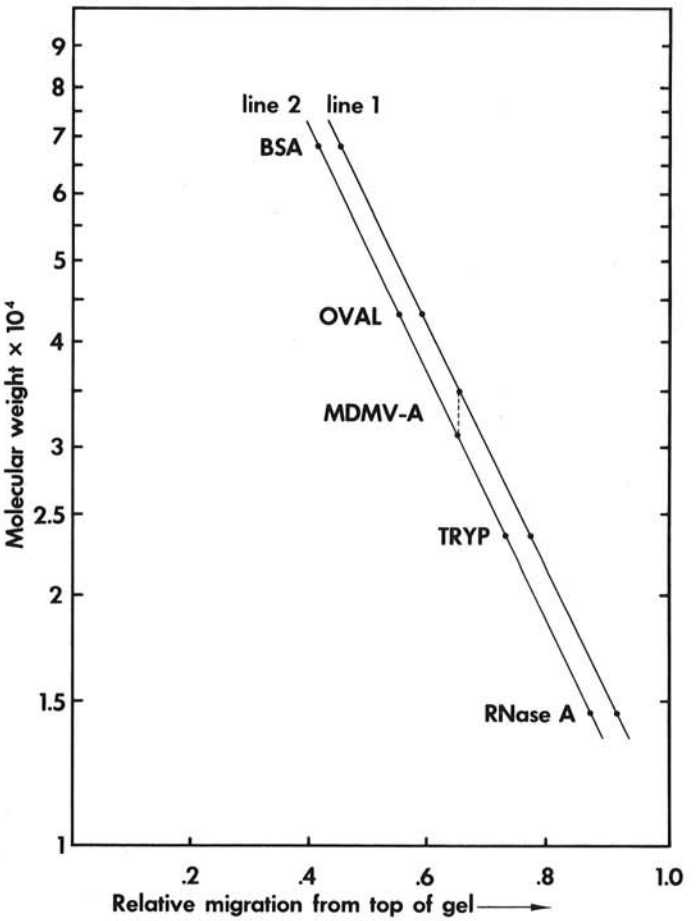

Fig. 3. Migration of carboxymethylated (*line one*) and noncarboxymethylated (*line two*) maize dwarf mosaic virus strain. A protein and protein standards on sodium dodecyl sulfate polyacrylamide gels (7.5% polyacrylamide plus 0.195% *bis*-acrylamide) electrophoresed at 6 mA for 5 hr. Standards and molecular weight were BSA (bovine serum albumin 67,000), OVAL (ovalbumin 48,000), TRYP (trypsin DPF [diisopropylfluorophosphate-treated] 23,300), and RNase A (ribonuclease A 13,700).

Selection of young infected leaves harvested 10–12 days postinoculation (18) and the use of a large extraction volume (4 ml/g fr. wt.) maximized yields of both strains. M-A was more difficult to purify than M-B.

Many combinations and concentrations of organic solvents have been tested to aid clarification of host extracts. Sehgal (13) found chloroform to markedly reduce infectivity of M-A; however, Tosic et al (17) and we found chloroform necessary to remove pigments and proteinaceous contaminants; 5% chloroform proved to be optimal for our purposes. Blending the mixture of sap from M-B infected plants and chloroform for 20 sec at high speed saved time, but we found no other advantages over emulsification by gently stirring for 30 min. However, blending was necessary to aid removal of proteinaceous material from M-A. Triton X-100 apparently facilitates separation of virus from chloroplast membranes (9), but that treatment in our experiments carried along more contaminant host proteins with the virus.

Generally, the darkness of the brown-colored supernatant was positively correlated with the loss of virus during purification. Sap containing NaDIECA remained light yellow and infectivity was preserved better than with 2-mercaptoethanol. Ten mM NaDIECA was adequate for young tissue; for older leaf tissue 20–30 mM NaDIECA was required to prevent oxidation. Phosphate buffers were used throughout since they preserve the highest infectivity(13).

Both low- and high-pH buffers have been used during extraction to assist rapid clarification. We prefer high-pH treatment since it enables extraction of more virus from infective sap than when it was adjusted to pH 4.7 (17). Sap from M-B infected plants was usually about 0.5 pH unit lower than sap from M-A infected plants when both plants were ground in an identical buffer. The initial

TABLE 1. Yield of nucleic acid and protein components from purified maize dwarf mosaic virus strains A and B after various preparation methods

Method	Virus strain	RNA Ratio $A_{260/280}$	RNA Yield (%)	Protein Ratio $A_{260/280}$	Protein Yield (%)
Phenol[a]	A	2.0	12	1.5	26
Phenol[b] single phase	A	0	0	0	0
Urea/SDS[c]	A	1.9	19	1.3	41
	B	1.5	37	1.4	98
LiCl-PO_4[d]	A	1.5	21	1.3	54
	B	1.8	42	1.4	69
LiCl-freeze 2×	A	...[e]	...	1.7	68
LiCl-Urea pH 7.0[f]	A	1.4	27	1.6	70
	B	1.9	80	1.6	90
Sodium perchlorate	A	2.0	36	..[g]	...

[a] Average of four experiments.
[b] M-A precipitated in three experiments.
[c] Wu and Bruening. 1971. Virology 46:596-612 with and without urea.
[d] Both at pH 7.0 and 7.5.
[e] Not measured.
[f] Average of six experiments.
[g] Protein is not recovered in the perchlorate method.

TABLE 2. Amino acid composition of the proteins of maize dwarf mosaic virus strain A (M-A) based on histidine = 7, of strain B (M-B) based on methionine = 11, compared with those of other potyviruses

Amino acid	M-A μ-moles amino acid/mg amino acids value used[a]	M-A Contribution to molecular weight[b]	Relative molar ratios M-A	M-B	M-B[d]	Tobacco[e] etch virus	Turnip[f] mosaic virus
Alanine	.301	1635	23	25	23	19	17
Arginine	.190	2187	14	14[c]	14	13	17
Aspartic acid	.337	2993	26	27	27	25	29
Cysteine	...	103	1[c]	1	1	1	1
Glutamic acid	.486	4777	37	32	29	23	23
Glycine	.231	1028	18	34	34	13	15
Histidine	.091	960	7	5[c]	5	6	8
Isoleucine	.083	792	7	9	8	5	11
Leucine	.178	1586	14	14	13	13	20
Lysine	.303	2944	22	12[1]	12	10	13
Methionine	.139	1443	11	11	11	10	10
Phenylalanine	.099	1178	8	8	7	5	9
Proline	.107	777	8	12	10	8	9
Serine[h]	.200	1305	15	25[g]	20	9	10
Threonine[h]	.241	1921	19	23	25	13	16
Tryptophan[h]	...	745	4[1]	4	4	2	3
Tyrosine[h]	.118	1469	9	10	9	7	8
Valine	.163	1288	13	12	12	12	12
Totals		29,131	256	278	264	194	231

[a] Based on the maximum average value of three analyses from 24 or 72 hr hydrolysates.
[b] Each number is the product of the relative molar ratio and the anhydrous molecular weight for the respective amino acid, rounded to the nearest integer.
[c] Assumed value based on Hill et al (7).
[d] Data of Hill et al (7).
[e] Data of Damirdagh and Shepherd (2).
[f] Data of Hill and Shepherd 1972. Virology 47:807-816.
[g] Baseline variation may have caused an exaggerated value.
[h] Values obtained by extrapolation to time zero from the data of 24- and 72-hr hydrolysates.

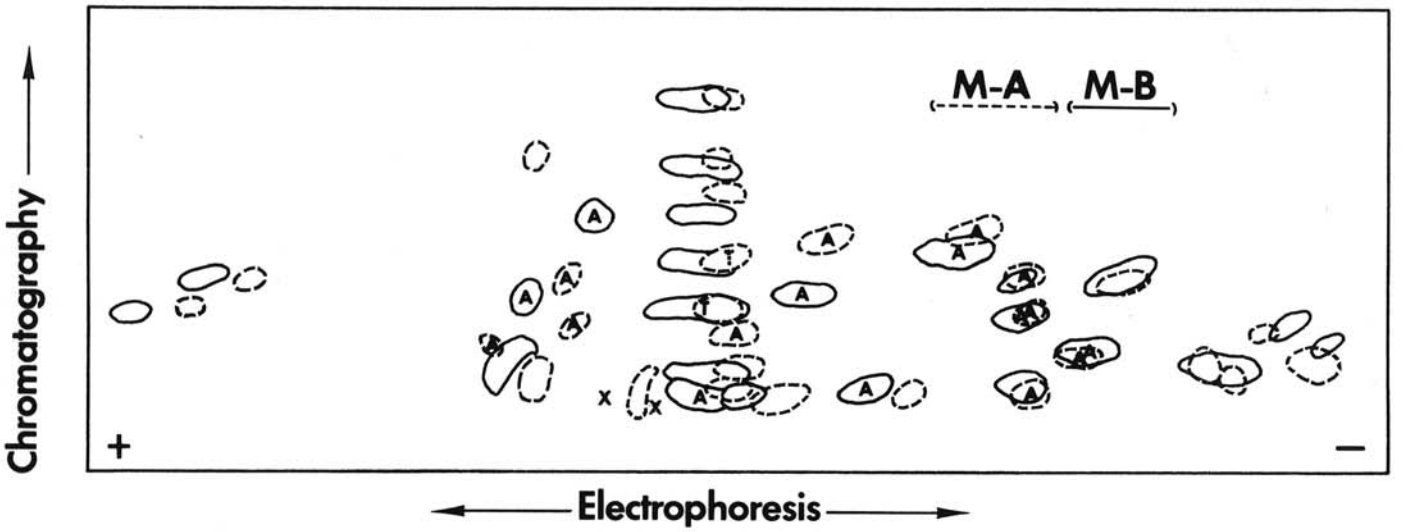

Fig. 4. Tracing of a representative tryptic peptide map of proteins from maize dwarf mosaic virus: ---- = strain A (M-A); —— = strain B (M-B); × = origin; T = tryptophan; and A = positive for arginine by ninhydrin test.

treatment for M-B should be about pH 8.5 rather than 9.0 since M-B was less stable than M-A at high pH. The final yield of M-A was enhanced by the addition of 0.4 M NaCl to the initial, clarified supernatant. However, since this concentration of NaCl induced significant contamination of the final product we obtained a cleaner preparation, but slightly less virus, when 0.3 M NaCl was used. Excessive contamination of M-B preparations from plants grown at higher summer temperatures also was prevented by the use of 0.3 M NaCl.

Most preparations of M-B precipitated by the various PEG concentrations yielded only one protein band in electrophoresis gels, even on overloaded gels. However, M-A consistently contained host protein contamination when either 6 or 8% PEG was used. Therefore, only 4% PEG should be used to ensure contaminant-free virus preparations despite a 12% loss in virus yield compared to that obtained by using 6 or 8% PEG.

Optimal time for 4–8% PEG to precipitate M-A and M-B was 20–30 min. Resuspension of M-B from the PEG pellet in phosphate buffer, pH 7.4, was satisfactory. Based on observations of flow birefringence, resuspension of M-A was optimal at pH 7.0–8.0 and M-B at pH 6.5–7.0. Buffers above pH 7.5 allowed more host contaminants to remain, although the addition of EDTA reduced that problem markedly (R. M. Goodman, *personal communication*). Thus, we recommend 10 mM EDTA-50 mM phosphate at pH 7.4 for M-B and at pH 7.8 for M-A.

Other workers have used two cycles of differential centrifugation before density gradient centrifugation (7,8,13) but Jones and Tolin (8) lost half of the virus at each cycle of centrifugation. We were unable to appreciably reduce host protein contaminants by differential centrifugation, but pelleting the virus through a 30% sucrose cushion aided removal of contaminants before a gradient centrifugation in CsCl.

M-A yields were always higher from CsCl gradients than from sucrose gradients. The CsCl method requires that the sample be purer than that used for sucrose gradients. We found that centrifuging the preparation from a 10–40% sucrose rate-zonal gradient through a 30–60% sucrose quasi-equilibrium gradient removed the remaining contaminants while minimizing aggregation. The width of the M-A band suggested some aggregation in spite of efforts to avoid it. Although passage through sucrose gradients largely solved the contamination problem, the pellets in the bottom of the tubes following rate-zonal centrifugation contained an average of 35% of the virus compared to 10% of the virus in pellets under quasi-equilibrium gradients.

Repeated centrifugations in CsCl gradients resulted in removal of 50–70% of the host contaminants during each cycle while losing only 10% of the virus. M-A could not be recovered from phosphate-EDTA, pH 7.8, CsCl gradients due to instability. When EDTA was eliminated and the pH lowered to 7.6, the CsCl gradient was a successful means of purifying M-A.

We avoided using urea in the buffers (7) because it reduced stability of both M-A and M-B and commonly allowed host contaminants to remain in the final product. Although EDTA was deemed necessary for maximum resuspension of PEG pellets, it was not used in later steps because it seemed to render both M-A and M-B less stable (50–100% losses on CsCl gradients).

When we carboxymethylated the M-B protein in order to compare our results with those of Hill et al (7), the 35,500 mol wt we calculated approximated their published value of 36,500. The carboxymethylated proteins used as standards when plotting and calculating the mol wt for M-A protein each had slightly larger relative mobility values. The carboxymethylation process seems to explain the discrepancy to our satisfaction since we duplicated the techniques, processes, and calculation methods of Hill et al (7). In our SDS-polyacrylamide gel experiments M-A and M-B co-migrated. Thus, we believe that M-A and M-B proteins have a nearly identical mol wt of ~30,700.

Peptide mapping suggests that M-A contains more tryptic peptides than M-B, that histidine = seven is probably correct, and that the number of arginines is close to the number predicted by amino acid analysis.

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