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Dissociation of Antigen-Antibody Complexes for some Plant Viruses in DAS-ELISA Tests

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Glycine-HCL buffers at pH 2.0 to pH 4.0 were used to study their effects on dissociation of immunoreagents in direct ELISA. Two spherical viruses, Cucumber mosaic virus (CMV) and Tomato black ring virus (TBRV) were used in polystyrene plates separately and two elongated ones Potato virus Y (PVY) and Potato virus S (PVS) were also used separately in our investigations.

Acid buffer between pH 2.0-2.5 dissociate about 50% of the conjugate of CMV and TBRV while PVS and PVY eluted only by 30% even by pH 2.0. Also, virus antigen of elongated viruses could not be eluted. The bond between plate and immunoglobulin G was less affected by acid buffer than other bonds.

Keywords: Acidification, CMV, dissociation, PVS, PVY and TBRV.

The difficulty to control phytopathogenic viruses by using pesticides, induction of systemic resistance or by biological control, make virus detection and identification received a great attention (Murphy *et al*., 2001). Science enzyme-linked immunesorbent assay (ELISA) was introduced in plant virology, it becomes one of the most wildly and sensitively serodiagnostic methods used to detect different plant viruses (Etieene *et al.,* 1991; Vaira *et al*., 1993; Hunter *et al*., 1995 and Elbeshehy and Sallam, 2010). A wide availability of ELISA method helped monitoring the crop healthy at fields (Watanabe, 2002 and Bobrovni, 2003). By using ELISA, it is possible to examine one sample against large number of different antiserum. Also, to obtain a reliable estimation of the degree of cross-reactivity existing between either two viruses (Van Regenmortel *et al*., 1988) or the antigen of the type strain (Bar-Joseph and Solomen, 1980). Although, it is considered one of the most sensitive serological methods, ELISA imposes several problems associated with sample preparation and test costs (Gase, 1990 and El-Afifi *et al*., 1997).

In DAS-ELISA, infected sap containing virus particles was used after a specific gamma globulin G (IgG) was applied to the polystyrene plates. Then, alkaline phosphatase conjugate (AP-conj) was added before substrate (Beemester and deBokx, 1987). The presence of virus particles that react positively with an added specific antibody will result in producing absorbance value (Lukasik *et al.*, 2000). Nevertheless, eluting the reacted immunoreagents will allow retesting the presence of another virus antibody (Orosz and Ovadi, 2002).

Converse and Martin (1990) reported that dissociation of antigen antibody complexes can accomplish by acid buffer treatments. Also, Hassan (1999) examined the effect of buffer molarities and time treatment of microtiter plates on dissociation of Potato virus S (PVS) from polystyrene plates.

This investigate was carried out on four viruses belong to different groups, two spherical (CMV & TBRV) and two elongated ones (PVY) and (PVS). The main aim was to find the possibilities of dissociation antigen-antibody complexes from ELISA plates by using glycine-HCL buffer to reduce the test costs and the probability to re-use ELISA plates more than one time.

Materials and Methods

Virus propagation:

Cucumber mosaic virus (CMV) was isolated and propagated in squash *Cucarbita pipo* L., cv. Eskandarany (El-Sagheer and Hassan, 2004). Tomato black ring virus (TBRV) was isolated from grapevine cv. Roomy Red and propagated in *Chenopodium amaranticolor* Cost & Reyn (Hassan, 2005). Potato virus Y (PVY) isolated from potato (*Solanum tuberosum* L.) cv. Diamant (Hassan and Youssef, 2006) and propagated in *Nicotiana debandii* L. Potato virus S (PVS) was propagated in *Chenopodium quinoa* Wild (Abdel-Aziz *et al*., 2000).

All plants were grown in air-condition insect-proof greenhouse at 27-30° C in porous clay pots (No. 20) containing fertile soils and using 16 hours photoperiod.

Sample preparation:

Leaf tissues of infected plant by CMV and TBRV were triturated 1:10 (w/v) in 0.05 M pH 7.4 phosphate buffered saline containing 0.05% Tween-20 and 2% polyvinyl pyrolidone (PVP). The triturate was pressed through two layers of cheese cloth and 0.2 ml aliquots pipetted into each well. The PVY and PVS infected tissues were triturated in 0.05 M sodium carbonate buffer (pH 9.6) containing 0.02% sodium azide.

DAS-ELISA procedure:

The procedure of DAS-ELISA proposed by Clark *et al*. (1986) was used during this investigation. Gamma globulins of CMV, TBRV, PVY and PVS or IgG-conjugated with alkaline phosphates (AP) were purchased as an ELISA kits from Agdia Inc. USA. Each gamma globulins were absorbed (1:10 v/v) with healthy plant extracts. Specific gamma globulin (IgG) for each virus was diluted 1:1000 in coating buffer (0.05 M sodium carbonate buffer, pH 9.6 and containing 0.05% sodium azide).

Each virus was performed in five-strips, 55-well (Nunc flat-bottomed microtiter strips), Dynatech Immunoassay system, Denmark. Absorbance at 405 nm (A_{405}) was recorded using Titertek Multiscan ELISA Microtiter plate reader.

Acid buffer treatments:

From each 5 strips used for each virus, a single strip was used to study a single pH value. Values were recorded after absorbance, then all microtiter plates were emptied, washed twice with PBS-Tween and filled (700 µl) with 0.1 M glycine – HCl buffer (Xu *et al*., 2010) adjusted to pH values between 2.0 and 4.0 with 0.5 intervals. The strips were incubated at room temperature overnight and washed twice with PBS-Tween.

To determine the effect of pH on removing the enzyme-conjugate from virus antigen, freshly prepared substrate was re-added to three wells in each strip. After 1 h incubation at room temperature the A_{405} values for all wells were re-determined.

To determine the effect of pH on cleavage the bond between virus antigen and specific antibodies, infectious sap was added to the second 3 wells and incubated for 24 h at refrigerator, washed for 3-min. Then fresh enzyme-conjugate was added. After 3 h incubation at 37° C the conjugate was removed. The strips washed three times with PBS-Tween and fresh substrate was added. The A_{405} values were recorded after one hour incubation.

To determine the effect of pH on breaking down the bond between IgG and polystyrene plate, the last three wells were treated sequentially with fresh dilutions of virus specific gamma globulins, enzyme-conjugate and substrate. After one hour incubation, the A_{405} values were recorded.

Healthy plants extracts of each host were used as a blank in the first and last well for each strip. The ELISA values for virus elution were expressed as percentage of dissociation for each treatment. All experiments were repeated three times and the data are presented as mean ± standard error for means.

$$\text{Dissociation (\%)} = \frac{\text{Value of 1}^{st}\text{ ELIZA - Value of 2}^{nd}\text{ ELIZA}}{\text{Value of 1}^{st}\text{ ELIZA}} \times 100$$

Results

ELISA measurement for infected plants:

Table (1) shows ELISA values for the tested viruses. It is clear that the A_{405} for spherical virus is higher than elongated ones. It is 2.72 and 2.06, respectively. The highest value is recorded in *Ch. amaranticolor* infected by CMV (2.95) whereas the lowest one was recorded in *C. quinoa* infected by PVS (1.96). The optical density (A_{405}) for the healthy tissues ranged from 0.038 to 0.052.

Table 1. ELISA values (A_{405}) for extracts of some host-plants infected by tested viruses

Virus			ELISA values for	
Name	Group	Shape	Infected tissue	Healthy tissues
TBRV	Nepovirus	Spherical	2.84±0.22	0.052±0.022
CMV	Cucimovirus		2.95±0.19	0.049±0.014
Mean			2.72	0.051
PVY	Potyvirus	Elongated	2.15±0.19	0.041±0.012
PVS	Carlavirus		1.96±0.20	0.038±0.019
Mean			2.06	0.040

Effect of pH on cleavage enzyme-conjugate from virus antigen:

Figure (1) show the effect of acidification on antigen-antibody complexes. It is obviously clear that low pH cleavage the bond between each virus and alkaline-phosphatase conjugate. These effects were studied by resubstrating the plates after the acid treatments and then measure the recovered absorbance at all pH's with all the tested viruses.

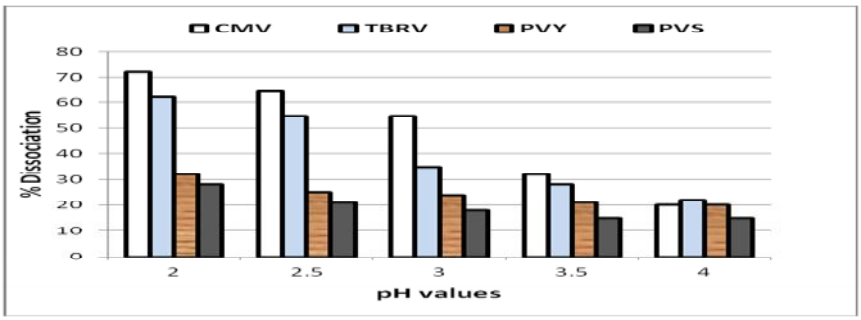

Fig. 1. Effect of pH values on cleavage the bond between alkaline-phosphatase and some plant virus.

Almost little dissociation was recorded at pH 3.50 and 4.0 especially for PVY and PVS. Nevertheless, more than 50% of CMV and TBRV were dissociated by buffer solution pH 2.0 and 2.5. In general, the highest elution was recorded by CMV while the lowest one was recorded by PVS.

Effect of pH on cleavage bonds between virus antigen and antibodies:

Figure (2) show the effect of pH values on cleavage the bond between immunoglobulin G (IgG) and each virus-antigen. Generally, the percent of dissociation is decreased by increasing the pH values. It is clear that the spherical viruses were eluted more than the elongated ones. It did not eluted more than 25% even at lowest pH value.

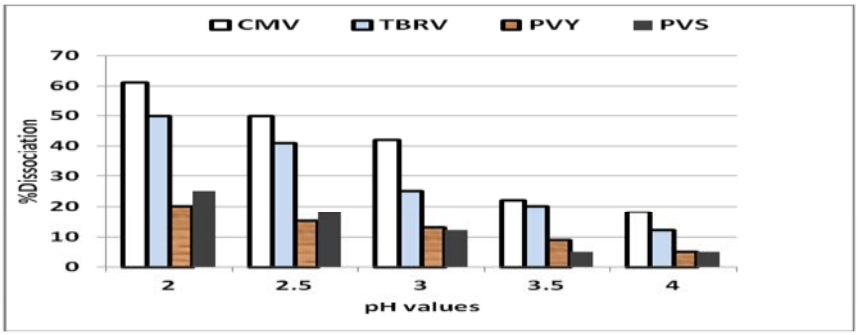

Fig. 2. Effect of pH values on cleavage the bond between immunoglobulin G and virus antigens during DAS-ELISA test, expressed as percent of dissociation.

It is also clear that dissociation percent for PVY is comparable with that for PVS. The maximum dissociation resulted from treatment CMV by buffer pH 2.0 (61%) while the lowest one was recorded in case of PVS treated with pH 4.0 (5%).

Effect of pH on cleavage bonds between IgG and polystyrene plate:

Figure (3) illustrate the effect of different pH (from 2.0 to 4.0) with 0.5 intervals on cleavage the bond between polystyrene plates and IgG during DAS-ELISA as indicated as dissociation percentage. These effects were studied by re-adding IgG, virus antigen, conjugate and substrate to the plates after the acid treatments and then measure the recovered absorbance at all pH's with all the tested viruses.

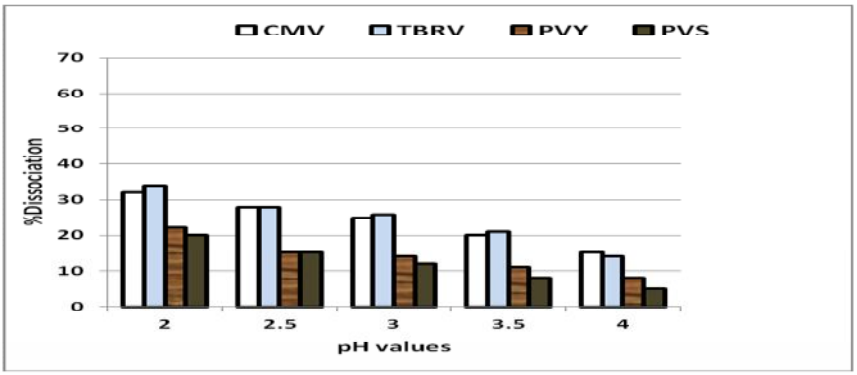

Fig. 3. Effect of pH values on cleavage the bond between polystyrene plates and immunoglobulin G during DAS-ELISA test expressed as percentage dissociation.

The effect of pH is increased by increasing the acidity. The most affected bond was recorded in case of CMV, but this bond is less affected than the bond between IgG and antigen or the bond between antigen and the conjugate. Percentage of dissociation ranged from 32 to 15. While the lowest one was recorded in case of PVS. Percentage of dissociation ranged from 20 to 5 at pH 2 to 4, respectively. Generally, the bond between ELISA plates and spherical viruses was more affected than that between the elongated virus and the plates.

Discussion

Usually, microtiter plates discarded after any ELISA test because the virus-specific antibodies complex interferes with detection of another test.

Elution of some viruses, *i.e.* Citrus tristeza virus (CTV), Carnation mottle virus (CarMV), Carnation yellow fleck virus (CYFV), Tobacco mosaic virus (TMV) and Potato virus S (PVS) and their respective conjugates from plates-bound antibodies have been demonstrated in ELISA double-sandwich (Kim *et al.*, 1990; Kummer and Li-Chan, 1998 and Hassan, 1999). According to them and another early report of Van Regenmortel (1982), dissociation of antigen antibody complexes can be accomplished by acid buffer treatments.

Glycine-HCL buffer was used to dissociate one or more immunoreagents in ELISA complex in the system of two spherical viruses (CMV) and (TBRV) and two elongated ones (PVY) and (PVS).

After the first test completion and the plates (strips) were washed with PBS, glycine-HCL buffer was added and left overnight to allow acidification to affect on the immunoreagent complexes. The effect of acidic buffer on the conjugate-antibodies complex was tested by reading the substrate to the plates (strips) directly after the final wash to detect the conjugates that still bound and active. It is evident that most but not all the activity was removed at pH below 2.5. When pH value increased, the dissociation decreased.

In order to relate those effects to its actual casual of the three immunoreagents components, each reagent and substrate afterwards were separately added. When the conjugate was added before the substrate step, an increase in the overall reading was obtained due to the conjugate. Again, the dissociation of the conjugate seems to follow the same pH pattern as when the substrate alone was added. Adding antibody, conjugate and then substrate was allowed to be regain most but not all the lost activity in plates (strips) within pH ranged from 2.0 to 2.5. This loss is obviously due to virus that had detached from the plates.

Most if not all activity was recovered at pH over 3.50 except with TBRV which seems to attach very weekly to the polystyrene plates. This data is in agreement with other reports of many researchers (Hoylaerts *et al*., 1990; Hassan, 1999; Lukasik *et al*., 2000 and Qingyou *et al*., 2007).

The increase in the second test reading over than the level of the first test at pH over 3.50 with CMV and PVY may be due to activation of binding sites after pH treatments. This could be attributed to the elimination of blocking elements at the binding sites as agreed with the cited information by Hull (2002) or exposing non-surface antigenic sites in the reaction media. Theses results are in agreement with data obtained by many researchers (Gase, 1990; Kim *et al*., 1990, Hassan, 1999, Stanker *et al*., 2008 and Xu *et al*., 2010) and almost disagree with Jana and Ali (1999) who mentioned that a competition between virus and non-viral protein cannot be eluted.

In conclusion, the use of acidic buffer to eliminate the first test effect would result in an inaccurate reading for the second test even when the pH is as low as 2.0.

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إزالة معقد الأنتيجين والأجسام المضادة لبعض الفيروسات النباتية في اختبارات الإليزا

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تم استخدام المحلول المنظم الجليسين - حمض الهيدروكلوريك على درجة حموضة من ٢.٠ إلى ٤.٠ لدراسة قدرته على إزالة معقد الأنتجين - الايميونوجلوبيولين في اختبارات الإليزا المباشرة. حيث أجريت الدراسة على فيروسين كرويين هما فيروس تبرقش الخيار وفيروس الحلقة السوداء في الطماطم وفيروسين عصويين هما فيروسي إس وواي البطاطس كلا على حدة.

أظهرت النتائج أن المحلول المنظم عند درجات حموضة ٢.٠ و ٢.٥ أزاح حوالي ٥٠% من الانتيجن المرتبط بالأجسام المضادة المعلمة بإنزيم اللكالين فوسفاتيز لفيروس تبرقش الخيار وفيروس الحلقة السوداء في الطماطم من الأطباق بينما لم يزاح فيروس إس البطاطس وفيروس واي البطاطس سوى بنسبة ٣٠% حتى عند درجة حموضة ٢.٠. أيضاً الأنتجين الفيروسى للفيروسات العصوية لم يتم إزاحته من الطبق. وكانت الرابطة بين مادة طبق الإليزا والإيميونوجلوبيولين-ج أقل الروابط تأثراً بالمحلول المنظم الحمضي بالمقارنة ببالقي الروابط.

ويمكن القول أن استخدام المحلول المنظم الجليسين - حمض الهيدروكلوريك لإزالة الفيروس المستخدم فى الاختبار الأول قد يعطى نتائج غير دقيقة فى الاختبار الثانى حتى عند درجة حموضة ٢.٠.