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Production and purification of antibody by immunizing rabbit with rice tungro bacilliform and rice tungro spherical viruses

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ABSTRACT

Rice tungro disease is the major disease caused by infection with the rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). In this study, New Zealand White rabbits were immunized with pure viruses for the production of antibodies against both species. The production of polyclonal antibodies against Tungro viral disease using ammonium sulfate precipitation and a protein A affinity column and their assessment are described. Two peaks were found from the protein A affinity column. Peak 1 represents the unbound compounds from the extracted serum and peak 2 represents antibody that bound to protein A, which was eluted using elution buffer. Peak 2 was collected for antibody titration. The amount of pure antibody in the titers was quantified by enzyme-linked immunosorbent assay (ELISA) to capture the tungro viruses. Antibody titer was analyzed by the ELISA method. For anti-RTBV, 1.696 mg/mL was highest at the second bleed and anti-RTSV was 2.3225 mg/mL was highest at the first bleed. These antibodies detected the tungro viral disease well and proved to be a potential probe for the detection of rice tungro disease.

1. Introduction

Plant diseases are a serious issue and significantly affect both the quality and quantity of agricultural products [1]. The infection of plants drastically reduces the yield [2] and causes a significant agronomic impact [3]. In addition, infection can cause catastrophes in large agricultural fields, which can lead to famine [4]. In this context, the tungro virus has caused major outbreaks and has caused serious production losses in various countries of South and Southeast Asia.

Generally, farmers detect tungro diseases by visual observation. However, it is quite difficult and not reliable to identify the symptoms through visual observation due to the difficulty in differentiating them from a non-pathogenic disorder such as excess water after a drought, nutritional deficiency, and insect injury, which have similar symptoms [5]. In addition, most farmers take a simple approach using pesticides to control and monitor tungro viral vectors. However, this approach is not effective, and it also affects the health of the operators [6]. Under these circumstances, it is imperative to develop a method to control against the independent or simultaneous occurrence of the two types of viral infections [rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV)] in order to prevent serious outbreaks. Detection of tungro disease requires a fast, simple and sensitive method compared to the current detection strategies, which are tedious and laborious.

The detection of analytes such as biological and chemical pathogens and contaminants plays an important role in the prevention of infection and disease [7]. It is important to develop a method with more specific detection. Recently, immunosensors that depend on antibody-antigen binding have been developed to substitute for traditional analytical techniques. Generally, immunological techniques require a purified antibody with low cost and good quality for long-term usage. Techniques for immunosensors are based on the combination of antibody and antigen specificity in solution or on a solid surface coupled with a transducer [8–11]. Antibodies are indispensable molecules for analyzing protein functions broadly in life sciences [12,13] and are particularly useful for immunohistochemistry, a technique that visualizes the specific tissue and cellular localization of proteins and other antigens. In this context, polyclonal antiserum is a primary source of antibodies due to its low cost and effectiveness in generating high-

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performance applications [14,15].

There are several techniques for testing the quality, quantity and functional aspects of the pure antibody. Using the enzyme-linked immunosorbent assay (ELISA), the specific binding between an antibody and antigen can be measured using an enzyme-labeled antibody. Antibody titer is the endpoint dilution that exhibits a reaction between the antigen and the pre-immune serum [16,17]. In the current study, New Zealand White rabbits were immunized with two viruses (RTSV and RTBV) for the efficient production of antibodies in order to develop detection systems. The antibodies that were produced were purified and the titers of polyclonal antibody were assessed against the tungro viral disease.

2. Materials and methods

2.1. Materials

Rabbit anti-mouse IgG-conjugated horseradish peroxidase was obtained from Abcam Ltd. (UK). Tetramethylbenzidine (TMB) substrate and blocking milk solution were purchased from KPL Ltd. (London, UK). Bovine serum albumin (BSA), phosphate buffered saline, Freund's complete adjuvant and Freund's incomplete adjuvant were procured from Sigma-Aldrich Co Ltd. (Dorset, UK). A HiTrap protein G column was obtained from Pharmacia Ltd. (Sweden). EZ-link Plus Activated Peroxidase was obtained from Pierce Ltd. (UK). Maxisorb ELISA plates were procured from Nunc (Germany). Six-month-old New Zealand White rabbits were obtained from a local rabbit farm (LTK Sdn. Berhad, Malaysia). The rabbit facility equipped with automated feeding and drinking systems, was located at the Malaysian Agriculture Research Institute, Malaysia. Polyclonal antibody production was conducted at the Malaysian Agriculture Research Institute, Malaysia. Other disposable items used in this study were purchased from Fisher Ltd. (UK). The Malaysia Agriculture Research and Development Institute (MARDI) Ethics Committee approved the procedures performed with animals. We obtained permission from the above committee and they have accepted all the approvals and retain the records.

2.2. Source and collection of tungro viruses

In this analysis, MR81 and Y1286 varieties were used as plants for infection by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). Both seeds were planted in 5 soil-filled pots, uniformly watered and fertilized to enhance seed growth. A plastic (Mylar) cage was used 20 days after planting. The upper portion of the cage was covered with a cotton ball after introducing 5 insect vectors (green leafhopper). *Nephotettix virescens* were introduced into each cage for 24–48 hours to initiate the inoculation. To maintain their health and expand their lifespan, the vectors were transferred to cultivar TN1. After 50–60 days of incubation and infection, infected rice plants exhibited rice tungro disease symptoms and were ready to use for the isolation and purification of rice tungro viruses.

The virus was purified according to a previous method by Omura et al. [18] with modifications. The artificially infected plant (100 g) was homogenized in 0.1 M sodium citrate, 0.01 M EDTA and 5% Celluclast using a heavy blender. Then, the sample was filtered through 2 layers of muslin cloth. The filtrate was added to a conical flask and shaken in an incubated shaker at 30 °C for 3 h. Afterwards, the sample was filtered again using 2 layers of muslin cloth, and then again, the filtrate was added to a conical flask and shaken in an incubated shaker at 40 °C for 3 h. Then, the sample was centrifuged at 19,000*g* for 15 min (Beckman Coulter Avanti J-26 XP1) with J-14 rotors, and the supernatant was collected. The supernatant was mixed with 7% polyethylene glycol (PEG8000), 0.2 M NaCl, 1% Triton X-100 at room temperature; after 1 h the mixture was centrifuged at 23,000*g* for 3 h at 4 °C. The pellet was resuspended in 5 mL of a mixture containing 0.1 M sodium citrate and 0.01 M EDTA. The mixture was stored overnight at −20 °C. Later, the supernatant was centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was then layered onto a 10% sucrose cushion and was centrifuged at 68,000*g* for 2.5 hours at 4 °C (Beckman Coulter Optima XPN-100 Ultracentrifuge) with a swing-out rotor. The pellet was collected and was resuspended in 0.5 mL of 0.1 M sodium citrate and 0.01 M EDTA. Finally, the suspension was centrifuged for 11,000*g* for 10 min at 4 °C (Rotina 420, Hettich).

2.3. Immunization procedure

Immunization of rabbits with the purified virus was achieved by the modification of a protocol described by Omura et al. [18]. Two different rabbits were used for immunization with RTBV or RTSV. The first and second injections were performed with 250 µg/mL and 500 µg/mL, respectively. Both injections were given intravenously for the first and second week after pre-immune blood collection. Subsequent injections were given subcutaneously with an emulsion consisting of 1.0 mg/mL in 1 mL of Phosphate Buffered Saline (PBS) and an equal volume of incomplete Freund's adjuvant (IFA). The injections were repeated one month after the third injection, substituting IFA with complete adjuvant (CFA). All injections with both viruses consisted of a virus suspension with an optical density of 1.0 at A_{260}. Afterwards, the rabbit was bled for antibody determination 1 week after the last injection.

2.4. Antibody purification

Antisera against Tungro viruses (RTBV/RTSV) were diluted in distilled water (1:10 v/v) and then precipitated using 80% saturated ammonium sulfate while stirring to obtain serum proteins. The serum mixture was centrifuged (5810 R, Eppendorf, Appendix A7) at 20,000*g* for 15 minutes at 4 °C. The pellet was resuspended in 0.01 M PBS, dialyzed three times and then passed through a protein G HiTrap column connected to an AKTA purifier (Pharmacia Ltd., Sweden) described in the following section. Fractions yielding the highest absorbance at OD_{280nm} were collected (IgG Stock). Then, IgG titers for anti-RTBV/RTSV were determined by indirect ELISA.

2.5. Fractionation of IgG using a protein G column

The purification steps were performed with the protein G column using AKTA-Prime plus following the manufacturer's instructions (Pharmacia). This instrument is a compact one-step lab-scale protein purification system combined with Prime View 5.0 software. First, the partially pure IgG sample (5 mL) was filtered (using a 0.45 µm filter) to eliminate the cell debris. The conditions for the IgG sample were adjusted to the ionic strength of the binding buffer by dialyzing against 0.02 M Phosphate (pH 7.0). The protein G column (5 mL) was first equilibrated with 5 column volumes of binding buffer before loading the IgG sample. After loading, the column was washed thoroughly with 5–10 column volumes of the binding buffer to eliminate unbound materials, and this process was continued until there was no protein present in the eluent (determined by UV absorbance at 280 nm). The IgG sample was eluted with 5 column volumes of elution buffer (0.1 M glycine-HCl, pH 2.7) at a flow rate of 2 mL/min, and then the column was re-equilibrated with binding buffer (5–10 column volumes) to stabilize the binding of protein G to the ligand. The IgG fractions were immediately neutralized with neutralization buffer (100 µL of 1 M Tris-HCl/mL of fraction, pH 9.0) to adjust the appropriate final pH of the IgG.

2.6. Indirect ELISA for the determination of antibody titer

The pure antibody was examined by the indirect ELISA method to determine the antibody titer using a microplate reader (Versamax). A higher antibody titer indicated a better quality of fractionated antibody. To perform the indirect ELISA, the surface microtiter plates were