PRODUCTION OF POLYCLONAL ANTIBODY TO THE COAT PROTEIN OF CITRUS TRISTEZA VIRUS IN CHICKEN EGGS

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ABSTRACT

Citrus tristeza virus (CTV) is one of the most destructive diseases in many citrus growing areas of Indonesia. Effective strategies for controlling CTV depend on diagnostic procedure namely enzyme-linked immunosorbent assay (ELISA). Study aimed to purify the CTV antigen and produced its polyclonal antibody. Virion of the severe CTV isolate designated UPM/T-002 was concentrated by polyethylene glycol (PEG) precipitation combined with low speed centrifugation. Semipurified antigen was further purified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The specific coat protein (CP) band of CTV with molecular weight of 25 kD was excised and eluted using elution buffer containing 0.25 M Tris-HCl pH 6.8 + 0.1% SDS, then used as antigen for injection into 6-month-old female of White Leghorn chicken. Results, showed than the specific polyclonal antibody raised against the 25-kDa CP had a titer of approximately 10^4, gave low background reaction with healthy plant sap and reacted specifically with CTV isolates. The reaction was equally strong for a severe, a moderate, a mild, and a symptomless isolate, suggesting a broad reaction range of this antibody toward different CTV isolates. Optimal virus titer can be obtained since virus loss during purification could be minimized and the highly purified antigen as an immunogen could be obtained by cutting out the CP band from SDS-PAGE gels. Large amount of highly titer of CTV antibody can be produced in chicken egg. The simplicity of the procedure makes it economically acceptable and technically adoptable because the antibody can be produced in basic laboratory.

[*Keywords*: Citrus, viroses, polyclonal antibody, proteins]

INTRODUCTION

Citrus tristeza virus (CTV) is one of the most destructive diseases in many citrus producing areas of the world. During 1986s, up to 78% citrus trees from various commercial varieties in Indonesia have been infected with CTV (Roesmiyanto *et al*. 1986). In Java, the failure of sweet orange grafted on sour orange rootstock variety formerly attributed to incompatibility between rootstock and scion (Toxopeus 1937), was later confirmed that it was due to CTV (Terra 1951).

CTV is endemic in citrus growing areas and exists in numerous strains. It is widely spread in West Kalimantan (Whittle *et al*. 1989), East Java, and West Sumatra. Most of the CTV strains severely infected commercial citrus varieties such as pomelos, oranges, tangerines, and mandarins (Muharam and Whittle 1991). The CTV strain caused declined inducing and stem pitting (Fig. 1) was the most destructive disease on limes. Infected plant developed very distinct vein clearing, vein corking, leaf cupping, severe stem pitting, and declining symptoms (Nurhadi *et al*. 2000). So far, there is no reliable data on the loss due to CTV in Indonesia, but the virus becomes a serious threat to the citrus industry.

Efforts to control CTV has been implemented under Citrus Rehabilitation Program since 1987. The program consisted of planting certified virus-free propagating material, early detection and eradication of infected plants. The success of the program depends on appropriate diagnostic procedure that is currently performed by enzyme-linked immunosorbent assay (ELISA). ELISA is the most convenient, reliable, fast, and relatively inexpensive procedure (Nikolaeva *et al*. 1995). However, the enormous scale of the indexing program requires large amount of specific antibody and a consistent supply of antigen for immunization purposes. Availability and consistent supply of CTV antibody in all CTV endemic areas are currently provided from costly commercial imported antibody.

The host range of CTV is limited to the Rutaceous family (Brunt *et al*. 1996). Like other closteroviruses, the virus is multiplied in the phloem of infected *Citrus* spp. (Bar-Joseph and Lee 1989), therefore, virus purification is difficult and the yields of purified CTV virions are usually low (Lee *et al*. 1987). Recent procedure for antigen preparation involves subject-
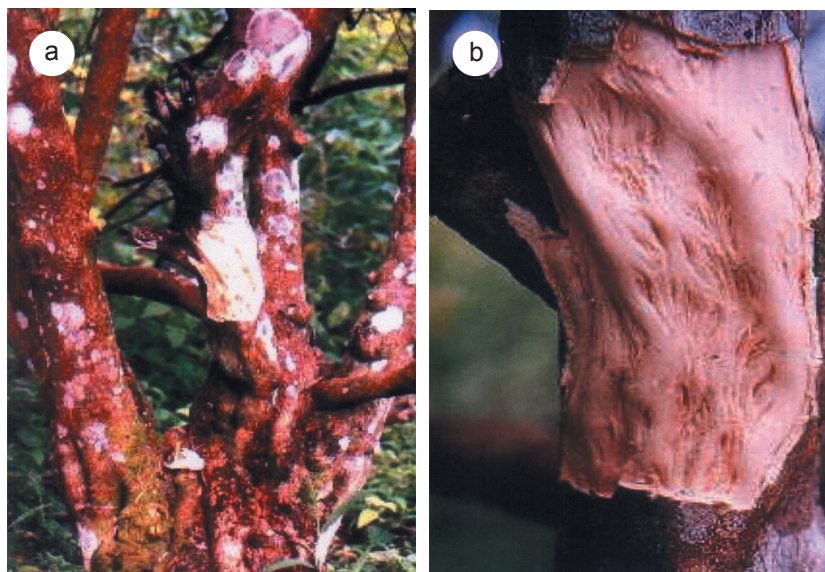
Fig. 1. Citrus tristeza virus infected *Citrus aurantifolia* tree showing severe stem pitting (a) with intense deep pits when the bark was peeled off (b), West Pasaman, Sumatra.

ing the concentrated virus suspension to one or more cycles of isopycnic density gradient centrifugation (Bar-Joseph *et al*. 1985; Lee *et al*. 1988). Virus purification includes peletting, resuspension and gradient formation in cesium salts or sucrose, or a combination of both. All these steps are sometimes repeated to obtain a more purified final product. Because of this intense manipulation, considerable amount of virus material are lost, and virus preparations usually still contain plant proteins that will also react with healthy plant in serological test.

Approaches have been developed to overcome these drawbacks. The CTV coat protein (CP) is antigenic and when purified virus is injected into animal, antibodies specific to the viral protein are formed (Pappu *et al*. 1994; Nikolaeva *et al*. 1995). This provided an alternative method to obtain the CTV-CP as an antigen. Compared with intact virion produced from conventional purification procedure, the use of CP antigen resulted in highly specific polyclonal antibody (Marco and Gumpf 1991). Dwiastuti *et al*. (1997) showed that upon SDS-PAGE, the CTV-CP was positioned at the 13.3 kDa band. However, antibody produced to this CP had very low titer and less sensitive. Nurhadi *et al*. (2001) found that the 25-kDa CP could be isolated using a simple purification technique. The objective of this study was to prepare highly purified protein containing the CP of the UPM/T002 CTV isolate, and produce specific polyclonal antibody against the CTV-CP.

MATERIALS AND METHODS

The study was conducted at microbiology laboratory of the Universiti Putra Malaysia during January-December 2002.

Virus Purification

The UPM/T-002, a CTV severe subisolate causing moderate stem pitting on West Indian (WI) lime (*Citrus aurantifolia* L.), and a representative of the most common type of tristeza present in West Pasaman and Riau were used in this study. The virus was isolated from young bark tissue and purified following the method described by Marco and Gumpf (1991) with little modification. Extraction buffer (0.1 M Tris-DIECA buffer pH 7.4, 0.5% 2-mercaptoethanol, 0.1% Triton) and addition of a 0.5 volume of a 1:1 mixture of chloroform + carbon tetrachloride in virus clarification step were used in this study.

The young bark tissues were cut into small pieces and ground to a fine fractions, to which 300 ml of extraction buffer was added. The extract was filtered, stirred, and centrifuged at 6,000 rpm for 15 minutes. The supernatant was filtered and polyethylene glycol (PEG) (6,000 MW) was added. After the PEG was completely dissolved, sodium chloride was added. The suspension was stirred and then centrifuged at 11,000 rpm for 20 minutes. The pellet was resuspended in 0.4 M KH_2PO_4 pH 8.0 for 1 hour at 4°C, and centri-

fuged at 7,000 rpm for 10 minutes. PEG was added to the supernatant for a final concentration of 6%. Suspension was stirred, centrifuged at 13,000 rpm for 15 minutes and the resulting pellet was resuspended in 0.4 M K_2HPO_4 pH 8.0. Preparation obtained was denatured by heating to 97.5°C for 4 minutes in the presence of two-fold discontinuous buffer system of Laemmli (1970). Proteins were electrophoresed on 16% separating gel and 4% stacking gel; each well was loaded with 30-40 ml of protein samples. Gels were electrophoresed for 3-4 hours with constant cooling to 6°C at 60 mA. Molecular weight of the protein bands was calculated based on the reference protein bands (Gibco BRL) as follows: ovalbumin from hen egg white 43 kDa, carbonic anhydrase (29.0 kDa), b-lacto-globulin 18.4 kDa, lysozyme 14.3 kDa, and bovine trypsin inhibitor 6.2 kDa.

CTV-CP was purified and isolated by the modified method of Waterborgh and Matthews (1994) except denaturing condition (sodium dodecyl sulphate = SDS elution) used in the elution procedure. After electrophoresis, the left and right borders of the gel were cut and stained for 10 minutes with Coomasie blue until the profile of protein bands was just barely visible. Once identified, unstained gel at the middle part containing the protein bands was excised using both stained borders as a marker. Sliced gels was minced into smaller pieces, then placed into a centrifuge tube to which the elution buffer was added and mixed by vortexing. The gel was then centrifuged at 15,000 rpm for 10 minutes and the supernatant containing the eluted protein was collected. The process was repeated 2-3 times to increase protein recovery.

Eluted protein was assayed by double antibody sandwich (DAS) ELISA described below, and protein reacted to commercial antibody against CTV was selected as antigen. The eluted protein was then kept at 20°C after measuring its concentration by UV-visible spectrophotometer using the extinction coefficient A_{280} = 1.4 for 1 mg ml^{-1} of CTV protein (Nikolaeva *et al.*, 1995). Recovery of the electrophoresed CP by elution using SDS elution buffer (0.25 M Tris-HCl pH 6.8) resulted in clean and pipettable eluted CP within 3 hours.

Production of Polyclonal Antibody Using Female White Leghorn Chicken

The standard procedure for producing polyclonal antibody in chicken followed Van Regenmortel (1982). The initial injection was emulsified CP antigen in Freund's complete adjuvant (1:1), each subsequent

injection was emulsified with Freund's incomplete adjuvant. Six-month-old female of White Leghorn chicken was injected three times at weekly intervals for a period of 4 weeks. The injections were made subcutaneous and intramuscularly in alternating thigh muscles at a concentration of 75, 75 and 100 µg ml^{-1} eluted CP, each for the first, second and third injection, respectively. The eggs were collected at one week after the last injection, and the titer of antibody on yolk against CTV was determined by micro-agglutination test and indirect ELISA.

Purification of IgY

The standard procedure for IgY purification was adopted from Polson *et al.* (1985). The yolks were equilibrated in 1/2 strength of PBS pH 7.4 containing 4.0 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, and 0.12 g KH_2PO_4, at a volume of 1:9 (yolk:buffer). PEG was added to a concentration of 3.5%, stirred at room temperature and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and made 8% with respect to PEG, stirred at room temperature, and left standing at room temperature for 10 minutes. The mixture was centrifuged at 8,000 rpm for 30 minutes and the resulting pellet was resuspended with 1/2 strength of PBS pH 7.4. The resuspended pellet was made 12% with PEG, stirred at room temperature and left standing at room temperature for 10 minutes. The mixture was centrifuged at 8,000 rpm for 30 minutes and the pellet was resuspended in 1/2 strength of PBS pH 7.4, cooled to 0°C and mixed 1:1 (v:v) with 50% prechilled ethanol and centrifuged in a prechilled rotor at 8,000 rpm for 30 minutes. The pellet which contained IgY was resuspended in 2 ml of 1/2 strength PBS pH 7.4, then purified using ammonium sulphate precipitation as described by Clark and Adams (1977).

Two milliliters of antibody suspension was diluted in 18 ml distilled water, to which 20 ml of saturated solution of ammonium sulphate pH 7.4 was added and stirred drop wise at room temperature for 30 minutes. The solution was kept at 4°C for 1 hour. The pellet was collected by centrifugation at 3,000 rpm for 30 minutes, dissolved in 1/2 strength PBS pH 7.4, and dialysed twice against two changes in 500 ml of 1/2 strength PBS pH 7.4 for 4 hours and overnight at 4°C. IgY was further purified by passage through a column of DE-52 cellulose equilibrated in 1/2 strength of PBS pH 7.4. The fractions were monitored at a wavelength of $A_{280\,nm}/A_{260\,nm}$ ratio and UV absorbing areas containing IgY was pooled.

Conjugation of IgY with Alkaline Phosphatase

Procedure of Avrameas (1969) was adopted for conjugation of antibody with alkaline phosphatase. Conjugation was done by dissolving 500 μg of alkaline phosphatase (Sigma) in 500 μl of the above immunoglobulin preparation. Half strength of 0.02 M PBS pH 7.4 was then added to the final concentration of 1 ml, and dialysed extensively against 500 ml of 1/2 strength of 0.02 M PBS pH 7.4 at 4°C. Glutaraldehyde was added to 0.05% final concentration and the mixture was incubated at 22°C for 4 hours. Glutaraldehyde was then removed by dialysis against three changes of 500 ml of 1/2 strength of 0.02 M PBS pH 7.4 and the conjugate with approximately 1% bovine serum albumin was stored at 4°C.

Serological Test

Sensitivity and specificity of antibody produced was evaluated by microagglutination and ELISA tests, respectively. The microagglutination test is economical in its use of antibody and is fairly sensitive since small precipitates are easily detected under the dark field microscope at 10-1000 time magnification, in which chloroplast and cell fragments present in a crude plant sap are agglutinated upon addition of virus (Van Regenmortel 1982). In microagglutination test, IgY was diluted at 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; 1:512; 1:1,024; 1:2,048; 1:4,096; 1:8,192 and 1:16,384 (v/v) in coating buffer pH 9.6 containing 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 per liter. A single drop of mixed reactants containing 20 μl antibody of each dilution was deposited on the microplate's cover grid, to which 20 μl semipurified antigen was added, mixed, and incubated at 37°C for 1 hour. Reaction was examined under dark field of stereo binocular microscope with 10-30 time magnification. Strong aggregation with cloudy part at central zone in the reactant drop, or chloroplast and cell precipitated agglutinated upon addition of virus antibody indicated a positive reaction.

ELISA procedure was performed as described by Clark and Adams (1977) unless otherwise indicated. Origins and biological properties of CTV isolates used in this study are presented in Table 1. Briefly, antigens were semipurified virus, bark of semidormant flush collected from WI lime infected with CTV, and bark of healthy WI lime (healthy control). Plant was harvested; bark was peeled off and ground in warring blender, diluted at 1:10 (v/v) in PBS and kept in 4°C until used. Microtiter plates (NUNC maxisorp)

Table 1. Origins and biological properties of citrus tristeza virus (CTV) isolates used in specificity test for polyclonal antibody produced from this study.

CTV isolates and origin	Biological properties
UPM/T-012, Serdang-Malaysia	This isolate was collected from a pomelo (*Citrus grandis*) tree in a 8-year-old citrus planting showing vein clearing caused by tristeza as it was confirmed by ELISA using commercial polyclonal antibody. Inoculated on West Indian (WI) lime induced only very mild vein clearing and no stem pitting.
UPM/T-015, Serdang-Malaysia	This isolate was collected from lime (*Citrus aurantifolia*) tree in a 6 year-old citrus planting showing vein clearing and stem pitting caused by tristeza as it was confirmed by ELISA using commercial polyclonal antibody. Inoculated on WI lime induced moderate vein clearing.
RIF/T-016, Riau-Indonesia	This isolate was collected from lime (*C. aurantifolia*) tree in a 6-year-old citrus planting showing vein clearing caused by tristeza as it was confirmed by ELISA using commercial polyclonal antibody. Inoculated on WI lime induced only moderate vein clearing.
RIF/T-052, West Sumatra-Indonesia	This isolate was collected from lime (*Citrus reticulata*) tree in a 6-year-old citrus planting showing vein clearing and stem pitting caused by tristeza, as it was confirmed by ELISA using commercial polyclonal antibody. Inoculated on WI lime induced only moderate vein clearing.

were first coated with the chicken egg immunoglobulin. The immunoglobulin was diluted at 1:10 up to 1:10,000 (v/v) in coating buffer pH 9.6 and 100 μl of the antibody was added to each well then incubated for 4 hours at 37°C. Following incubation in a moisture box at 37°C for 2 hours, the plates were then washed 3-5 times with PBS-T pH 7.4. Following the addition of 100 μl of antigen to each well, blocking of the microtiter wells was carried out with plain pasteurized milk 1% (w/v) in PBS pH 7.4 for 30 minutes at room temperature. The milk was removed by patting them on paper towels without washing with PBS-T before the addition of 100 μl chicken egg immunoglobulin alkaline phosphatase conjugate diluted at

1:500 (v/v) in PBS-T, and incubated for 3-4 hours. After washing the plates with PBS-T 3-5 times, 100 µl of p-nitrophenyl phosphate (Bio-Rad Laboratories) (1 mg^{-1} ml) in substrate buffer (Bio-Rad Laboratories) was added to each well. Reaction was determined by absorbance values at 405 nm (METERTECH-ELISA reader Σ 960). Samples were considered positive when the mean absorbance of duplicate well exceeded twice the mean absorbance of appropriate healthy control.

RESULTS AND DISCUSSION

Virus Purification and Elution of Coat Protein for Use as Antigen

In general, procedure developed for antigen preparation involves subjecting the concentrated virus suspension to one or more cycles of isopycnic density gradient centrifugation. All these steps are sometimes repeated to obtain a more purified final product. As a result, considerable amount of virus material are lost, and virus preparations usually still contain plant proteins that will also react with healthy plant in serological test. Attempt to remove plant contaminants during the various stages of virus purification developed in this study showed an effective result.

Addition of 6% PEG + 0.125 M NaCl at the first and second precipitation combined with low speed centrifugation reduced virus loss during preparation and had significant effect on antigen recovery (Fig. 2). Recovery of detectable antigen by ELISA varied between 80.53%, 75.50%, and 60.40% following the first, second and third PEG precipitation, respectively, of that present in the original clarified extract. The protein content monitored before and after the first and second PEG precipitation is shown in Fig. 3. Protein bands of plant origin before PEG precipitation (lane 2) and after the first PEG precipitation (lane 3) were scattered above 43 kDa zone and around 29 kDa zone, obscuring any specific CTV-related proteins. However, CTV-related proteins after the second PEG precipitation was clear (lane 3). Very distinct CTV-related proteins were exhibited after the second PEG precipitation (lane 4). These two bands indicated that selective precipitation of the virions occurred resulting in the exclusion of other plant proteins from the supernatant. Obviously, subsequent use of 6% PEG + 0.125 M NaCl combined with low speed centrifugation resulted the most selective and effective CTV recovering during purification steps. SDS-PAGE showed that CP was the major band, which indicated an antigenic response when checked by commercial
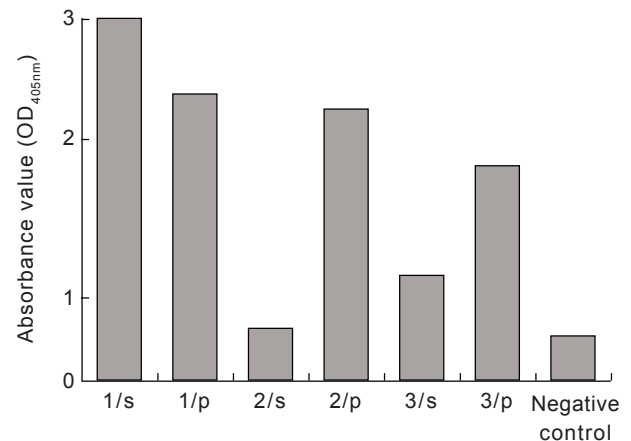

Fig. 2. Antigen recovery by ELISA; 1/s: clarified extract before PEG precipitation, 2/s and 3/s: supernatant after the first and second PEG precipitation, 1/p and 2/p: pellet after the first and second PEG precipitation, 3/p: resuspended pellet before further purified by SDS-PAGE.
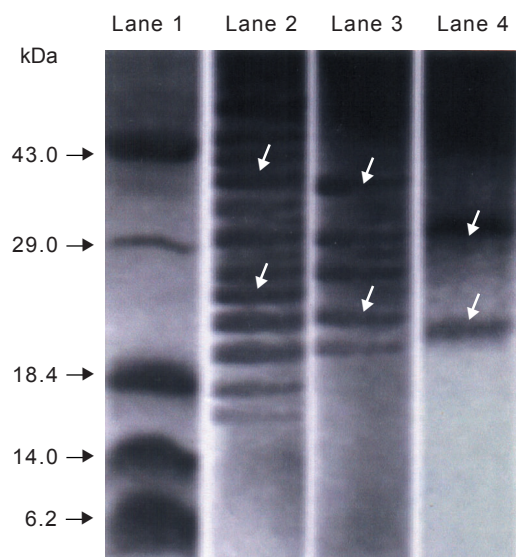

Fig. 3. SDS-PAGE pattern of viral proteins; lane 1: molecular weight standard, lane 2: purified before the first PEG precipitation, lane 3: purified after the first PEG precipitation, lane 4: purified after the second PEG precipitation; arrows indicate CTV-related protein bands.

antibody for CTV by ELISA. Therefore, semipurified CTV-CP could be obtained by using elution buffer followed by two to three low speed centrifugations.

Purification of semipurified preparations through SDS-PAGE resulted two protein bands specific to CTV with molecular weight of 25 kDa (p25) and 33 kDa (p33). The 25 kDa protein strongly reacted with commercial polyclonal antibody in DAS-ELISA,

whilst, the p33 kDa protein band reacted weakly. Compared to CTV-CP from previous studies (Bar-Joseph *et al*. 1972; Pappu *et al*. 1994; Febres *et al*. 1996) the calculated molecular mass of present CTV-CP was estimated 22-28 kDa. Since p25 reacted strongly with the commercial CTV polyclonal antibody, it is assumed that p25 is a coat protein which has antigenic determinant. Therefore, it was selected as the best candidate for use as the immunogen. This result was better than that reported by Dwiastuti *et al*. (1997).

Elution of a major band containing CP using elution buffer containing 0.25 M Tris-HCl pH 6.8 + 0.1% SDS followed by low speed centrifugation could separate the CP band from SDS-PAGE gels thus avoiding the presence of contaminating proteins of plant origin. At the final step, it yielded a characteristic nucleo-protein by UV absorption spectrum, although it was slightly flattened with no pronounced peak at 280 nm. Protein recoveries following the first and second elution using native and SDS elution buffer clearly increased the efficiency of the elution with three times more protein being eluted in the presence of SDS (42% and 8% of protein were eluted with and without SDS, respectively). In both cases, the amount of protein eluted in the second elution decreased to 20% with SDS and 5% without SDS. Combining the results of both elution runs, 50% of the protein was eluted in the presence of SDS, while only 13% was eluted without SDS. Specific antigen can be produced since SDS-PAGE is able to separate CTV-CP, a specific antigen which consists of antigenic determinant for CTV. In addition, it eliminated several virus purification steps, the protein preparation obtained had no detectable contaminant, and a less degraded CP preparation is obtained since the use of electro-elutor in ultrapurification was avoided. The simplicity of the method makes it economically acceptable because the antigen can be prepared without more sophisticated equipments.

The starting material used for purification in this work consisting of 50 g of bark tissue of semidormant flush yielded 750 μg of eluted CP as an antigen, which means that the amount of starting material should be enough for immunization up to 3-4 chickens. The contribution and advantage of this work are: firstly sufficient CTV-CP could be recovered to use as an immunogen following a minimal purification schedule, and secondly the highly purified antigen obtained by cutting out the CP band from SDS-PAGE gels avoided the presence of contaminating proteins of plant origin.

Production of Specific Polyclonal Antibody Against CTV

Fifteen grams of egg yolk purified by ammonium sulphate precipitation, membrane dialysis, and DE-52 cellulose chromatography yielded 28 fractions containing IgY with concentration ranging from 0.920 to 1.340. Fractions were then pooled, dialysed and re-examined spectrophotometrically for their $A_{280 nm}/A_{260nm}$ value. At this final step, from 15 g of yolk yielded 18.41 mg of purified IgY. Upon microagglutination test, the appearance of cloud as well as cell fragment and/or protoplast precipitation was first observed 1 hour after grid complete titration (AGCT) (Fig. 4). The monospecific antibody reacted strongly with CTV antigen diluted at 1:1,024. A medium reaction was performed at the dilutions of 1:4,096 and 1:8,192, and those above 1: 8192 reacted weakly (figure not shown). Visible reaction gradually increased within 8 hours following AGCT when all of the antibody dilution showed reaction with variable degree of intensity. Obviously, antibody with 1:1028 dilution still reacted strongly against antigen with 1:10,000 dilution, and antibody diluted up to 1:4,096 was still giving reaction against antigen at 1:1,000 dilution. The immunized chicken gave high titer antibody after three injections. Compared to previous study, by
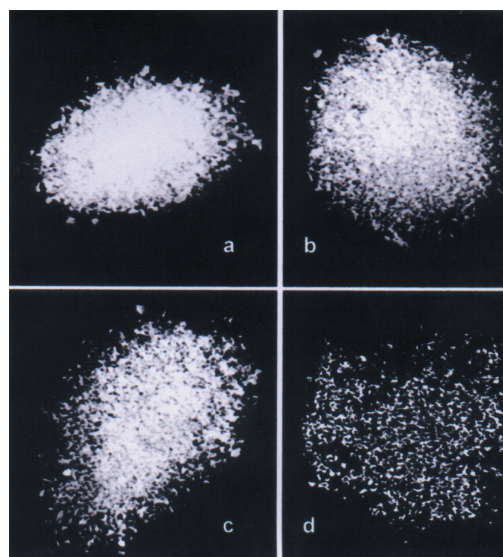

Fig. 4. Reaction of chicken polyclonal antibody generated from CTV-CP against purified virus in microagglutination test showing cloudy appearance as well as cell fragment and/or protoplast agglutination; a: dilution of 1:1,024, b: dilution of 1:4,096, c: dilution of 1:8,192, d: absence of cloudy appearance indicating healthy plant sap.

using 13.3 kDa SDS-PAGE protein band slices of purified CTV immunized onto rabbit, Dwiastuti *et al.* (1997) found that antibody produced had very low titer, less sensitive in agglutination test, and resulted negative reaction at 1:4 antibody dilution.

The titer of antibody recovered from eggs was high and had to be diluted for use in ELISA. By using semipurified virus preparation, in an indirect ELISA, the specific polyclonal antibody produced using the procedures described in this study had a titer of approximately 10^4. The titer was calculated on the assumption that this is the highest antibody dilution at which the signal in ELISA is at least two-fold higher than the signal for the healthy extract (Table 2). It was demonstrated by the OD ($A_{405\ nm}$) value following ELISA reading 2-24 hours after substrate addition (HASA), that an initial reaction was performed at 2 HASA, in which the effect of immuno-globulin vs. conjugate increased gradually up to 18 HASA and constant response prolonged up to 24

HASA. Optimal reading was obtained from reaction of the immunoglobulin diluted at 1:1,000 and conjugate diluted at 1:500. To illustrate the specificity of the antibody produced, we further confirmed in ELISA of samples from CTV-infected and healthy plants. The antibody gave a strong specific reaction with the CTV-CP band of approximately 25-kDa from CTV-infected plants. The reaction was equally strong for a severe, moderate, mild, and symptomless isolate of CTV, suggesting a broad reaction range of this antibody toward different CTV isolates. With the IgY diluted at 1:1000 and conjugate diluted at 1:500, disease/healthy ratio of four different isolates of CTV was ranging from 2.31 to 3.35, showing a significant difference as compared to negative control (Table 3).

The egg yolk laid by immunized chicken has been recognized as an excellent source of polyclonal anti-body for over decade. Specific antibody produced in chicken egg offers several important advantages over producing antibodies in other mammals. Because a

Table 2. Reaction of a 1:10,000 dilution of chicken antibody collected after three immunizations with citrus tristeza virus coat protein, obtained using PEG precipitation of bark extracts followed by SDS-PAGE purification.

Dilution of		OD ($A_{405\ nm}$) value (hour after substrate addition)[1]					
IgY	Conjugate	1	2	18	20	22	24
10^{-1}	1:100	0.160	0.297**	2.496***	2.536***	2.693***	2.695***
	1:500	0.135	0.207	1.623**	1.737**	1.857**	1.887**
	1:1000	0.136	0.195	1.437**	1.532**	1.640**	1.671**
	1:1500	0.117	0.153	1.364**	1.475**	1.594**	1.626**
	1:2000	0.137	0.195	1.376**	1.446**	1.565	1.601
10^{-2}	1:100	0.141	0.235	1.933**	2.089**	2.216**	2.239**
	1:500	0.165	0.263**	2.175***	2.306***	2.497***	2.540***
	1:1000	0.137	0.204	1.692**	1.820**	1.947**	2.035**
	1:1500	0.155	0.235	1.865**	1.980**	2.123**	2.178**
	1:2000	0.146	0.208	1.598**	1.704**	1.839**	1.907**
10^{-3}	1:100	0.133	0.255	2.060***	2.174***	2.283**	2.364**
	1:500	0.165	0.266**	2.046***	2.138**	2.259**	2.310**
	1:1000	0.111	0.149	1.031	1.078	1.167	1.192
	1:1500	0.149	0.176	0.958	1.014	1.112	1.140
	1:2000	0.143	0.190	1.254	1.337	1.459	1.503
10^{-4}	1:100	0.161	0.209	1.745**	1.820**	1.947**	1.949**
	1:500	0.159	0.246	1.759**	1.846**	1.958**	1.996**
	1:1000	0.124	0.180	1.333	1.412	1.517	1.549
	1:1500	0.116	0.150	0.953	1.020	1.110	1.132
	1:2000	0.137	0.178	1.098	1.167	1.264	1.287
Negative control (NC)[2]		0.106	0.128	0.670	0.721	0.789	0.805
	2 x NC	0.211	0.256	1.340	1.442	1.578	1.610
	3 x NC	0.318	0.384	2.010	2.163	2.367	2.415

[1]Values correspond to the average of three wells in each two replicated 96-well Numemicrotiter plates.

[2]West Indian lime maintained at glass house.

**OD ($A_{405\ nm}$) value exceeded 2 times than those of negative control.

***OD ($A_{405\ nm}$) value exceeded 3 times than those of negative control.

Table 3. Absorbance (OD$_{405nm}$) values of IgY diluted at 1:1,000 and conjugate diluted at 1:500 against different citrus tristeza virus (CTV) isolates.

CTV isolate	Variety/cultivar	Symptom[1]	Absorbance (OD$_{405nm}$) Means + Sd[2]	Disease/ healthy ratio
UPM/-T012	*Citrus grandis*	VC	2.693 + 1.06	3.35
UPM/-T015	*Citrus aurantifolia*	VC, SP	2.834 + 0.90	3.52
RIF/T-016	*Citrus aurantifolia*	VC	1.857 + 1.03	2.31
RIF/T-052	*Citrus reticulata*	VC, SP	2.140 + 0.81	2.66
Negative control			0.805 + 0.82	

[1]VC: vein clearing, SP: stem pitting.

[2]Values correspond to the average of three wells in each two replicated 96-well Nunc-microtiter plates, read 60 min after substrate addition to the wells.

single egg contains as much antibody as an average bleed from rabbit, simple, non-invasive approach presents an appealing alternative to conventional polyclonal antibody production methods. Purification of IgY does not require animal bleeding, in addition, the eggs from immunized chicken provide a continual, daily source of polyclonal antibody, and this convenient approach offers greater compatibility with animal protection regulations (Van Regenmortel 1982).

Due to the phylogenetic distance between birds and mammals, there is a greater potential of producing a higher percentage of specific antibody against mammalian antigens when using chicken. Highly conserved mammalian proteins sometimes fail to elicit a humoral immune response in animals, such as rabbits, that are traditionally used for generating polyclonal antibody. Since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial or mammalian crystallizable fragment, non-specific binding is reduced, and the need for cross-species immunoabsorption is also eliminated (Polson *et al*. 1985).

CONCLUSION

Procedure described in this study was able to optimally recover high purity CTV-CP antigen, and effective to minimize virus loss during purification processes and the possible contamination of the final immunogen with plant components. Total amount of CTV-CP eluted was 750 µg from 50 g bark of diseased plant. Amount of polyclonal antibody produced from 15 g of immature eggs was 18.41 mg. The antibody reacted strongly with semipurified CTV antigen diluted at up to 1:8192 in microagglutination test, whereas by indirect ELISA, the highest titer was approximately 10^4. This is the first study reporting production of specific polyclonal antibody for CTV-CP using Indo-

nesian isolate, which showed effective in detecting CTV from mild, medium, and strongly diseased symptoms. The simplicity of the procedure makes it economically acceptable and technically adoptable as the antigen can be prepared with limited chemical and equipment.

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