Citrus Tristeza Virus: Characterization of Coat Proteins

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ABSTRACT

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The coat proteins of several aphid-transmitted Florida isolates of citrus tristeza virus (CTV) were characterized by polyacrylamide gel electrophoresis, western blotting followed by serological probing, and polypeptide mapping on sodium dodecyl sulfate polyacrylamide gels following treatment with proteolytic enzymes. All isolates had a major coat protein (CP1) of about M_r 23,000 and a minor coat protein (CP2) of about M_r 21,000, both of which reacted with CTV-specific antisera when using western blot and enzyme-linked immunosorbent assays. The amino acid composition of CP1 was similar to that reported for the coat proteins of other closteroviruses. Polypeptide maps of CP1 and CP2 were similar. In vitro translation of RNA extracted from purified virions produced proteins of various sizes, but only one peptide of about M_r 26,000 was immunoprecipitated with CTV-specific antiserum.

Additional keywords: amino acid analysis.

Tristeza is the most important virus disease of citrus. The disease is caused by citrus tristeza closterovirus (CTV), a phloem-limited, flexuous filamentous virus approximately 2,000 × 11 nm in size (1). There is a need to quickly differentiate between mild and severe isolates of CTV (4,16). Because of the inherent problems of low yields of purified virus, susceptibility of the virus to shearing, and lack of an efficient infectivity assay, the closteroviruses are not well-characterized. Furthermore, CTV has been especially difficult to work with because its hosts are woody perennials.

In recent years, however, several advances have been made that overcome some of these limitations. Mechanical transmission of CTV is now possible (15,22). The virus has been purified (3,17,22) and antisera produced for development of serology-based sodium dodecyl sulfate (SDS)-immunodiffusion (17), enzyme-linked immunosorbent assays (ELISA) (2), and radioimmunosorbent assays (23). Serologically specific electron microscopy (11,22) has been used for qualitative detection of CTV. Inclusion body staining techniques have been developed (12), and partial purification of these inclusion bodies is now possible (21). Virus purification procedures have been improved to yield the amounts of virus necessary for viral characterization and antisera production (3,17,22), whereas other purification procedures have been modified to yield virions with high specific infectivity when mechanically transmitted (15) and for the extraction of CTV RNA for in vitro translation (26). Thus, it is now possible to assess more readily and completely the biochemical properties of the purified virions.

The purpose of this study was to characterize the properties of the CTV coat proteins. The coat proteins were compared from different CTV isolates by polypeptide mapping to determine if CTV isolates could be differentiated. A preliminary report of this work has been published (20).

MATERIALS AND METHODS

Viruses. The T3, T4, T30, and T36 aphid-transmitted isolates of CTV were used. Biological properties of these isolates have been described previously (27).

Virus purification. Tristeza virions were purified by a method similar to that reported by Garnsey et al (15). Bark tissue was stripped from young flush tissue, chopped into fine pieces (about 1–2 mm wide), and added to the extraction buffer (1 g of tissue to 4 ml of buffer) that contained 0.1 M sodium citrate, pH 6.0, with 2% Driselase (Sigma Chemical Co., St. Louis), 10% sucrose, 0.5% 2-mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride. The preparation was shaken at about 200 rpm at 6 C for 1–2 hr. After incubation, the pH was increased to neutrality by addition of 1/200 volume of 3.0 N NaOH. The preparation was frozen at −20 C for at least 1 hr, then thawed, filtered through four layers of cheesecloth, and centrifuged for 10 min at 9,000 g at 4 C. The supernatant was collected, and Triton X-100 was added to a final concentration of 0.2%. Using 25 × 89 mm ultracentrifuge tubes, 27 ml of the virus preparation was layered onto 5 ml of 25% sucrose and 5 ml of 60% sucrose, both of which were in 0.05 M Tris-Cl, pH 8.0. The tubes were centrifuged in a Beckman SW28 rotor for 15 hr at 4 C at 19,000 rpm. At the end of the centrifugation, three fractions of 1.5 ml each were collected from the bottom of the tubes. The CTV, located by ELISA, was concentrated in the second and third fractions. These fractions were pooled and layered onto a 1.5 × 7 cm column of Bio-gel A-15, 100–200 mesh (BioRad, Richmond, CA). The virus eluted in the void volume. One-ml fractions were collected in tubes containing 0.25 ml of 25% sucrose in 0.05 M Tris-Cl buffer, pH 8.0.

The virus containing fractions from the Bio-gel column were pooled and brought to 22.5 ml with 0.05 M Tris-Cl buffer, pH 8.0, then mixed with 17.5 ml of 3 molal Cs_2SO_4 made in 0.05 M Tris-Cl buffer, pH 8.0. This preparation was centrifuged in a Beckman VTi50 rotor for 18 hr at 50,000 rpm at 4 C. The tubes were fractionated after centrifugation by using an ISCO fractionator; the fractions containing CTV, located by ELISA, were dialyzed against 0.05 M Tris-Cl, pH 8.0, containing 10% sucrose.

After dialysis, the virus preparation was centrifuged at 9,000 *g* for 10 min at 4 C. The supernatant was layered onto a preformed step gradient made up of 2 ml each of 0.5, 1.0, and 1.5 molal Cs_2SO_4 in 0.05 M Tris-Cl buffer, pH 8.0. Gradients were centrifuged in a Beckman SW41 rotor at 36,000 rpm for 15 hr at 4 C. The tubes were then fractionated by dripping from the bottom and the opalescent, virus-containing band collected, dialyzed against 0.05 M Tris-Cl buffer, pH 8.0, containing 10% sucrose, and centrifuged

at 8,000 *g* for 10 min at 4 C.

In some experiments, different purification procedures (3,15,17,22) were followed to determine what effect these procedures might have on the apparent size and ratio of viral proteins.

Labeling of proteins. Purified CTV virions were dissociated and labeled with dansyl chloride by using the procedure of Falk and Tsai (14). The dansylated proteins were visualized by viewing over a 302-nm UV transilluminator. Gels containing dansylated proteins were photographed on Kodak Contrast Process Pan film with UA 1 and number 22 orange filters (Kodak, Rochester, NY).

Alternatively, purified CTV virions were dissociated by heating to 50 C for 10 min in Tris buffer with 0.1% SDS, then labeled with ^{125}I by using the chloramine T procedure (24). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gels containing ^{125}I-labeled proteins were dried and fluorograms made as described by Bonner and Laskey (8).

SDS-PAGE. Proteins from dissociated, denatured virions were electrophoresed on 15% polyacrylamide slab gels as described by Bethesda Research Laboratories, Inc. (7). Preparative gels without urea were used for purification of CTV coat proteins and were run at 100 V for 24 hr at room temperature. The polypeptide products of CP1 and CP2 after digestion by proteolytic enzymes were electrophoresed at 70 V for 16–20 hr at room temperature. Bovine serum albumin (66,000 M_r), ovalbumin (45,000 M_r), trypsinogen (24,000 M_r), β-lactoglobulin (18,400 M_r), lysozyme (14,300 M_r), and bovine trypsin inhibitor (6,500 M_r) (all from Sigma Chemical Co.) were used as molecular weight markers. The gels were silver stained using the procedure of Morrissey (25).

Gel pieces containing proteins were placed in a siliconized test tube with 5 ml of distilled water and ground with a Tekmar Tissuemizer. The proteins were eluted from the suspension of gel fragments by shaking them overnight at room temperature. They were filtered through a 0.2 micron filter, and the filtrates were lyophilized in siliconized bottles and stored at −20 C.

Polypeptide analysis of CTV coat proteins. The SDS-PAGE purified proteins labeled with either dansyl chloride or ^{125}I were resuspended in sterile distilled water and dispensed into reaction tubes immediately before digestion by proteolytic enzymes. The enzymes used for polypeptide analysis of CP1 and CP2 proteins were V8 protease, thermolysin (both from Pierce Chemical Co., Rockford, IL), and trypsin. Clostripain, trypsin (both from Sigma), V8 protease, and thermolysin were used to produce polypeptide maps with purified CTV virions. An enzyme-protein ratio of 1:20 was used; reactions were for 30 min at 25 C. V8 protease and thermolysin reactions were run in 50 mM Tris-Cl buffer, pH 7.8; clostripain reactions in 50 mM Tris-Cl buffer, pH 7.6, plus 2.5 mM dithiothreitol; and trypsin reactions in 46 mM Tris-Cl buffer, pH 8.1, plus 11.5 mM calcium chloride. Proteins labeled with either dansyl chloride or ^{125}I before separation of CP1 and CP2 on preparative gels were not relabeled before proteolytic digestion for comparison of the resultant polypeptides by SDS-PAGE.

Protein concentrations of CP1 and CP2 were estimated by the BioRad protein microassay procedure with bovine gamma globulin as a standard. Virus concentrations were estimated by spectrophotometry by using an extinction coefficient of 2.0 $(mg/ml)^{-1}$ cm^{-1} at 260 nm (17).

Serological assays. Western blot analyses of SDS-PAGE gels using ^{125}I-labeled protein A were performed as described by Zabel et al (28) after electroblotting onto diazophenylthioether paper (Schleicher and Schuell, Keene, NH) that was converted from 2-aminophenylthioether paper to the diazo form immediately before use (27). The double-antibody sandwich ELISA procedure (2) was used. Antisera prepared against unfixed CTV (T4 isolate) and SDS-dissociated CTV (T4 isolate) were used (11).

In vitro translation of CTV RNA. RNA was extracted from purified CTV virions by adding double-strength dissociation buffer (10) and 10 μg of proteinase K per milligram of virus and heating the mixture at 60 C for 7 min. This mixture was overlayered onto log-linear sucrose gradients (9), centrifuged in a SW41 rotor for 45 min at 41,000 rpm at 13 C, and fractionated.

The RNA was translated in an in vitro rabbit reticulocyte lysate system (13) containing ^{35}S-methionine. Portions of the in vitro translation products were immunoprecipitated (18) by using antiserum to SDS-dissociated CTV virions. After electrophoresis on 7.5 to 15% gradient SDS-PAGE gels (19), translation products and immunoprecipitated products were visualized by fluorography (8).

Amino acid analysis. Unlabeled coat proteins from purified CTV virions were collected and lyophilized from SDS-PAGE without urea and with 0.05 M potassium phosphate buffer, pH 7.2, instead of a buffer containing glycine as used by Laemmli (19). Alternate gel lanes contained dansyl chloride-labeled CTV proteins to aid in identification of the unlabeled proteins. The protein samples from two different purifications of CP1 protein were resuspended in 6 N HCl and hydrolyzed under nitrogen in sealed tubes for 24 and 48 hr at 110 C. Samples from CP2 were from one purification and 24 hr hydrolysis. Three replications were run on each hydrolysis. Samples were concentrated in a rotary evaporator and the residue dissolved in 0.2 M sodium citrate buffer, pH 2.2, for analysis in a Beckman Model 120 C amino acid analyzer equipped with an automatic integrator. The results are expressed as molar ratios extrapolated to zero time of hydrolysis and are rounded for best fit for amino acid residues per molecule of protein.

RESULTS

When purified CTV virions were dissociated and examined by SDS-PAGE, two proteins were detected (Fig. 1, lanes 2–4). The larger coat protein (CP1) had an estimated molecular weight of 23,000 and was present in much greater quantity than the smaller coat protein (CP2), which had an estimated molecular weight of 21,000. When CP1 and CP2 were recovered by preparative SDS-PAGE, both reacted in ELISA tests with polyclonal antisera made against either whole unfixed or SDS-dissociated CTV virions. Both CP1 and CP2 reacted with polyclonal antiserum to SDS-dissociated CTV when the proteins from dissociated CTV virions were analyzed by SDS-PAGE and subjected to western blot analysis (Fig. 1, lanes 5–7), but they did not react against pre-immune serum (Fig. 1, lanes 8–10). All Florida CTV strains examined contained both CP1 and CP2 (Fig. 1). The CP1

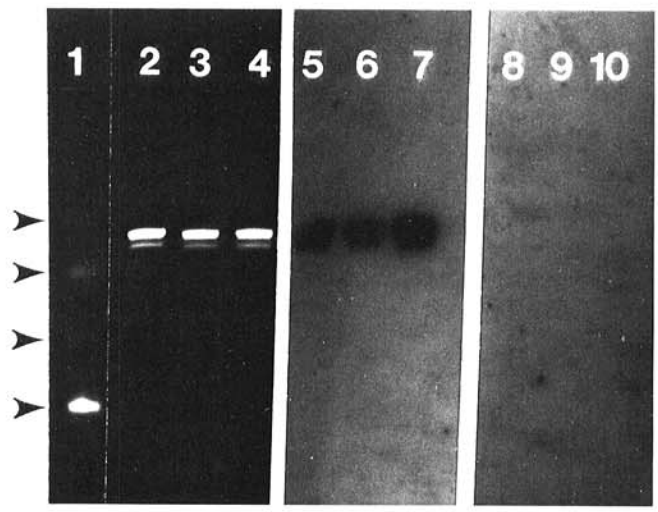


Fig. 1. Western blot analysis of Florida CTV isolates after labeling with dansyl chloride and electrophoresis on SDS-PAGE. Lane 1 contains trypsinogen (M_r 24,000), β-lactoglobulin (M_r 18,400), lysozyme (M_r 14,300), and bovine trypsin inhibitor (M_r 6,500) from top to bottom as indicated by arrows on left. Lanes 2–4 contain CTV isolates T3, T4, and T30, respectively, after dansylation and SDS-PAGE. Lanes 5–7 are from a fluorogram of the same isolates after transfer to APT paper, incubation in antiserum made against SDS-dissociated CTV virus, then incubation in ^{125}I-labeled protein A. Lanes 8–10 are from a fluorogram of the same isolates from the same paper after stripping, incubation in pre-immune serum, and then in ^{125}I-labeled protein A.

and CP2 could be repeatedly recovered from gels, and their apparent molecular weights did not change upon re-electrophoresis. The longer run times and higher voltage of the preparative SDS-PAGE achieved good separation of CP1 and CP2 and permitted recovery of each with no apparent contamination with the other (Fig. 2). The ratio of CP1 to CP2 recovered from preparative gels was about 5:1 based on ELISA values and estimates of protein concentrations by the BioRad protein test. Several methods of purification, which varied in time and complexity, were used to purify virus. The method of purification used to obtain the virus had no apparent effect on the size or ratio of CP1 and CP2.

The polypeptide maps of CP1 and CP2 were compared by using proteins collected from preparative gels and digested with several different proteolytic enzymes. Usually, the purified CTV virions were dissociated and labeled with either dansyl chloride or ^{125}I before preparative SDS-PAGE. After CP1 and CP2 were recovered from the preparative SDS-PAGE gels and lyophilized, they were resuspended in sterile water, dispensed into reaction tubes, and subjected to proteolytic digestion. The resultant polypeptides were compared by SDS-PAGE. Reproducible and consistent results were obtained from numerous purifications of the virus isolates and by using different lots of proteolytic enzymes.

When dansylated CP1 and CP2 from CTV isolate T30 were digested with V8 protease, similar polypeptides were obtained for both CP1 and CP2 (Fig. 3). There were two major polypeptides that migrated between the bovine trypsin inhibitor and lysozyme after digestion with V8 protease (Fig. 3, lanes 1 and 2). The molecular weight of these two polypeptides approximates that of CP2. CP1 yielded a large polypeptide that comigrated with CP2 upon partial digestion with V8 protease.

Further comparisons were made of CP1 and CP2 by using ^{125}I as the label because this method offers greater sensitivity in detecting the resultant labeled polypeptides. When digested with V8 protease, only one major polypeptide was seen (Fig. 4, lanes 3 and 4); the smaller polypeptide that was readily seen with dansylated polypeptide maps of CP1 and CP2 was not detected. The trypsin digestion yielded a polypeptide map similar to that of V8 protease (Fig. 4, lanes 5 and 6). Thermolysin produced two polypeptides (Fig. 4, lanes 7 and 8). The polypeptide maps for CP1 and CP2 were similar regardless of the proteolytic enzyme used.

Amino acid analyses of the CP1 protein of CTV isolate T36 revealed that the molar ratio of amino acids is similar to that reported for beet yellows virus (1), the type member of the

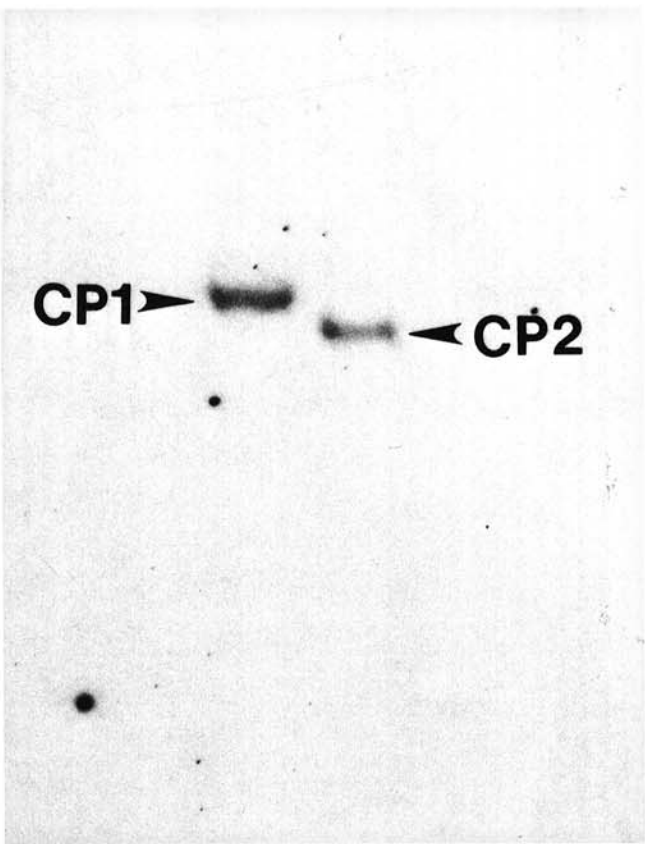


Fig. 2. Western blot of CP1 and CP2 from CTV isolate T30 after elution of separated CP1 and CP2 from preparative SDS-PAGE and re-electrophoresis on a second preparative SDS-PAGE. Antiserum was prepared against whole purified CTV isolate T4.

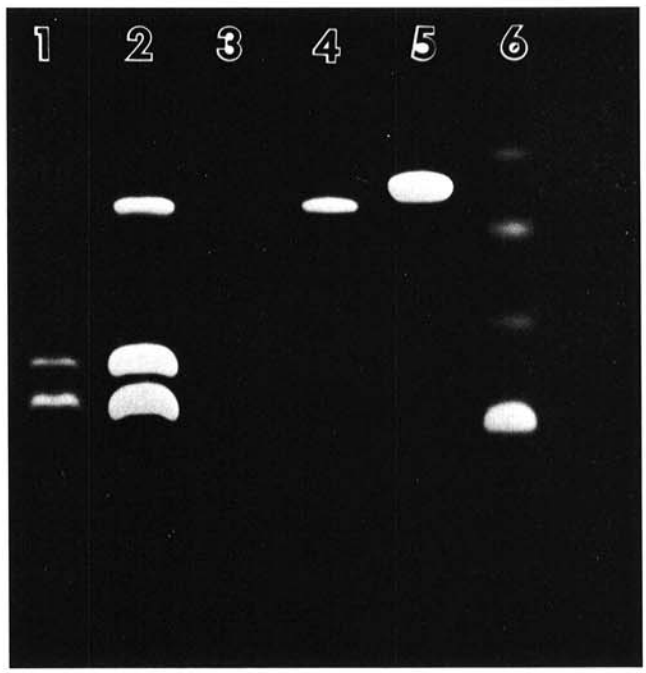


Fig. 3. Comparison of polypeptide maps of dansylated purified CP1 and CP2 from CTV isolate T30 after incubation with V8 protease. Lanes 1 and 2 contain CP2 and CP1, respectively, after incubation with V8 protease. Lane 3 contains V8 protease only, without CTV proteins. Lanes 4 and 5 contain CP2 and CP1, respectively, each of which was incubated without added proteolytic enzyme. Lane 6 contains the same marker proteins as in Figure 1.

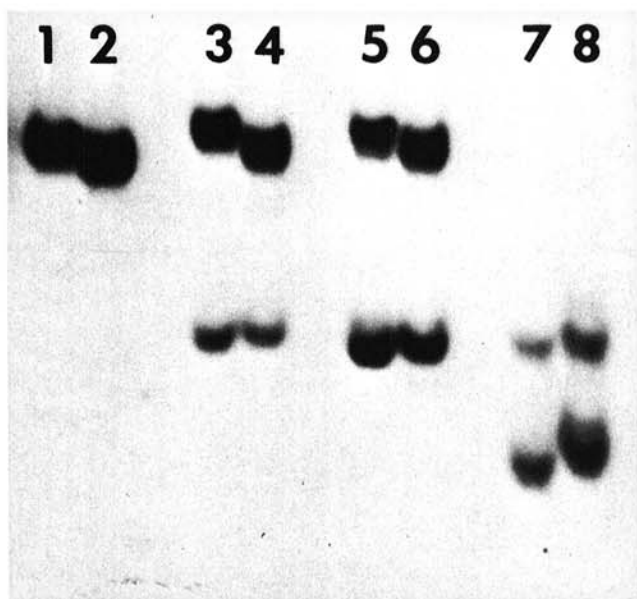


Fig. 4. Comparison of polypeptide maps on autoradiogram of gel with ^{125}I-labeled CP1 and CP2 from CTV isolate T30 after incubation without proteolytic enzymes (lanes 1 and 2, respectively), incubation with V8 protease (lanes 3 and 4), incubation with trypsin (lanes 5 and 6), and with thermolysin (lanes 7 and 8).

closterovirus group (Table 1). The data are averages from two different purifications of CP1 protein. The amino acid molar ratios for CP2 were similar to CP1 except it lacked 2 Lys, 4 Asp, 2 Val, 1 Ile, 4 Leu, and 1 Phe residues. However, this information is based on only one purification and a 24-hr hydrolysis with three replications.

Tristeza RNA preparations were not efficient messengers under the conditions used for in vitro translation. The maximum level of ^{35}S-methionine incorporated into trichloroacetic acid precipitable products was four times the incorporation level of translation lysate without added mRNA. RNAs extracted from citrus variegation virus and citrus leaf rugose virus yielded incorporation levels 10 times this background when used as messenger RNAs in the same system (unpublished data). The translation products from RNA extracted from CTV isolates T36 and T3 were similar (Fig. 5). Several products were apparent with major translation products having approximate molecular weights of 65,000, 50,000, 33,000, and 26,000. Portions of the translation products were immunoprecipitated with CTV polyclonal antisera. A product of about 26,000 daltons was immunoprecipitated from translates of both RNA preparations (Fig. 5, lanes 3 and 6). A larger product in the range of M_r 200,000 was also present in the immunoprecipitations. No products could be detected by immunoprecipitation with pre-immune serum.

The polypeptide maps of several CTV isolates were compared by labeling the purified viruses with dansyl chloride after dissociation with SDS, then incubating with various proteolytic enzymes. Each proteolytic enzyme produced a characteristic polypeptide map. Clostripain and thermolysin produced polypeptide maps having numerous bands, whereas V8 protease and trypsin produced relatively few bands (Fig. 6). Although the polypeptide maps for each enzyme produced similar maps for all CTV isolates examined, there were some consistent minor differences. Isolate T3 was consistently slower to be digested by any of the proteolytic enzymes used in this study, but the resultant polypeptide map was similar to other isolates (Fig. 6). Isolate T36 consistently had a distinct doublet instead of the broad single upper polypeptide found in the other isolates (Fig. 6B). The polypeptide maps formed after digestion with V8 protease and trypsin were similar for all isolates (Fig. 6C).

DISCUSSION

Purified CTV virions contained two proteins. The larger coat protein (CP1), about 23,000 daltons, was present at concentrations of about five times that of the smaller protein (CP2), about 21,000 daltons. The two proteins were found with all CTV isolates examined from Florida and reacted with antisera specific for purified CTV. The ratio or size of CP1 and CP2 was not affected by method of purification of the virions or by repeated electrophoresis and recovery on SDS-PAGE. Both CP1 and CP2 reacted with

TABLE 1. Comparison of the molar ratios of the CP1 protein of T36 isolate of citrus tristeza virus and of the coat protein of beet yellows virus, the type member of the closterovirus group

Amino acid	Molar ratios	
	CP1	Beet yellows[a]
Lys	17	14
His	4	5
Arg	8	12
Asp	22	16
Thr	12	15
Ser	16	16
Glu	13	22
Pro	5	8
Gly	24	17
Ala	16	17
Cys	1	6
Val	13	7
Met	1	1
Ile	8	7
Leu	20	26
Tyr	4	4
Phe	4	11
Try	...[b]	...[b]

[a] As reported by Carpenter (1).
[b] Not determined.

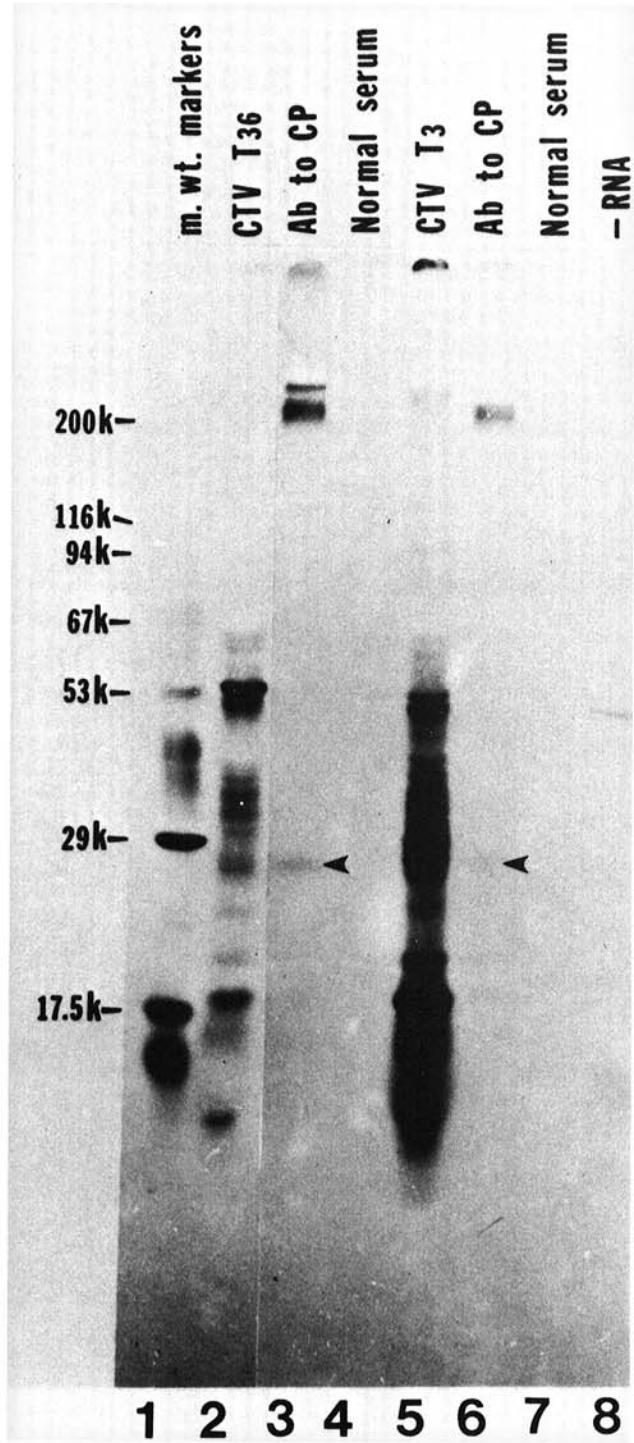
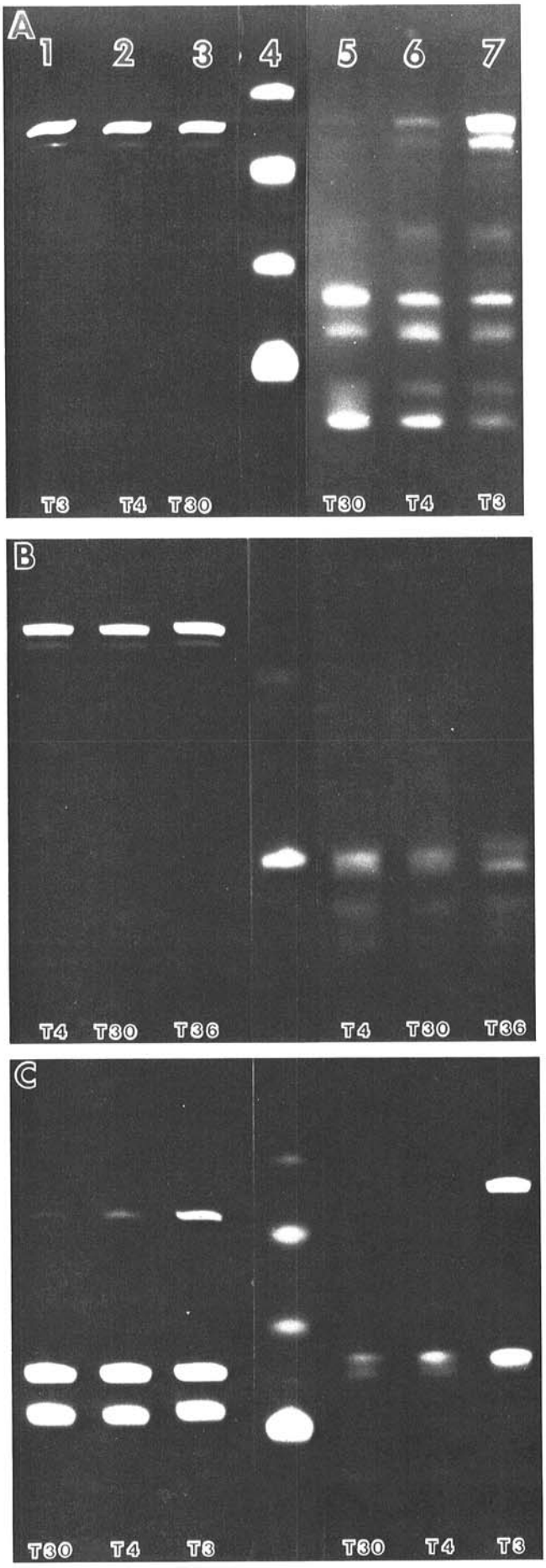


Fig. 5. Autoradiogram of SDS-PAGE gel containing the in vitro translation products of CTV RNA from two isolates. Lane 1 contains molecular weight marker proteins with M_r as indicated in kilodaltons. Lane 2 contains total products translated from CTV isolate T36 RNA. Lane 3 contains the T36 products immunoprecipitated with antiserum made against SDS-dissociated virus. Lane 4 contains the T36 products immunoprecipitated with normal (pre-immune) serum. Lane 5 contains total products translated from CTV isolate T3 RNA. Lane 6 contains the T3 products immunoprecipitated with CTV antiserum. Lane 7 contains the T3 products immunoprecipitated with pre-immune serum. Lane 8 contains total products translated in the in vitro system without added messenger RNA.



CTV-specific antisera by ELISA and western blot analysis. Polypeptide maps of CP1 and CP2 after digestion with trypsin, V8 protease, or thermolysin were similar, and thus the amino acid composition of CP1 and CP2 was similar.

Gonsalves et al (17) observed a protein smaller in size than the major CTV coat protein in some preparations stained with Coomassie brilliant blue. They speculated that this smaller protein was a breakdown product of the coat protein. We did not observe the smaller protein (CP2) when gels were stained with Coomassie brilliant blue unless the gel was overloaded (Lee, unpublished). It was only when CTV coat proteins were analyzed with more sensitive staining techniques of silver staining, fluorescent-labeling with dansyl chloride, and labeling with ^{125}I that it became apparent that CP2 was consistently associated with CTV and of a specific size.

When CTV RNA was translated in vitro, one protein, estimated at 26,000 daltons, was immunoprecipitated with CTV antisera. The size estimate of 26,000 daltons is near the size of CP1. The size estimate of the 26,000 dalton immunoprecipitated product was made on a gradient SDS-PAGE Laemmli gel, whereas the 23,000 dalton size estimate of CP1 was made on a 15% SDS-PAGE gel containing 7.0 M urea. Therefore, it was difficult to directly compare sizes of these proteins. Some large proteins near 200,000 daltons also were immunoprecipitated. The significance of these larger proteins is unknown, but similar large products in potyvirus translation immunoprecipitations have been interpreted as polyproteins associated with a monocistronic genome organization (6). It is possible that CP2 is derived from CP1 as a result of post-translational processing, as it has not been observed as an immunoprecipitated product in any of the translations. Alternatively, it could be translated independently from CP1, but is present in such small amounts that it is not detectable by immunoprecipitation.

It does appear that CTV RNA is plus-stranded because the RNA has messenger activity in an in vitro translation system, and one of the products formed reacts serologically with CTV antisera. Also, purified virions have been shown to be infectious (22).

The difference in specificity of labeling between the dansylation method and the ^{125}I-labeling method can be observed in the polypeptide maps. Dansyl chloride labels tyrosine, lysine, and the N-terminal amino acid (5). The method used for ^{125}I-labeling labels tryosine and, to a lesser degree, histidine (24). For example, dansylated CTV when digested with V8 protease has two major polypeptides (Fig. 3), but ^{125}I-labeled CTV had only one major polypeptide labeled (Fig. 4). This suggests that the smaller polypeptide from the dansylated preparations may contain a high ratio of lysine to tyrosine. An alternative possibility is that the lysine sites are more exposed and are thus more easily labeled.

Based on the similarity of SDS-PAGE polypeptide maps after incubation of whole viruses with different proteolytic enzymes, the CTV coat proteins appear to be conserved among isolates. Although there are some consistent differences, as previously mentioned in the results, these differences are minor and would not provide a means to readily and reliably differentiate CTV isolates, especially in plants infected with more than one CTV isolate. However, because subtle differences do occur among some isolates, it may be possible to differentiate given CTV isolates by serological means, with properly selected monoclonal antibodies.

Fig. 6. Comparison of dansylated CTV isolates and resultant polypeptide maps on SDS-PAGE after incubation with various proteolytic enzymes. **A**, Lanes 1, 2, and 3 contain CTV isolates T3, T4, and T30, respectively, after incubation without added proteolytic enzyme. Lanes 5, 6, and 7 contain T30, T4, and T3, respectively, after incubation with clostripain. **B**, Lanes 1, 2, and 3 contain T4, T30, and T36, respectively, after incubation without added proteolytic enzymes. Lanes 5, 6, and 7 contain T4, T30, and T36, respectively, after incubation with thermolysin. **C**, Lanes 1, 2, and 3 contain T30, T4, and T3, respectively, after incubation with V8 protease. Lanes 5, 6, and 7 contain T30, T4, and T3, respectively, after incubation in trypsin. Lane 4 in A, B, and C contains the same marker proteins as in Figure 1.

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