

2種類の間接ELISA法によるズッキーニ黄斑モザイクウイルス(ZYMV)の検出

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Comparison of Two Indirect Enzyme-linked Immunosorbent Assay Procedures for Detecting Zucchini Yellow Mosaic Virus*

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Abstract

Two indirect enzyme-linked immunosorbent assay (I-ELISA) procedures employing intermediate antibodies of Japanese quail (*Coturnix coturnix japonica* Temminck *et* Schlegel) and hen immunoglobulins were compared on their sensitivity for detecting zucchini yellow mosaic virus (ZYMV). The I-ELISA employing quail immunoglobulin on plates coated with gamma-globulin from rabbit antiserum against ZYMV was sensitive as that on non-coated plates, capable of detecting ZYMV in purified preparation at detection limitation of 10–50 µg/ml and in crude extracts of infected pumpkin leaves at dilution end point of 10^4 – 10^5 . In the assay with hen immunoglobulin, both procedures permitted to detect purified ZYMV at minimum concentration of 5–10 ng/ml. However, when antigen in crude extracts of infected pumpkin leaves was assayed, the dilution end point obtained by non-precoated I-ELISA was 10^6 – 10^7 , compared with 10^5 – 10^6 of precoated I-ELISA procedure. Of the procedures tested, non-precoated I-ELISA employing hen immunoglobulin showed the highest non-specific absorbance values. Despite of the relatively low sensitivity of non-precoated I-ELISA employing quail immunoglobulin, this procedure was relatively simple and useful tool for disease diagnosis of field samples. Quail appears to have potential as a source of anti-viral immunoglobulins in non-precoated and precoated I-ELISA for detecting ZYMV and perhaps for other plant viruses.

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Key words : indirect ELISA, quail, zucchini yellow mosaic virus.

Introduction

Since enzyme-linked immunosorbent assay (ELISA) was introduced into plant virology in 1976¹⁴⁾, it has become a key research tool of serodiagnosis. Several workers have reported a narrow strain specificity with double antibody sandwich (DAS)-ELISA that has led to its rapid adoption for separating strains of viruses^{1-3,7,11)}. However, indirect ELISA (I-ELISA) has been superior for a large-scale program where thousands of samples must be tested. Not only this procedure possesses a broad specificity that enable us to detect the viruses which occur in many serotypes, but also offers the use of a single anti-species IgG conjugate which is commercially available for all kinds of viruses^{8,13)}. The major constraint in using I-ELISA is the requirement for two virus antisera prepared in different animal species.

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Hen was usually employed to produce egg yolk immunoglobulin for use either as coating ("first")¹³⁾ or intermediate ("second")^{2,5,6,8)} antibodies of I-ELISA. Recently we succeeded in producing yolk immunoglobulins against ZYMV using quail (*Coturnix coturnix japonica* Temminck *et* Schlegel) and hen. This communication deals with the comparison of non-precoated and precoated I-ELISA procedures employing intermediate antibodies of quail and hen immunoglobulins on their sensitivity for detection of ZYMV.

Materials and Methods

Virus and purification. ZYMV (isolate 169) was propagated and purified as described previously^{9,12)}.

Birds and antisera. Throughout the following experiments, the term of quail was used for Japanese quail. All quails were between 15 and 17 weeks old and were kindly provided by Dr. M. Kobayashi, Saga University. A white Leghorn hen was kindly supplied by Saga Prefectural Livestock Experimental Station. All birds were raised in single battery cages with feed and water *ad libitum* at 25-28 C, and the photoperiodicity was 14 hr lighting: 10 hr darkness.

Three quails were employed to produce immunoglobulin against ZYMV. Each bird was twice given intravenous injection into a wing vein with 0.2 ml of 75 μ g/ml of ZYMV in 0.02 M potassium phosphate buffer, pH 7.0 at 7 days interval. To produce hen immunoglobulin, the hen was twice given intravenous injection spaced a week apart at a wing vein with total purified ZYMV of 300 μ g at the first injection and 400 μ g at the second injection.

Rabbit antiserum against ZYMV was the same as in the previous studies^{9,12)}. Rabbit anti-chicken IgG (whole molecule) was purchased from Miles Laboratories, Inc.

Immunoglobulins preparation. The group of three quail eggs and individual hen egg were used to prepare partially purified yolk immunoglobulins using a method as described by Polson *et al.*¹⁰⁾, except that after the final addition of polyethylenglycol 6,000, washing procedure was done three times and the final solution was centrifuged at 3,000 rpm for 15 min. The supernatant was stored at -20 C until use. The titer of the immunoglobulins from quail and hen was determined by I-ELISA using 100 times dilution of crude extracts of infected leaves and 10 μ g/ml of purified ZYMV, respectively. In these assays, all partially purified immunoglobulins were used as coating antibody at the concentration of 100 μ g/ml. The quail and hen immunoglobulins collected at the 30th and 35th days, respectively, after injection were used in all the following experiments.

Gamma-globulin from rabbit antisera against ZYMV or chicken IgG was prepared by the method described by Clark and Adams⁴⁾, except for the use of DE₂₃ instead of DE₂₂ cellulose.

Conjugate. 1.5 mg of alkaline phosphatase (Grad 1, from calf intestine, Boehringer, Mannheim GmbH, W. Germany) was conjugated with 1 mg of gamma-globulin from rabbit anti-chicken IgG immunoglobulin using glutaraldehyde procedure⁴⁾ and the conjugate was used at dilution of 4,000.

Indirect enzyme-linked immunosorbent assay (I-ELISA). Unless otherwise

stated, I-ELISA procedures were based on those described by Koenig⁸⁾ and used in the earlier work¹²⁾. The plates were sequentially incubated with: (1) 200 μ l of coating ("first") antibody in 0.05 M sodium carbonate buffer, pH 9.6 for 4 h at 37 C; (2) 200 μ l of antigen in 0.02 M PBS, pH 7.4 containing 0.05% Tween 20 and 0.05% polyvinylpyrrolidone 40,000 (PBS-TPO) for 18 h at 6 C; (3) 200 μ l of intermediate ("second") antibody in PBS-TPO containing 0.1% of bovine serum albumin (BSA) for 4 h at 37 C; (4) 200 μ l of alkaline phosphatase-labeled rabbit anti-chicken IgG immunoglobulin in PBS containing 1% of BSA for 4 h at 37 C; and (5) 250 μ l of 1 mg/ml of *p*-nitrophenyl phosphate (Wako Pure Chem. Ind. Ltd.) in 10% diethanolamine, pH 9.8 for 1.5 h at 25 C. The reaction was terminated with 50 μ l of 3 M NaOH. The I-ELISA absorbance values were measured with a spectrophotometer (Hitachi Model 200-20) using a micro-cell with a 1 cm light path at wavelength of 405 nm.

Results

Interspecies cross-reactivity

The conjugate used in these experiments was not ZYMV-specific, but specific for the intermediate antibodies. Before using the yolk immunoglobulins as intermediate antibodies in I-ELISA, it was hypothesized that rabbit anti-chicken IgG immunoglobulin may cross-react with quail immunoglobulin. The hypothesis was verified by the following experiments. Microtiter plates were firstly coated with immunoglobulin from quail (10 μ g/ml) or hen (4 μ g/ml), and 0.05 M sodium carbonate buffer, pH 9.6, as control for 4 h at 37 C; then with alkaline phosphatase-labeled rabbit anti-chicken IgG immunoglobulin for 4 h at 37 C; finally with substrate for 1.5 h at 25 C. The mean absorbance values in the wells coated with immunoglobulins from hen, quail, and control buffer were 2.25, 1.525, and 0.074, respectively. These data suggested that rabbit anti-chicken IgG immunoglobulin could actually cross-react with quail immunoglobulin. Thus, in the following experiments quail immunoglobulin was employed as intermediate antibody of I-ELISA using heterologous conjugate of alkaline phosphatase-labeled rabbit anti-chicken IgG immunoglobulin.

Yolk immunoglobulins as intermediate antibodies of I-ELISA

The two kinds of yolk immunoglobulins were compared for their suitability for use as intermediate antibodies in precoated I-ELISA. The assays were done on plates coated with 2 μ g/ml gamma-globulin from rabbit antiserum against ZYMV. The immunoglobulins from quail and hen were used at concentration of 10 μ g/ml and 4 μ g/ml, respectively. The minimum detectable concentration of purified ZYMV by I-ELISA using quail immunoglobulin was 10-50 ng/ml compared with 5-10 ng/ml for hen immunoglobulin. The dilution end points of the crude extracts from infected pumpkin leaves assayed by I-ELISA using intermediate antibodies from quail and hen were 10^4 - 10^5 and 10^5 - 10^6 , respectively (Fig. 1).

Comparison of two procedures of I-ELISA

The sensitivity of precoated I-ELISA using intermediate antibodies of yolk immunoglobulins in detecting ZYMV was compared to that of non-precoated I-ELISA. To remove the unsuitable antibody from hen immunoglobulin, it was diluted with extracts of

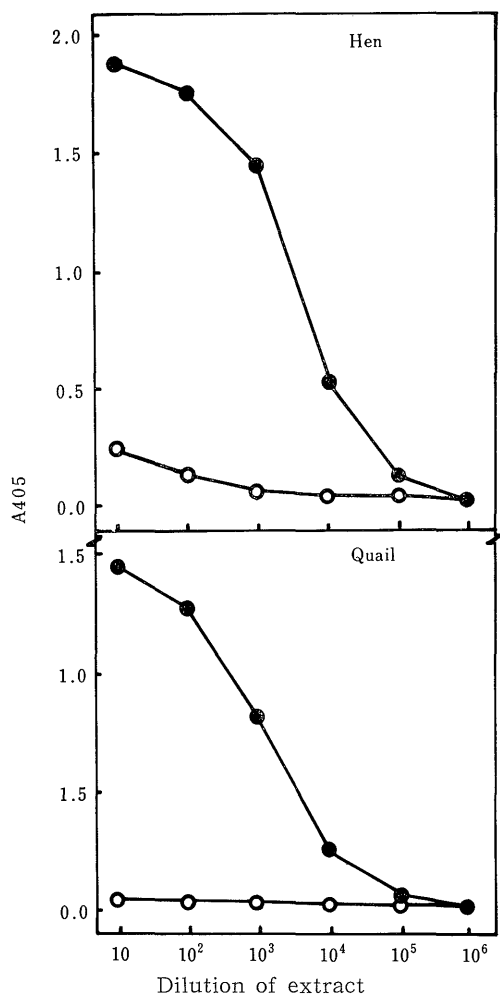


Fig. 1. Relative effectiveness of yolk immunoglobulins as intermediate antibodies of I-ELISA for detecting ZYMV in crude extracts of healthy (○) and infected (●) pumpkin leaves. Antigen was trapped on plates by using 2 μ g/ml of gamma-globulin from rabbit antisera against ZYMV, then the trapped antigen was reacted with yolk immunoglobulin of hen (4 μ g/ml) or quail (10 μ g/ml) diluted with 0.1% of bovine serum albumin (BSA) in PBS-TPO. Each point is the mean of the three experiments using three wells in each experiment.

healthy pumpkin leaves (1: 100) in PBS-TPO to the concentration of 2 μ g/ml. The mixture was then incubated overnight at refrigerator before being used as intermediate antibody in I-ELISA.

The I-ELISA employing intermediate antibody of 10 μ g/ml quail immunoglobulin on plates coated with 2 μ g/ml gamma-globulin from rabbit antiserum against ZYMV was as sensitive as that on non-coated plates, capable of detecting ZYMV in purified preparation at minimum concentration of 10–50 ng/ml and in crude extracts of infected pumpkin leaves at dilution end point of 10^4 – 10^5 (Fig. 2a). Both procedures gave low non-specific absorbance values in healthy control.

When 2 μ g/ml of hen immunoglobulin was employed as intermediate antibody, both procedures permitted to detect purified ZYMV at minimum concentration of 5–10 ng/ml. In the assays with antigen in crude extract of infected pumpkin leaves, the non-precoated I-ELISA resulted in dilution end point of 10^6 – 10^7 compared with 10^5 – 10^6 for precoated I-ELISA procedure. However, the non-precoated I-ELISA showed also higher non-specific absorbance values in healthy control (Fig. 2b).

Discussion

In I-ELISA using conjugate of anti-chicken IgG immunoglobulin, intermediate antibody of quail immunoglobulin gave lower sensitivity than that of hen immunoglobulin (Fig. 1). It can probably be improved by using homologous conjugate with enzyme-labeled anti-quail IgG immunoglobulin. In the cross-reactivity stud-

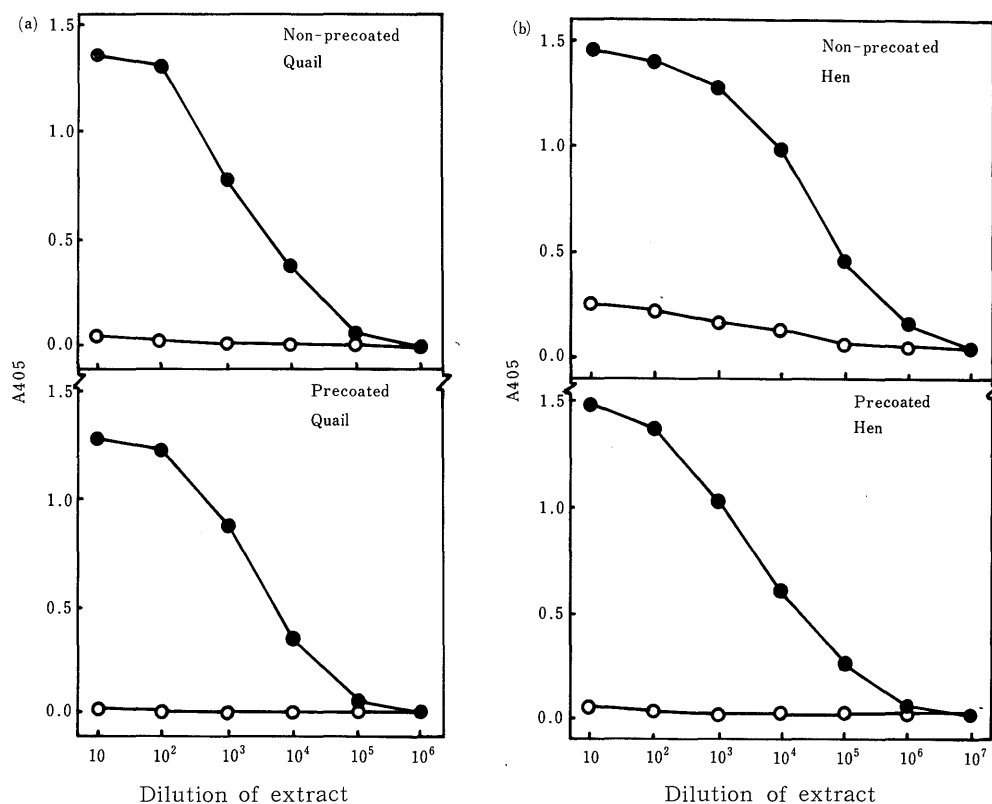


Fig. 2. Comparison of the sensitivity of non-precoated and precoated I-ELISA using intermediate antibody of quail or hen immunoglobulin for detecting ZYMV in crude extracts of healthy (○) and infected (●) pumpkin leaves. Antigen was immobilized directly on plates (non-precoated) or on plates previously coated with 2 μ g/ml gamma-globulin from rabbit anti-serum against ZYMV. The immobilized antigen was reacted with 10 μ g/ml of quail immunoglobulin in 0.1% BSA or 2 μ g/ml of hen immunoglobulin in extracts of healthy pumpkin leaves. Each point is the mean of the three experiments using three wells in each experiment.

ies, it was found that the heterologous reaction between quail immunoglobulin and conjugate of rabbit anti-chicken IgG immunoglobulin resulted in about 70% of absorbance values to those of homologous hen immunoglobulin.

Non-precoated and precoated I-ELISA procedures differ fundamentally in the way of immobilizing antigen to the surface of polystyrene plates. In non-precoated I-ELISA, the antigen is immobilized directly to the polystyrene surface, whereas in precoated I-ELISA it is selectively immobilized to the polystyrene surface by using coating ("first") antibody.

In the assay using quail immunoglobulin, the non-precoated and precoated I-ELISA procedures offered the same sensitivity of virus detection either in crude extracts of infected pumpkin leaves (Fig. 2a) or in purified preparation, and showed no problems of non-specific reaction with extracts of healthy pumpkin leaves. It was a further evi-

dence that the non-precoated I-ELISA procedure was also useful tool for detecting ZYMV. A similar result was obtained by using intermediate antibody of gamma-globulin from rabbit antiserum against ZYMV¹²⁾.

The use of extracts from healthy plants for diluting intermediate antibody of hen immunoglobulin could almost completely avoid the non-specific reaction in precoated I-ELISA but not in non-precoated I-ELISA. Avoiding non-specific anti-host reaction in precoated I-ELISA by mixing hen immunoglobulin with crude extracts of healthy plants was previously reported by Bar-Joseph and Malkinson²⁾ with citrus tristeza virus. However, the high non-specific absorbance values obtained by non-precoated I-ELISA using hen immunoglobulin might result from the presence of residual antibodies against plant components in the immunoglobulin. Selectively immobilizing antigen by gamma-globulin from rabbit antiserum against ZYMV in precoated I-ELISA appeared to have a great effect in decreasing the non-specific reaction.

Despite of the demand for virus antisera prepared in two different animal species, precoated I-ELISA using coating antibody of gamma-globulin from rabbit antiserum against ZYMV and intermediate antibody of hen immunoglobulin was the most sensitive and specific tool for diagnosis of ZYMV. Though non-precoated I-ELISA using quail immunoglobulin showed rather low sensitivity, it proved to be as specific as the precoated I-ELISA. Furthermore, non-precoated I-ELISA using quail immunoglobulin was relatively simple because it required for producing immunoglobulin only in one species of animal.

Finally, it seems that the use of quail as a source of anti-viral immunoglobulins in non-precoated and precoated I-ELISA is also applicable for other plant viruses.

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和 文 摘 要

Susanto SOMOWIJARJO・佐古宣道・野中福次：2種類の間接 ELISA 法によるズッキーニ黄斑モザイクウイルス (ZYMV) の検出

本研究では、ウサギから作製した抗体の他に、ニワトリとウズラの卵黄から採取した抗 γ -グロブリンを中間抗体として用いる間接 ELISA 法によって、ZYMV の検出試験を行った。その結果、ウサギ抗体を処理し、次にウズラ抗体を処理した場合、その検出限界は純化ウイルスで $10 \sim 50$ ng/ml、罹病葉汁液では $10^4 \sim 10^5$ 倍希釈で、ウサギ抗体を処理しなかった区と差はみられなかった。中間抗体としてニワトリ抗体を用いて、ウサギ抗体処理区と無処理区の比較を行ったところ、罹病葉汁液ではそれぞれ $10^5 \sim 10^6$ 倍、 $10^6 \sim 10^7$ 倍希釈まで検出可能であったが、純化ウイルスでは両区とも $5 \sim 10$ ng/ml であった。供試した各種の抗体処理のうちでは、ウサギ抗体を処理せずにニワトリ抗体を処理した時に、健全葉汁液による非特異反応が最も高かった。ウサギ抗体を処理しないでウズラ抗体のみを処理する間接 ELISA 法は、その検出限界が低いが、比較的簡便であり、圃場試料の診断法にも有用であろう。ウズラは本ウイルスだけでなく、他のウイルスの本法による検出に必要な抗体を作製する動物として使用に耐えると考えられる。