Antigenic Properties of Fixed and Unfixed Particles of Some Cucumber Mosaic Virus Strains

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Abstract. The virus particle of Cucumber mosaic virus (CMV) is unstable and degrades during and after virus preparation. For longterm storage, either 0.25% glutaraldehyde or 0.2% formaldehyde are used as protein cross-linking reagents for stabilising the antigenic binding sites of the viral protein. The glutaraldehyde effect on the stability of purified CMV strains preparation after longterm storage were investigated by serological reactions with either fixed and unfixed CMV antisera. These preparations were also analysed by sucroce density gradient centrifugation and ISCO density gradient fractionation, then by gel electrophoresis and by electron microscopy. During longterm storage (more than one year) some fixed and unfixed virus strains had degrade partially as shown by the appearance of double precipitine lines in gel immunodiffusion tests and the shape of absorbance peaks of ISCO density gradient fractionation. However, the degradation of virus particles was not apparent by electron microscopy. On the other hand, in agarose gel electrophoresis the virus particle of CMV strains produced pattern of mutiple bands that shown that virus was certainly degraded. It appeared that the fixed virus had faster mobility bands than unfixedvirus.

Keywords: fixed and unfixed particle, CMV strains, degradation, longterm storage

1. INTRODUCTION

Cucumber Mosaic Virus (CMV) particle is unstable virus, its protein as a coat of RNA is degradable. Therefore for long term storage usually used either 0.25% glutaraldehide[1] or 0.2% formaldehyde [2]. These reagent used as protein crossed linking to stabilised antigenic binding sites of the viral protein and it called fixed antigen. When the fixed antigen used as the immunogen, increases the titre of the antiserum as demonstrated of CMV [2]and Alfalfa mosaic virus [3].

Both fixed and anfixedantigen were tested by the fixed and unfixed *antisera. These* was investigated becaused the particles seem to be degrade in gel immunodiffusion[4]. Serological used in this study was done by gel immunediffusion and by plate trapped antigen (PTA) indirect ELISA whereas antibody and antigen complex was detected with commercial goat antirabbitIgG alkaline phosphatase conjugate. It is more sensitive for detecting distantly related viruses when using purified virus rather than crude extract of leaf sap [5]. In this study, it was investigated whether the glutaraldehyde effected on the stability of purified CMV strains preparation after longterm storage by serological reactions with either fixed and unfixed CMV antisera.

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These preparations were also analysed by sucrose density gradient centrifugation and ISCO density gradient fractionation, then by gel electrophoresis and by electron microscopy.

2. MATERIALS AND METHODS

2.1 Reactivity of homologous fixed and unfixed antigens with fixed and unfixed antisera in gel immunediffusiontests.

The fixed antigen prepared by dialysed purified virus with 0.3% glutraldehyde in borate buffer pH 8.0 and stored at 4 °C, and the unfixed antigen prepared by adding 50% glycerol in borate buffer pH 8.0 of purified virus and stored at -20°C. A tests were done in a 0.7% gel in 10 mM PO4 buffer pH 7.0 in a petridish as shown in a pattren of Fig. 1. Virus of 10 ml at a concentration of 1 mg/ml were placed at peripheral wells. The homologous antiserum was placed at the central antigen wells. Gels were incubated at room temperate for 2hrs.

2.2 Reactivity of fixed and unfixed virus in plate trapped antigen(PTA)

Microtiterplates were pre-coated directly with either fixed and unfixed antigen of Twa (subgroup I), L ny

(subgroup II) or Ywa (intermediate serogroup) in twofold dilution series. The diluted series was startedat 0.5 ug/ml, then were blocked with BSA solution. Either fixed or unfixed antisera to Twa or Lny were used at 1/2000 dilution as the first antibody.GoatantirabbitIgG AP-ase conjugate was used as a second antibody at 1/9000 dilution. Theabsorbency was read at 405 nm

2.3 Analysis of physical changes in fixed and unfixed virus by sucrose density gradient centrifugation

Three mg of purified viruses (fixed and unfixed preparations) which 20-30 months stored were layered in 13 ml (SW 41) tubes containing 5-30 % sucrose density gradient and run for 2 ½ hours at 25.000 rpm. The physical changes in particle virus was analysed with an ISCO density fractionator.

2.4 Analysis of physicochemical changes of fixed and unfixed virus by agarosegelelectrophoresis

Virus preparation used in this experiment were the same with no. 3 above. Two mg/ml of fixed or unfixed virus were loaded on 1.5% agarose gel containing either TAE or TBE buffer then subjected to electrophoresis at 100 V for 70 minutes. The gel was firstly stained with ethidium bromide to observe RNA pattern, then stained with coomassie blue to observe virus protein pattern [3].

2.5 Analysis of virus particle by electron microscopy

Particles of some CMV strains were observed under electronemicroscope to determine whether particles degraded during longterm storage. Virus particles were stained 2% uranyacetat or a mixture of 0.2% uranyl acetate with 3% K-phosphotungstic acid pH 7.0.

3 RESULTS AND DISCUSSION

3.1 Reactivity of homologous fixed and unfixed antigens with fixed and unfixed antisera in gel immunodiffusion tests.

Figure 1 showed that some of unfixed virus at the same months of stored had degraded by double precipitate lines and they all appeared the homologous reaction except antigen Y2 against Y2 antiserum. That was also appear by the fixed virus (A1). It was concluded that both unfixed and fixed virus protein degraded after several times storaged.

Fig 1. Reaction of homologous fixed and unfixed antigens with antiserum prepared against either fixed or unfixed antigens in gel immunodiffusion. The reaction was recorded after 40 hrs incubation. The letter with subscip 1 refers to fixed virus, and subscrip 2 refers to unfixed virus. The antiserum wells were labelled with lowercase letter.

In gel immunodiffusion tests, the same antigen prepareation produced different precipitinlines when tested against homologous fixed or unfixed antiserum (Fig 1). Some antigen produced double precipitinlines which indicated that degradation of virus particles had occured. It was possible that degradation occured either when the virus prepared ,stiored or in the rabbit during immunisation (fig. 1 and 2). It was interesting to note that some strains produced double precipitin lines where the outer line formed a spur to the neighbouring distantly related antigen, whereas some produced spur to the neighbouring closely related antigen of the same serogroup. Double precipitinines commonly procused in the interaction of fixed antigen to the antiserum to unfixed virus (Fig 2). The degradation of unstable virus (CMV) into its constituent subunits may lead the production of antibodies which do not react into intact virions,[2]found difficulties in making antiserum from unfixed virus in mice rather in rabbit. A possible explanation, was the unfixed virus was degradate soon after injecting to mouse, resulted in the inability ofmice to produce antibodies specific to injected strains [6].

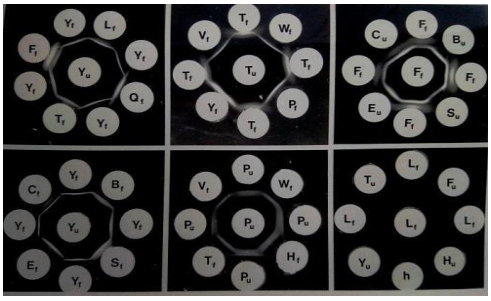

Fig 2. Reactivity between combined placement of fixed and unfixed antigens with either fixed and unfixed antiserum in gel immunodiffusion tests.

3.2 Reactivity of fixed and unfixed virus in plate trapped antigen(PTA)

This tests were to determine the dilution end point of fixed and unfixed antigen in PTA indirect ELISA (Fig I). The lowest concentration of virus reacted with both fixed and unfixed antiserum in this test was 31.5 ng/ml. Fixed antiserum gave slightly stronger reaction by homologous fixed unfixed antigen (Fig. 3 IA, C). The same reaction were shown also with unfixed antiserum by unfixed antigen (Fig.3 I B,D) than by fixed antigen.

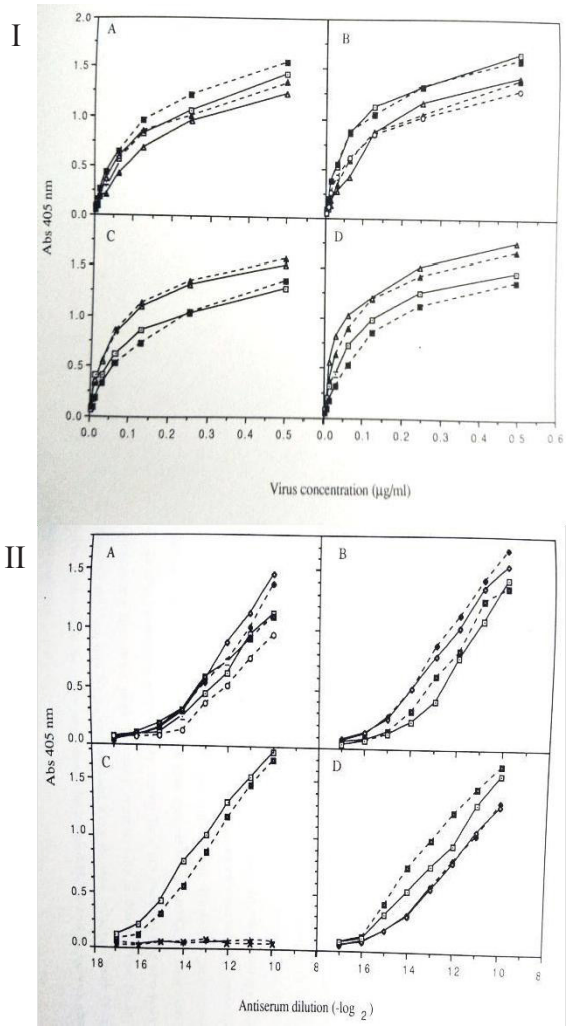

Fig 3. Plate trapped antigen (PTA) indirect-ELISA. I. Reactivity the homologous antisera with fixed and fixed antigen in twofold dilution. II.Reactivity the homologous antigenwiththeantiserawereused inatwofolddilution. ▲—▲ Fixedantigen, ●- - -● unfixedantigen,. ■-·-■TMV-U1 and ◆- - -◆TAV-V.

Another test was to determine the dilution end point antisera against fixed and unfixed antiserum in PTA. Wells ofmicrotiter plate were pre-coated with either fixed or unfixed antigen 0.5 mg/ml. Unfractionatedunfixed and fixed antisera in twofold dilution series were used as the first antibody, starting with dilution of 1/1054. Goat antirabbit AP-ase was used at 1/900 dilution. With this experiment, a very low dilution antiserum could still detect the homologous antigen at a concentration 0.5 mg/ml. Fixed antigens reactedmore strongly to homologous fixed antiserum than did to unfixed antigen (Fig. 3 II A,C).

3.3 Analysis of physical changes in fixed and unfixed virus by sucrose density gradient centrifugation

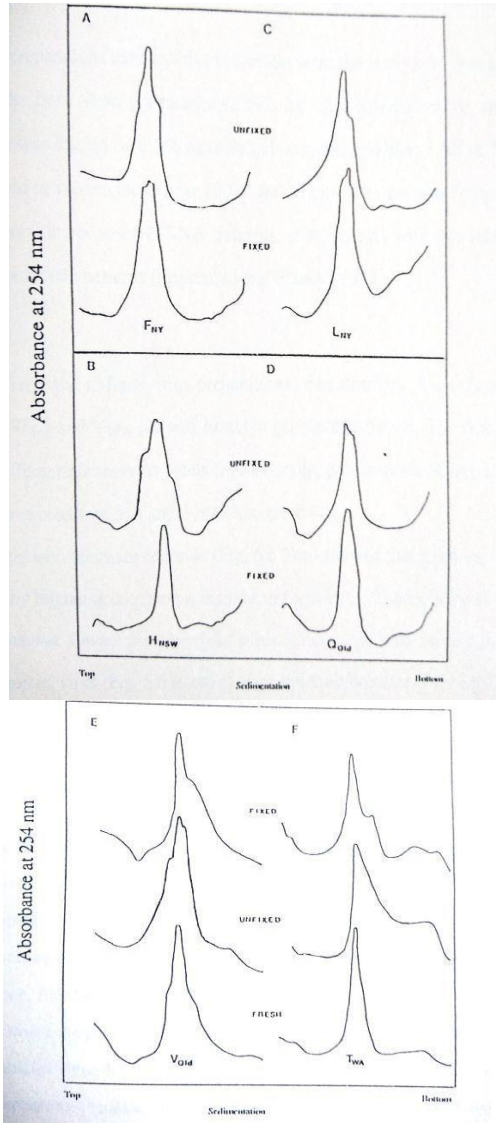

Fig. 4 Sucrose density gradient sedimentation analysis of fixed and unfixed virus after storege for more than one year, using ISCO density gradient fractionation.Preparation of (A) fixed Fny (26 months) and unfixed Fny (23 months), (B) fixed Hnsw (32 months) and unfixed Hnsw (23 months), (C) fixed Lny (28 months) and unfixed Lny (27 months), (D) fixed Qqld (34 months) and unfixed Qqld (30 months), (E) both fixed and unfixed Vqld (21 month), (F) fixed Twa (25 months) and unfixed Twa (25 months) were compared with fresh virus preparation.

On the other hand, this experiment was good enough to detect and separate CMV strains with TAV-V and TMV-U1 (Fig. 3 IIC). Therefore, the PTA-indirect ELISA technique it is economical, practical and suitable for virus detection of a wide range of plant viruses. This four families contained six species virus had been tested were from the family

Secoviridae, Comovirus genus (*Squash mosaic virus*, SQMV and *Cowpea severe mosaic virus*, CPSMV), family *Bromoviridae, Cucumovirus* genus (*Cucumber mosaic virus*, CMV), family *Potyviridae, Potyvirus* genus (*Cowpea aphidborne mosaic virus*, CABMV and *Zucchini yellow mosaic virus*, ZYMV) and *Sobemovirus* (*Papaya lethal yellowing virus*, PLYV) in infected plant tissues (12)ELISA plate wells were also treated with extracts from healthy plants of cowpea [*Vignaunguiculata* (L.) Walp subsp. *unguiculata*], papaya (*Carica papaya* L.) and melon (*Cucumismelo* L.) to function as control.(13) considered that the new developed PTA-ELISA kit could be used to detect simultaneously up to four different plant virus families, in the same ELISA plate, instead of the use of kit for each virus like the commercial kits available by multinational companies.

Figure 4 showed the sucrose gradient density analysis of fixed and unfixed virus after longterm preparations (A, B, C, D) and comparison with fresh virus preparation (E, F). The UV absorbance profiles for fixed preparation ofFnyandLnyviruswhichhadbeenstoredfor 26and28 months, respectively, showed heterogeneity. Preparations of fixed Hnsw and Qqld which were kept for 30months were more stable than unfixed of the same strain of virus after storage for 23, 27, and 30 months, respectively. Fixed Twa (25 months) had two peaks, the peak preceding the virus peak possibly representing a dimer. The fresh virus preparation of Twa and Vqld and unfixed Twashowed only one peak. The peak above the virus peak shown by unfixed Hnsw and Qqld was possibly degradeparticles.

Some CMV strains varied in stability after fixation, some It was concluded that strains of CMV have different stabilities after fixing with glutaraldehyde, as shown by the different shapes of the absorbance peak although glutaraldehyde used for crossed linking of protein to stabilised antigenic binding sites [2]. Thus, fixed virus of some strains were stable and some degraded partially during storage and detected by appearance of more slowly sedimented component after analysis by sucrose density gradient sentrifugation (Fig. 4). This results was in unagreement with [2] that glutaraldyde increased the stability CMVparticles.

The structure of antigenic many plant viruses have been investigated through epitopes recoqnised by monoclonal and policlonal antibodies. The rod shape virion, i.e. Tobamovirus (TMV) the epitopes has been localized along the surface virion[7,8]. Coat protein CMV and other genus of Cucumovirus are interest antigenicdeterminant sites because CP is primary determinant of aphid transmission [9]. So the epitopes is a useful analysis tool of topography of CP structural and antigenic of CMV [10].

3.4 Analysis of physicochemical changes of fixed and unfixed virus by agarose gelelectrophoresis

Both fixed and unfixed virus preparations of strains Fny, Twa, Hnsw, Cnsw, Lny, Vqld, Wtas, and Ywa showed bands in gel electrophoresis (Fig. 5). Strains of CMV produces different numbers of bands. For example, preparations of fixed Twa, Vqld, and Ywa virus produced 5, 4, and 3 bands,respectively.

Staining with ethidium bromide (Fig. 5 top) showed that fixed viruscontained a more rapidly migrating component than the unfixed virus. The mobility of bands from unfixed virus was slower than of fixed virus. Coomassie blue stained the bands of fixed and unfixed virus (Fig 5 Bottom) after ethidium bromide stained (Fig. 6 ~~Top~~). The used of TBE as electrophoresis buffer were separated virus component better than TAE buffer. The mobility of some component of unfixed virus appears to be more slightly slower than that of fixed virus [4]. This result was in agreement with observation on the mobility of AMV particles. The fixed AMV particles produce a sharp bands while the unfixed particles produced diffuse bands, indicating that the unfixed virus particles had a lost their integrity as a result of an effect of their protein-RNA interaction [3]. However, inmyexperiment both fixed and unfixed CMV particles produced sharp bands. The fixed virus was distinguish by producing an additional more rapidly migrating band but the composition of this band was not determine (Fig. 5). The binding affinity of MAb against different strains of CMV is specific key to identify the epitope mapping of the CP site [10].

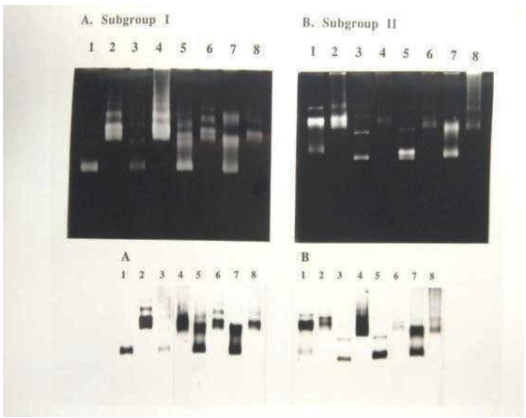

Fig 5.Analysis of the electromobilities of fixed and unfixed virus from (A) subgroup I (Fny, Twa, Hnsw, Cnsw) and (B) subgroup II (Lny, Vqld, Wtas, Ywa. Track no 1, 3, 5, 7 were fixed virus and tracks no. 2, 4, 6 and 8 were unfixed virus. The gels were subsequently with ethidium bromide to visualize the viral RNA (Top) and coomassie blue to visualize the viral protein (Bottom).

3.5 Analysis of virus particle by electronmicroscopy

The electron microscopy seemed that the particle of neither fixed nor unfixed CMV particles were degrade (Fig 5 A, B, C, D). Apparently the unfixed of Vqld particles were smaller than the fixed particles (Fig.5 C, D), because it stained with the positively stain partially, and the diameter of particle were the same and all the particles appeared as intact particles after longterm storage. This could be caused the stained prevent the fixed and unfixed particles more degraded. [11] especially the addition stain of 0.2% uranyl acetate, pH 7 than with phosphotungstic acid (PTA) alone.

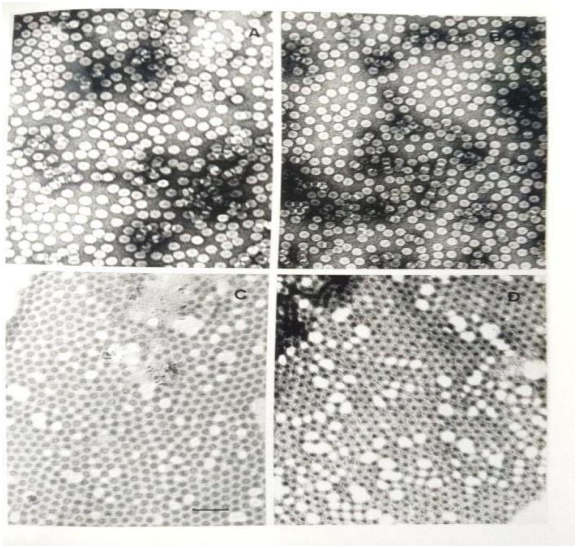

Fig 5.Electrom micrograph off virus particles from fixed and unfixed purified virus after longterm storage. Preparationof(A) unfixed and fixed virus (B) of strain Hnsw were stained with 2% uranyl acetate and (C) unfixed and (D) fixed virus of strain Vqld were stained with a mixture of 2% uranyl acetate and 3% phosphotungstic acid (pH 7.0). Both fixed and unfixed particles had the same diameter. Bar scale 100 nm.

4 CONCLUSION

Some fixed (with 0.25% glutaraldehyde) and unfixed (with adding 50% glycerol) CMV strains has dedraded partially after stored more than one year as shown by the appearance of double precipitin lines in gel immunediffusion tests and by the shape of absorbance peaks in sucrose density gradient sedimentation analysis and in electromobilities analysis. However, this degradation of virus particles was not apparent by electron microscopy.

References

1. A.H. Korn, S.H..Fearheller, E.M. Fillachione. Glurahdehyde, nature of the reagen. *J.Mol.Biology*65:525-529 (1972).
2. R.I.B. Francki, N. Habili. Stabilization of capsid structure and enhancement of immunology of Cucumber mosaic virus (Q strain) by formaldehyde. *Virology*48:309-315 (1972).
3. M.R. Hajimorad, R.I.B..Francki. Serological diffenciation of some strains of Alfalfa mosaic virus with polyclonal antibodies. *Phytopathology* 8:1603-610 (1991).
4. W.S. Wahyuni, R.G..Dietzgen, K. Hanada, R.I.B. Francki. Serological and biological variation between and within subgroup I and subgroupII strains of Cucumber mosaic virus (CMV). *Plant Pathol.* 41: 282-297 (1992).
5. M. Jaegle, M.H.V. Regenmortel. The use of ELISA for measuring the extent of serological cross-reactivity between plant viruses. *J. Virol. Methods* 11:189-198 (1985).
6. M.H.V. Regenmortel. Serology and Immunochemistry of plant Viruses. New York: Academic Press Inc (1982).
7. M.H.V. Regenmortel. Tobacco mosaic virus: antigenic structure. In: Van Regenmortel, M.H.V., Fraenkel-Conrat, H. (Eds.), In The Plant Viruses. The Rod-Shaped Plant Viruses. Plenum Press, New York, pp. 79–104 (1986)
8. I.E. Dore, D. Weiss,. M.H.V. Altschuh,. V. Regenmortel. Visualization by electron microscopy of the location of tobacco mosaic virus epitopes reacting with monoclonal antibodies in enzyme immunoassay. *Virology* 162, 279–289.
9. K.L. Perry. L. Zhang, M.H. Shintaku, P. Palukaitis. Mapping determinants in Cucumber mosaic virusfor transmission by Aphis gossypii. *Virology* 205:501-595 (1994).
10. H.S. Zein, A. Zeina, T. Jaime, D. Silva, K. Miyatakec. Antigenic properties of the coat of Cucumber mosaic virus using monoclonal antibodies. *J. Virological Methods* 162:223-230 (2009).
11. R.I.B. Francki. J.W. Randles, T.C. Chambers, S.B. Wilson. Some properties of purified Cucumber mosaic virus (Q strain). *Virology* 28:749-741 (1966).
12. A.K.Q. Nascimento, *G.Silva-Barbosa, AQ. Kelly*.Biological, physical, and molecular properties of a *papaya lethal yellowing virus* isolate. *Plant Disease* 94:1206-1212, 2010.
13. A.Q. Kelly, AKQ Nascimento,G. Silva-Barbosa, JA.Araújo Lima.. A simple kit of plate-trapped antigen enzyme-linked immunosorbent assay for identification of plantviruses**.** *Rev. Ciênc. Agron***.**48: 42-47˙2017**.**