# A Simple Technique for the Production of Highly Specific Polyclonal Antisera for Citrus Tristeza Virus

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ABSTRACT. Highly specific polyclonal antiserum against citrus tristeza virus (CTV) was produced using one of the viral coat protein subunits. Virus was concentrated by a single polyethylene glycol precipitation followed by low speed centrifugation. Purification of the coat protein was done by SDS-PAGE. The most abundant coat protein subunit—unit II or CP 1 according to previous work was used as antigen for injection into chickens. Chicken lgY recovered from egg yolks had a very high titer and reacted specifically with CTV isolates available in our collection. Healthy plant sap gave no background reaction. The simplicity of this method makes it attractive because the antigen can be prepared without a wide range of equipment.

Serological detection of CTV using polyclonal antibodies is normally carried out with antisera that have been developed using highly purified virus as the immunogens. This usually involves density gradient centrifugation in cesium salts or sucrose or a combination of both. Centrifuged gradients are subsequently fractionated and virus containing fractions, as determined by spectrophotometry, serology and/or electron microscopy are collected (3). Unfortunately, some material of plant origin frequently accompanies the "purified" virus even after density gradient centrifugation. Even though methods have been developed for the removal of contaminating host plant impurities present in the virus suspension they are not always successful (10). Antiserum from animals immunized with virus preparations containing plant proteins will also react with healthy plant controls in serological tests frequently making it necessary to cross absorb the serum to eliminate the unwanted reaction (4). The purpose of this work was to develop a method for the production of a highly specific polyclonal antiserum for CTV using limited equipment.

## MATERIALS AND METHODS

**Virus concentration.** Etrog citron seedlings were inoculated with isolates of CTV designated "T-516" and "SY-575" (2) and maintained in a greenhouse at the University of California, Riverside (UCR). Plants were harvested, the bark peeled off, cut into small pieces and kept at –20C until used for purification. Fifty grams of bark tissue divided in 10-g portions were ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was added to 300 ml of 0.1 M Tris buffer, pH 7.6, stirred in an ice bath for 20 min and centrifuged at 5K RPM for 15 min. The supernatant fluid was filtered through 3 layers of Miracloth by vacuum in a Buchner filter. Polyethylene glycol (PEG) 6,000 MW was added to the clarified extract while stirring slowly in an ice bath until a final concentration of 6% PEG was obtained. After the PEG was completely dissolved, sodium chloride was added to a final concentration of 0.125 M and the suspension stirred for at least one hour. This suspension was centrifuged at 11K RPM for 20 min, the pellet resuspended in 0.4 M $K_2HPO_4$, pH 8.0, and stirred for 1 hr at 4C. Following an additional centrifugation at 7K for 10 min, PEG was added to the supernatant for a final concentration of 6%, stirred for 1 hr, centrifuged at 13K RPM for 15 min and the resulting pellet resuspended in 3 ml of 0.4 M $K_2HPO_4$, pH 8.0. Three ml of 2X treatment buffer containing 0.125 M tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol was added to the suspension, which was aliquoted into 250 µl, boiled for 1 min and kept at –20C. This material consisted of an impure preparation containing many dif-

ferent proteins that precipitate with PEG including CTV particles.

**Isolation of viral coat protein.** CTV coat protein bands in the SDS-PAGE (5) gels were stained with silver nitrate (11) or Coomasie blue. Viral coat protein bands were identified by Western blots using monoclonal and polyclonal antibodies for CTV prepared in our laboratory. A Minibloter® (Immunetics, 145 Bishop Allen Dr., Cambridge, MA 02139) with 45 channels that permits the simultaneous testing of several antisera was used to prepare blots on nitrocellulose paper sheets. Once the positions of CTV coat protein bands in gels was known it was not necessary to run additional Western blots. When needed, immunogen was recovered from SDS-PAGE gels as described later.

**Chicken immunization.** Viral coat protein from isolates T-516 and SY-575 purified by SDS-PAGE was injected into chickens using a slight modification of the procedure described by Powdell and Vacquier (8). Gels were stained for a short time with Coomasie blue until the coat protein band was just barely visible. The bands were excised with a sterile razor blade and stabilized in PBS buffer at 4C for at least 24 hr, then ground in petri dish using a glass spatula before emulsifying with an equal volume of Freund's complete adjuvant. The chickens were immunized three times at 10 day intervals. Eggs were collected daily, stored at 4C and immunoglobulin purified from egg yolks using the technique of Polson *et al.* (9). All solutions used for antigen preparation were kept sterile.

**ELISA.** Well characterized isolates of CTV belonging to UCR's collection were used to develop an indirect DAS-ELISA protocol for the polyclonal antiserum prepared in chicken. Coating of the microtiter plates was done using a goat anti-CTV antiserum available in the laboratory diluted at 2 μg per ml in buffer containing 1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$ and 0.2 g $NaN_3$ per liter at a final pH of 9.6. Coating was completed in 2 hr at 32C. Antigen consisted of green bark of Etrog citron inoculated with known CTV isolates, powdered in liquid nitrogen and diluted 1:10 in PBS buffer. Bark from uninoculated Etrog citron was used as the healthy control. Antigen was incubated overnight at 4C. Blocking of the microtiter wells was carried out with plain pasteurized milk during 30 min at room temperature. The milk was removed by patting them on paper towels without washing with PBST before the addition of the anti-CTV chicken antiserum diluted 1:5,000 in PBS buffer and incubated for 2 hr at 32C. Conjugate was Sigma #A-1043 diluted 1 mg per ml in PBS buffer and incubated for 2 hr at 32C.

## RESULTS

SDS-PAGE protein profiles are shown in Fig. 1. The protein content of crude extracts of healthy and CTV-infected Etrog citron bark in Tris buf-

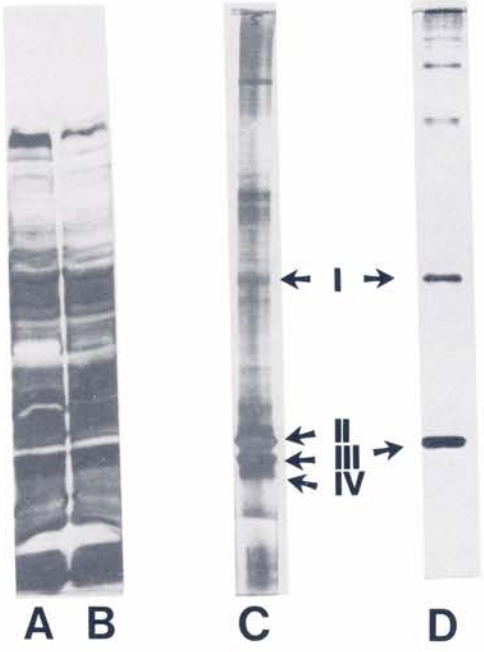


**Fig. 1. The protein content of crude extracts of healthy and CTV-infected Etrog citron bark in Tris buffer as they appear before PEG precipitation (lanes A and B), after PEG precipitation (lane C) and following cesium sulfate isopycnic gradient centrifugation (lane D). Numbers I to IV indicate CTV related proteins as described in the text.**

fer as they appear before PEG precipitation are shown on lanes A and B. Protein bands of plant origin are found throughout the gel from top to bottom obscuring any specific CTV-related proteins. Infected plant extracts were treated with PEG resulting in preparations containing CTV at concentrations that allowed detection of viral coat proteins (Fig. 1, lane C). These bands were not distinguishable in crude extracts before PEG precipitation of the virus which indicates that a selective precipitation of virions has occurred resulting in the exclusion of other plant proteins from the supernatant fluid after low speed centrifugation. However, this preparation could not be used as antigen to immunize animals because it still contained many plant proteins in addition to CTV proteins when compared to a purified preparation obtained after isopycnic density gradient in cesium sulfate (Fig. 1, land D).

Western analysis using a polyclonal antiserum made in rabbit against the non-denatured virus showed reactivity with all four CTV-related protein bands found in SDS-PAGE (12) (Fig. 2). One major protein (II) reacted positively with monoclonal and polyclonal antibodies for CTV. A second band (III) present in gels sometimes as abundant as the first one did not react with the monoclonal antibodies used for this test (Fig. 2, lanes P and M). The molecular weight of these two proteins, based on the electrophoretic mobility of molecular weight markers loaded in parallel channels (data not shown), are similar to those reported by previous authors (7) and referred to as CP 1 (23K $M_r$) and CP 2 (21K $M_r$). CTV-related proteins I and IV, according to Zeineh *et al.* (12) are also detected by the polyclonal antiserum, but only I reacted with the monoclonal antibodies. Since the molecular weight of I is approximately 45K (twice the molecular weight of II) and it reacted with monoclonal antibodies that recognized only II, suggests that I is a dimer of II (or CP 1). Band II was selected as the best candidate for use as the immunogen because of its reactivity

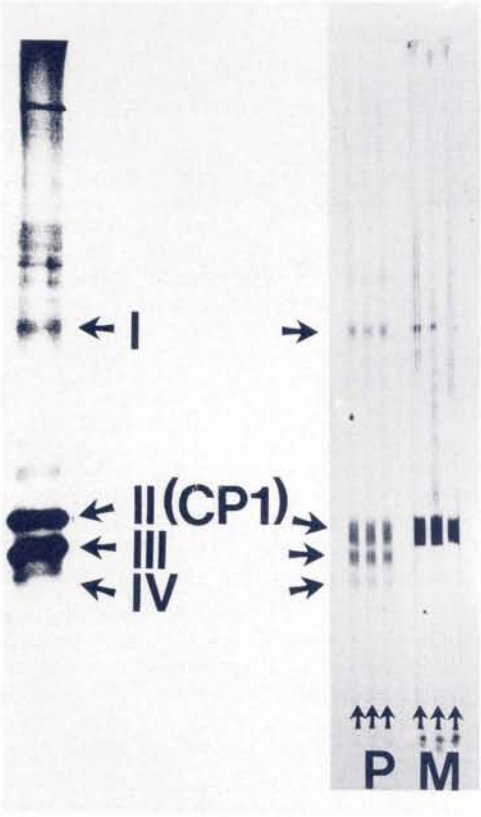


**Fig. 2. SDS-PAGE protein pattern after silven staining (left) and the corresponding Western blot in nitrocellulose paper (right) for a clarified CTV preparation. P and M indicate lanes of Miniblotter® reacted with anti-CTV polyclonal and monoclonal antibodies, respectively. Labels I to IV (and CP 1) indicate protein units related to CTV virions.**

with monoclonal antibodies specific for CTV. This band was cut out of the gels and injected into chickens as described under Materials and Methods.

The titer of the antiserum recovered from eggs laid during a period of 2 months was high and had to be diluted between 1:5,000 and 1:20,000 for use in ELISA tests. More concentrated dilutions developed color too fast and the reactions had to be stopped with 3 M NaOH 5 min after substrate addition to avoid non-specific reaction with healthy plant controls. Table 1 shows typical results obtained using one antiserum batch extracted from one egg collected 40 days after the first immunization injection of the chicken. The specificity of the antiserum is demon-

TABLE 1
REACTIONS OF A 1:5,000 DILUTION OF CHICKEN ANTISERUM FOR CTV SERUM WAS COLLECTED AFTER TWO IMMUNIZATIONS WITH CTV COAT PROTEIN I (OR CP 1) OBTAINED USING PEG PRECIPITATION OF BARK EXTRACTS FOLLOWED BY SDS-PAGE PURIFICATION.

| CTV isolate | OD@405nm[z] | Disease/ healthy ratio |
|---|---|---|
| SY575 | 1.05/1.06 | 13.2 |
| SY551 | 0.83/0.90 | 10.8 |
| T514 | 0.90/0.90 | 11.3 |
| T516 | 0.75/0.81 | 9.8 |
| Healthy Etrog | 0.08/0.08 | |

[z]Values correspond to the average of 3 wells in each two replicated 96-well microtiter plates read 15 min after substrate addition to the wells.

strated by the disease vs. healthy ratio after spectrophotometric reading of the plates (Table 1).

## DISCUSSION

The standard procedure used by citrus virologists for antigen preparation when working with CTV involves subjecting the concentrated virus suspension to one or more cycles of isopycnic density gradient centrifugation (1, 3, 6). Virus purification includes pelleting, resuspension and gradient formation in cesium salts or sucrose, or a combination of both. All these steps are sometimes repeated in order to obtain a more purified final product and as a result of this intense manipulation considerable amounts of virus material are lost.

The starting material for purification used in this work consisted of 50 g of bark tissue which yielded sufficient antigen for 21 immunization doses. The immunized chicken gave high titer antiserum after only two injections which means that 50 g of plant tissue used as the starting material should be enough for immunization up to 10 chickens.

The contributions of this work are two fold: first we were able to recover sufficient CTV coat protein to use as an immunogen following a minimal purification schedule and secondly the highly purified antigen obtained by cutting out the coat protein band from SDS-PAGE gels avoided the presence of contaminating proteins of plant origin.

The decision to use only protein unit II as the antigen was fortunate. This decision was made because at the time this work was started there was no reliable information regarding the SDS-PAGE pattern of CTV coat protein. Even though units I through IV are CTV-related proteins (7, 12) we assumed that proteins not detected with the monoclonal antibodies might have been pathogenesis related (PR) or other plant proteins not related to CTV virions. It is well known that proteins of plant origin can elicit an immune response in immunized animals resulting in a non-specific antiserum.

## LITERATURE CITED

1. Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, A. Rosner, and I. Ginzberg
1985. A simple purification method for citrus tristeza virus and estimation of its genomic size. Phytopathology 75: 195-198.
2. Garnsey, S. M., D. J. Gumpf, C. N. Roistacher, E. L. Civerolo, R. F. Lee, R. K. Yokomi and M. Bar-Joseph
1987. Towards a standarized evaluation of the biological properties of citrus tristeza virus. Phytophylactica 19: 151-157.
3. Gonsalves, D., D. E. Purcifull and S. M. Garnsey
1978. Purification and serology of citrus tristeza virus. Phytopathology 68: 553-559.
4. Harlow, E. and D. Lane
1988. Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, 726 pp.
5. Laemmli, U. K.
1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680-685.
6. Lee, R. F., S. M. Garnsey, R. H. Brlansky and A. C. Goheen
1987. A purification procedure for enhancement of citrus tristeza virus yields and its application to other phloem-limited viruses. Phytopathology 77: 543-549.

7. Lee, R. F., L. A. Calvert, J. Nagel and J. D. Hubbard
1988. Citrus tristeza virus: characterization of coat proteins. Phytopathology 78: 1221-1226.
8. Podell, S. B. and V. D. Vacquier
1984. Inhibition of sea urchin sperm acrosome reaction by antibodies directed against two sperm membrane proteins. Expt. Cell Res. 155: 467-476.
9. Polson, A., T. Coetzer, J. Kruger, E. von Maltzahn and K. J. van der Merwe
1985. Improvements in the isolation of lgY from the yolks of eggs laid by immunized hens. Immunol. Investigations 14: 323-327.
10. Van Regenmortel, M. H. V.
1982. Serology and immunochemistry of plant viruses. Academic Press, New York, 302 pp.
11. Wray, W., T. Boulikas, B. P. Wray and R. Hancock
1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118: 197-203.
12. Zeineh, R. A., G. Kyriakidis, R. Acey and C. Smith
1986. Detection and assessment of citrus tristeza virus antigens by computer-aided scanning densitometry. Appl. Biochem. Biotechnol. 13: 119-126.