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RAISING OF POLYCLONAL ANTIBODY AGAINST THE *ASPERGILLUS ORYZAE* PROTEINS IN ALBINO RAT

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ABSTRACT

Aspergillus oryzae is one of the common plant pathogens in wheat, sorghum and other crops. Its toxic activity has been proved. Hence, a sensitive, rapid, and inexpensive screening test to detect *Aspergillus* in agricultural commodities is necessary to protect human health. *Aspergillus oryzae* proteins were prepared, characterized, and used as an antigen. 6-month-old white albino rat were immunized by the antigen in combination with Freund's adjuvant. The antiserum of immunized rat was collected after 35 days. Proteins present in antiserum were precipitated by ammonium sulphate precipitation and purified by dialysis, ion exchange chromatography and SDS PAGE. Immuno blotting analysis was performed to determine antibody specificity towards the antigen. The optimum dilution of prepared IgG was 1:32. Dot strip ELISA test confirmed the presence of specific Antigen-Antibody interaction

KEY WORDS

Aspergillus oryzae, antigen, antibody, purification

INTRODUCTION

Fungi are important pathogens of plants and cause more significant yield losses than bacteria or viruses. However, bacteria and viruses are more important than fungi as pathogens of animals; indeed, whether or not a fungus even becomes pathogenic on an animal often depends on the immune status of the host. Until the rapid rise of opportunistic fungal infections in humans, pathogenicity mechanisms in plant pathogens were better understood than those in animal pathogens. In fungi, the hydrophobic surface with a hydrophobic coating facilitates the attachment of hyphae to the hydrophobic surfaces, aerial growth of the hypha, dispersal of aerial spores, and proper gas exchange in fungal air channels (Wessels, 1994; Wo sten *et al.,* 1994; Wo sten *et al.,* 1999; Wo sten *et al.,* 2000). Moreover, some hydrophobins implicated in the pathogenicity of several fungi are involved in the interaction between the pathogenic fungi and their host plants (Bowden *et al.,* 1994).

Increased research activity in medical mycology has coincided with the development of molecular genetics and genomic resources, which are being exploited to develop a detailed understanding of fungal pathogenesis in both animals and plants. Several constraints and peculiarities govern the types of information that can be derived from such studies. For instance, analyses of human-pathogenic fungi generally rely on cell lines and experimental animal models, in contrast to plant pathogens, which can be studied directly on their hosts. Many more fungal species infect plants than animals, and thus, more plant-fungus systems than animal-fungus systems are studied. This is mainly because there are far more plant hosts than animal hosts that are of economic importance, with the obvious exception of human disease, and because plants can be manipulated without the ethical issues associated with animal experimentation.Purification of immunoglobulins (Ig) is required for many applications in numerous fields of science and technology. Antibodies are important tools used by many investigators in their research and have led to many medical advances. Mammalian sera represent a remarkable and economical source of immunoglobulins widely used in diagnostic and therapeutic applications (Gallacher, 1993; Gathumbi *et al.,* 2001). In biochemical and biological researches, polyclonal antibodies are routinely used as ligands for the preparation of immunoaffinity columns (Shin *et al.,* 2001) and as coating or labeling reagents for the qualitative and quantitative determination of molecules in a variety of assays, such as enzyme linked immunosorbent assay (ELISA), double diffusion, radial immuno-diffusion, western blot and radioimmunoassay (Calabozo *et al.,* 2001; Cheung et *al.,* 2002; Verdoliva *et al.,* 2000). A growing list of purification procedures has emerged, reflecting the heterogens nature of this group of molecules and also different researchers, demands for varying levels of purity. Most papers deal with monoclonal antibodies from mouse ascites as a starting material (Clezardin *et al.,* 1985; Danielsen et *al.,* 1988; Hwang *et al.,* 1988), and for the isolation of murine monoclonal IgG antibodies, affinity chromatography on immobilized protein A or G is probably the most commonly used technique (Oppermann, 1992). In the present study, we have performed the production, purification and detection of polyclonal immunoglobulins against *Aspergillus oryzae* proteins in rat.

MATERIALS AND METHODS

Preparation of A.oryzae glycoprotein antigens

Aspergillus oryzae strain was obtained from the Wingene Research laboratory, Bangalore, India. Strains used this study were isolated from infected maize plant in Bangalore region. Cultures of *A. oryzae were* grown in stationary 500 ml conical flask containing 50 ml of potato dextrose broth. Cultures were incubated at 22°C until mycelial mats covered the surface of the broth (about 10 days). Formaldehyde used to kill the spores. By using filtration through glass fiber filters (Whatman GF/A) the mycelia were removed. NaoH used to precipitate the proteins and concentrated 30-fold by ultrafiltration (Erwin and Katznelson, 1961). This concentrate was used directly or after dialysis against phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) for immunization. It was lyophilized for all other purposes, resuspended in Tris buffer (5 mM, pH 6.8), dialyzed against the same buffer, and brought to a concentration of 1mg protein/ml. Yield of protein (Peterson, 1977) was typically 8 to 10mg/jar. These preparations were previously found to contain primarily glycoprotein (Wade and Albersheim, 1979) and are referred here as extracellular protein. Protein concentration was quantified by a Coomassie dye binding assay (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

Immunization of rat

Attenuation fungal cultures were grown 2-3 days on a solid medium to determine their liveliness. Intra muscular (I.M) injection of two hundred micro liters of prepared immunoglobulins (1mg/mL) in PBS was emulsified with equal volumes of Freund's complete adjuvant (Sigma). Blood was collected from animal on day 35 by cardiac puncture under terminal anesthesia. All animal experiments were carried out under protocols reviewed and approved by the animal ethical committee. A one-tailed Student's *t* test was used to detect statistically significant differences in survival.

Purification of rat immunoglobulins

Anti sera was collected from immunized rat and precipitated by 50% ammonium sulfate. Dialysis performed against Tris-HCl buffer about 8 hrs and further purified by using ion-exchange chromatography was done on DEAE cellulose in a column apparatus in a desired flow rate.

SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to check the purity of various IgG preparations under the conditions described by Laemmli (1970). After separation, the proteins were stained with Coomassie Brilliant Blue G 250 and destaining was carried out. (Blakesley and Boezi, 1977).

Determination of titer value and specificity of immunoglobulins

Ouchterlony Double Diffusion (ODD) and Single Radial Immunodiffusion (SRID) method was used to estimate the titer value of antibody and titer value of antigen respectively (Bailey, 1996). Dot strip ELISA was used to detect the presence polyclonal antibodies against *A.oryzae* proteins.

RESULTS AND DISCUSSION

Polyclonal antibodies represent a group or mixture of antibodies produced by different B-lymphocytes in response to the same antigen; thus, different antibodies in the group recognize different parts of the antigen. The diversity of antibodies provides an advantage by allowing the detection of multiple epitope sites on the protein of interest (Abolade Afolabi, 2009).

In this study rat were immunized with precipitated *Aspergilllus oryzae* fungal proteins. After multiple immunizations, immunoglobulins present in blood was collected and purified by ammomium sulphate precipitation, dialysis and ion-exchange chromatography.At this stage, the titre and the specificity of the antibody were checked by immuno blotting test and dot strip ELISA respectively.

In SDS-PAGE the purified IgG shows single band. Molecular weight of the purified IgG was 66KDa (Figure1). Ouchterlony double diffusion bands were indicated that the antibody

was specific against the antigen and the antibody titre was found to be 1:31. Bands in single immuno diffusion indicated that the antibody was specific against the antigen and the antigen titer also 1:32. An orange colour formation appeared on ELISA strip. It confirmed the presence of antibody against *A.oryzae* proteins.

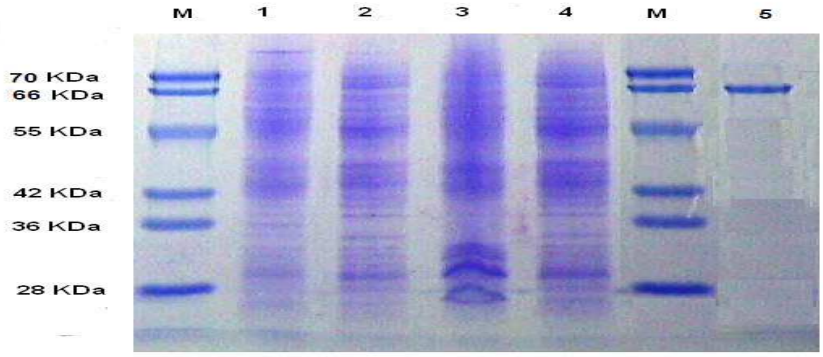

Figure 1

Proteins were separated by SDS polyacrylamide gel electrophoresis in 12% acrylamide gels. Lane M – Marker, Lane 1, 2, 4 – serum sample (IgG) after ammonium sulphate precipitation, Lane 3 – IgG obtained after dialysis, Lane 5– pure IgG obtained after Ion exchange chromatography.

The production of antibodies in laboratory animals has become an essential part of many research projects (Robson, 1995). Monoclonal antibodies that are discreet in certain epitopes have also been very useful (Agindotan *et al.,* 2006). In some cases such as, electron microscopy, polyclonal antibody acts better than monoclonal antibody where detection of antigen with various epitopes is the target of study, since polyclonal antibody can connect to the more connective sites resulting in better sensitivity.

The purification of immunoglobulins presents several practical complications, especially for polyclonal antibody production (Verdoliva *et al.,* 2000). Ion exchange chromatography was used here for the purification of rat IgG polyclonal antibody. Some factors were affect the separation and recovery of proteins from ion exchange chromatography. The selection of ideal conditions for protein purification involves changing some or all of these parameters (Tishchenko, 1998). This technique is well established for the purification of IgG antibody (Baradaran *et al., 2006*; Javanmard *et al.,* 2005; Majidi *et al.,* 2005). Furthermore, ion-exchange chromatography is considered as an economical alternative to affinity and immunoaffinity chromatography. 98% pure protein obtained after the purification step.

Immunoblotting tests such as Ouchterlony Double Diffusion, Single Radial Immunodiffusion used for rapid and sensitive detection of antigen and antibody titre value. In ouchterlony double diffusion method both antigen and antibody are diffused in agarose matrix and intract one another. Precipitation band was observed at zone of equivalence. In single radial immunodiffusion, antibodies mixed along with agarose and used as stationary phase (Bailey, 1996). Here antigen used as a mobile phase. Precipitation was appeared around the well. Because of antigen diffuse through the gel and interact with the antibody.

Mab-based immunoassays have been developed to detect *Botrytis cinerea* (Bossi and Dewey, 1992; Salinas and Schots, 1994), *Pseudocercosporella* (Priestley and Dewey, 1993), *Rhizoctonia solani* and *Pythium ultimus* (Yuen *et al.,*1993). DAS-ELISA method also were used to detect *Erwinia chrysanthemi pv*. Dianthicola (Nassar *et al.,* 1996), barley yellow streak mosaic-virus (Skaf and Carroll, 1995) and *V. dahliae* using rabbit polyclonal antiserum as the capturing antibody. However, in this system,

Dot Strip ELISA test was applied to evaluate the detection of specificity of immunoglobulins (Figure 2). Because of direct evaluation of antigen-antibody complex, the test has a high degree of precision (Howard and Bethell, 2000)

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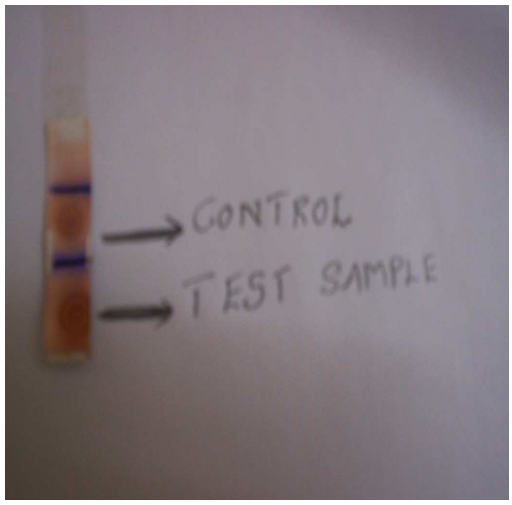
Figure 2
DIP STRIP-ELISA



CONCLUSION

The production of antibodies to specific and non specific antigens is a tool utilized in nearly all diagnostics fields, biomedical research. In this study, the production, purification polyclonal IgG against *Aspergilllus oryzae* proteins in white albino rat were carried out. Ammomium sulphate precipitation, dialysis and ion-exchange chromatography techniques were used to purify the protein present in the serum of immunized rat. Antigen – antibody titre value and specificity was checked by immuno blotting test and dot strip ELISA respectively

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