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Chicken Antibodies: A Useful Tool for Antigen Capture ELISA to Detect Bovine Leukaemia Virus without Cross-reaction with Other Mammalian Antibodies

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ABSTRACT

The 24 kDa protein from the gag of the bovine leukaemia virus was cloned and expressed as a fusion protein GST-p24. This recombinant protein was then used to immunize a Leghorn chicken. The partially purified chicken anti-GST IgY was used to develop a solid-phase assay by binding the IgY to an ELISA plate. When the fusion protein contacts the antibody, it binds it by its N-terminal, leaving the C-terminal, which carries the sequence that acts as a capture antigen in solution maximally exposed, reducing the risk of epitope masking. The conditions of the fusion protein on the solid phase maximize the presentation of the antigens' epitopes in solution. For the first time, a system has been developed with a non-mammalian coating antibody. Besides optimizing the recognition of low-molecular-weight antigens synthesized as fusion proteins, it avoids cross-reactions with commonly used secondary antibodies, mostly raised in mammalian hosts.

Keywords: BLV, chicken IgY, ELISA, glutathione *S*-transferase, pGEX recombinant peptides

Abbreviations: BLV, bovine leukaemia virus; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; FLK, fetal lamb kidney; GS4B, glutathione agarose 4B; IPTG, isopropyl-β -D-thiogalactopyranoside; PMSF, phenylmethylsulphonyl fluoride; FCA, Freund's complete adyuvant; BCI/TNBT, 5-bromo-4-chloro-3-indolyl phosphate/tetranitroblue tetrazolium; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine.

INTRODUCTION

The problem of restricted availability of purified eukaryotic proteins has been partially solved with the possibility of expressing them in bacteria. Cloning and expression of eukaryotic cDNA in *E. coli* provides an abundant source of polypeptides that are often limited by the polypeptide's low natural availability. The production of recombinant proteins using the pGEX plasmid has been described previously (Smith and Johnson, 1988; Kovacs-Nolan *et al.*, 2001; Naz and Zhu, 2001; De Carlos *et al.*, 2003). In this system the foreign protein is expressed fused to the C-terminus of the glutathione *S*-transferase (GST; EC 2.5.1.18) from *Schistosoma japonicum*. After expression, purification through an immobilized matrix of glutathione, and cleavage with thrombin or blood coagulation factor Xa, purified proteins can be obtained with a yield of 15 μg protein/ml of culture (Smith and Johnson, 1988).

Bovine leukaemia virus (BLV), which belongs to the genus *Deltaretrovirus* of the family *Retroviridae* (Van Regenmortel *et al.*, 2000), is responsible for enzootic bovine leukosis, probably the most damaging and common malignancy of dairy cattle (Ghysdael *et al.*,

1985). The disease is characterized by a strong immunological response against several viral proteins (Desahes *et al.*, 1980). This response is directed mostly against the major envelope glycoprotein, gp51, and against gag proteins, which participate in the formation of the structure of the virion. Of these, p24 is the major capside component and has several antigenic sites detectable by monoclonal antibodies (Ghysdael *et al.*, 1985).

There are several reasons to apply a p24-specific test in addition to the commonly used gp51 test during a BLV eradication campaign. Some cattle that develop an antibody response exclusively to p24 may not be detected during routine testing. Furthermore, a p24-specific test could be especially useful as a confirmatory method when an eradication campaign reaches its final stage, when false-positive reactors to gp51 are more likely to ocur. Finally, where gp51-based vaccines are used, p24-specific tests can discriminate between vaccinated and naturally infected animals (Kittelberger *et al.*, 1999).

Since monoclonal antibodies are specifically directed to a single epitope, when they are used for diagnostic purposes some infected individuals may not be detected in immunoassays. This is due to antigen masking or antigen conformational changes (Bán *et al.*, 1991; Rosati *et al.*, 2003). To address this issue, we designed a method that maximizes the availability of an epitope to incoming antibodies by attaching an antigenic protein to a solid phase. In this way, epitopes on the protein are presented to antibodies in the least obstructed and most desirable way. Several attempts have been made to immobilize pure GST to the bottom of the microwell (Cartwright *et al.*, 1995), covalently link GST to another protein such as haemoglobin (Murray *et al.*, 1998) or casein (Sehr *et al.*, 2001) or to directly bind mammalian antibodies against GST (monoclonal or polyclonal) to the bottom of the plate (Kilty *et al.*, 1998; Yamauchi *et al.*, 1998).

In the present study, we present a novel method that includes a GST fusion protein bound by its glutathione-binding moiety to an anti-GST IgY raised in chicken. In this way, the foreign peptide part of the fused protein, anchored to the solid phase by the antibody, remains free in solution and its epitopes are optimally presented to specific identifying antibodies. The use for the first time of a non-mammalian system avoids any kind of interspecies cross-reactions between the second antibody and the capturing antibody.

MATERIALS AND METHODS

DNA cloning of p24

The coding region for the p24 gene of the BLV was amplified by PCR from the stable infected cell line FLK (fetal lamb kidney) (ATCC, Rockville, MD, USA). The forward primer carried a restriction site for *Bam*HI 5′GCCGGATCCCCAATCATATCTGAAGG 3′, and the reverse primer carried a restriction site for *Eco*RI 5′TGGGAATTCGACGAGAAGTGCAGGCTG 3′. Primers were designed with two different restriction sites to facilitate insertion of the fragment in the correct orientation. PCR was carried out using 35 cycles of denaturing at 94°C for 75 s, annealing at 55°C for 100 s, extension at 72°C for 100 s, followed by an extension period at 72°C for 400 s.

The amplification product was loaded for electrophoretic separation in a 1% low-melting-point agarose (Promega, Madison, WI, USA). After the run, the expected fragment was sliced from the gel and purified by precipitation with 3 mol/L ammonium acetate, pH 5, and ice-cold absolute ethanol.

Both the purified fragment and the pGEX-2T glutathione *S*-transferase (GST) expression vector (Amersham Pharmacia Biotech, Piscataway, NY, USA) were digested with *Eco*RI (Promega), and then with *Bam*HI (Promega). Purification was carried out with the PCR preps DNA purification system (Promega, catalogue no. A7170). Ligation was carried out at 16°C ON, in the presence of T4 ligase (Promega). This mix was used to transform *E. coli* DH5α F′IQ competent cells (Invitrogen, Carlsbad, CA, USA) Transformed bacteria were selected by plating in the presence of 100 μg/ml ampicillin (Roemmers, Buenos Aires, Argentina). Colonies were picked and grown in the presence of antibiotic; plasmid isolation was done using the Wizard SV96 plasmid DNA purification System (Promega, catalogue no. A2250). The resulting plasmid was named pGEX/p24. To check for the presence of the cloned fragment, the resulting plasmid was digested with *Eco*RI and/or with *Bam*HI and the products were run in a 0.8% agarose gel.

Recombinant plasmid pGEX/p24 sequencing

Plasmid pGEX/p24 was sequenced in an automatic sequencer to establish correct insertion. GST vector primers for sequencing were used corresponding to the C-terminal of the GST protein and to the pGEX plasmid (Amersham Pharmacia): forward primer (catalogue no. 27-1410-01), 5′GGGCTGCAAGCCACGTTTGGTG 3′; reverse primer (catalogue no. 27-1411-01), 5′CCGGGAGCTGCATGTGTCAGAGG 3′.

Expression of the fusion protein

Sterile LB broth (5 g/L yeast extract, 10 g/L NaCl, 5 g/L tryptose) containing 100 μg/ml of ampicillin was inoculated with an overnight culture of transformed *E. coli* at a dilution of 1/100, and incubated at 37°C with vigorous shaking. After 1 h, the expression of the fusion protein GST-p24 was induced by the addition of 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St Louis, MO, USA). Three hours after induction, cells were pelleted by centrifugation at 3100*g* for 10 min, washed once with cold phosphate-buffered saline (PBS) and resuspended in 20 ml of lysis buffer (1% PBS, 0.5% Triton X-100). The resuspended cells were disrupted by sonication for three cycles of 15 s each, using a 6 mm diameter microtip. To the lysate was added 1 mmol/L PMSF (phenylmethylsulfonyl fluoride) (Sigma) and the mixture was centrifuged at 4180*g* for 10 min. The pellet was discarded.

Purification of the fusion protein

Glutathione agarose 4B (GS4B) (Sigma) was prepared according to the manufacter's protocol. The GS4B was previously equilibrated with PBS-1%Triton X-100, and 2 ml of a

50% slurry was added to the supernatant. The mixture was incubated for 15 min with gentle shaking, washed three times with PBS-1%Triton X-100, and then three times with PBS to remove unbound material. The bound fusion protein was eluted by adding 2 ml of 5 mmol/L glutathione (Sigma) in 50 mmol/L Tris pH8. Protein determination was carried out with the BioRad protein assay (catalogue no. 500-0006, Richmond, CA, USA) and showed a yield of approximately 7 mg/ml.

SDS-PAGE electrophoresis and Western blot

Sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS-PAGE), using 14% acrylamide in vertical slab gel, was carried out according to the method of Laemmli (1970). One gel was stained with 0.1% (w/v) Coomasie brillant blue R-250, and destained with 25% (v/v) methanol–10% (v/v) acetic acid solution. Another gel was transferred overnight onto a nylon membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membrane was blocked for 20 min with MTBS (PBS, 5% non-fat milk, 0.05% Tween 20) and incubated to 1 h with MoAb against p24 (VMRD, Inc.) (1:50 000 MTBS). Four washes were done with PBS–0.05% Tween 20 (PBST), followed by a 30 min incubation with a mouse biotin-conjugated secondary antibody (biotin-SP-conjugated affiniPure goat anti-mouse IgG H+L, Jackson Immunoresearch, West Grove, PA, USA), 1:10000 in PBST. After four washes with PBST, avidin-alkaline phosphatase was added (1:10000 in PBST for 15 min), followed by four washes with substrate buffer (0.1 mol/L NaCl, 0.1 mol/L Trizma, 0.05 mol/L $MgCl_2$). Immunoreactive bands were detected with BCI/TNBT alkaline phosphatase substrate (Moss, Inc., Pasadena, MD, USA). Reaction was stopped by addition of distilled water. All the reaction was carried out at room temperature.

Thrombin cleavage

Recombinant protein was incubated for 20 min at 30°C in a mixture containing 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.05 mmol/L EDTA, 50 mmol/L Tris-HCl pH7.9 and 20% bovine thrombin (Sigma) (1/1000 w/v in distillated water). The incubation time was sufficient to cleave more than 90% of the added fusion protein.

Production of anti-GST polyclonal antibodies (IgY)

Bacteria transformed with the pGEX plasmid were induced to produce GST under the same conditions as described above. Briefly, the bacterial extract was prepared and mixed with a 50% slurry of GS4B, to retain the induced protein. On the addition of 5 mmol/L glutathione in 10 mmol/L Tris pH8 to the agarose beads, the protein was released. The purified GST was dialysed against double-distilled water, lyophilized and stored at −40°C prior to immunization.

The protein was resuspended in 1% sterile saline and emulsified with an equal amount (1:1) of Freund's complete adjuvant (FCA) (Sigma). A Leghorn chicken was injected intramuscularly with approximately 0.1 mg of GST each time. The inoculation was repeated for six more times, every 2 weeks. Each time after injection, the chicken was bled from a wing

vein, and antibody titres were monitored by ELISA and boosters administered as required. After the ninth inoculation, plasma from the chicken was delipidized using the EGGstract Chicken IgY Purification System (Promega). After the treatment, antibodies showed a high capture titre.

Enzyme-linked immunosorbent assay

A 96-well microwell plate (Nunc, Kamstrup, Denmark) was coated with 100 μl of a 1:4000 dilution of purified IgY-anti GST in sodium bicarbonate buffer (pH 9.6), and incubated at 4°C overnight. The plate was washed twice with PBS containing 0.05% Tween 20 (PBST) and 100 μl of recombinant GST-p24 was added, diluted 1:100 in 5 g/L pluripeptone, 3 g/L meat extract containing 0.05% Tween 20 (PMET). Incubation was performed at 37°C for 60 min. The microwell plate was washed four times with PBST, and incubated at 37°C for 60 min with 100 μl monoclonal antibody against p24 (VMRD), diluted 1:2000 in PMET. The plate was washed four times with PBST, and incubated 45 min at 37°C with 100 μl of anti-mouse-peroxidase diluted 1:4000 in PBST. The plate was washed again with PBS, and detected with 90 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) for 30 min at room temperature. The reaction was stopped by addition of 30 μl of 2 mol/L H_2SO_4. Absorbances were read at 450 nm, using a Bio-Tek model EL311sx plate reader.

RESULTS AND DISCUSSION

Recombinant proteins expressed as glutathione *S*-transferase fusion proteins can be specifically and easily purified under nondenaturing conditions using glutathione affinity columns. Following the purification, and after specific cleavage with thrombin or factor Xa, glutathione *S*-transferase is readily removed by rechromatography on a glutathione affinity column (Smith and Johnson, 1988). Here, we selected for the design of the ELISA the 24 kDa protein from the gag of the bovine leukaemia virus. DNA from FLK, a stable BLV-infected cell line, was extracted. The coding region corresponding to the p24 protein was amplified by PCR. Following amplification, the cloned fragment was run in an agarose gel. A band of approximately 724 nucleotides was observed. The PCR product was then purified and subcloned into the expression plasmid pGEX-2T. The resulting plasmid (pGEX/p24) was sequenced, and the nucleotide sequence of the cloned gene was identical to that published for this region (Sagata *et al.*, 1985) (data not shown).

The recombinant protein was expressed in the bacterial strain *E. coli* DH5α′, as a fusion protein with *Schistosoma japonicum* GST. After cleavage with thrombin, the electrophoretic mobility of the resulting proteins was shown to be 50 kDa and 24 kDa respectively (Figure 1). The antigenic activity of the GST-p24 fusion protein, and the cleaved recombinant p24 were verified by western immunoblot analysis, using an anti-p24 monoclonal antibody. Both the GST-p24 and p24 bands were identified by the anti-p24 monoclonal antibody (Figure 1).

To raise chicken anti GST IgY, *E. coli* bacteria carrying the pGEX plasmid were induced to produce GST in the same way as described previously. The bacterial lysate was loaded into a GST-agarose column, eluted with glutathione, and dialysed against distilled

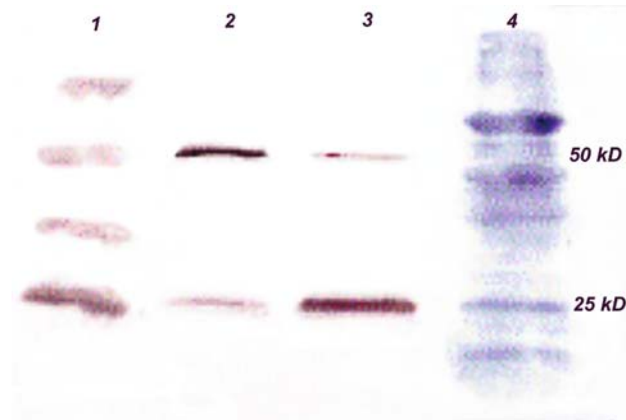

Figure 1. Western blot analysis of the recombinant protein. Lane 1, biotinilated molecular weight markers; lane 2, GSH-agarose-purified GST/p24; lane 3, thrombin digestion of GSH-agarose-purified GST/p24; lane 4, Coomasie blue-stained molecular weight markers

water. The purified protein was mixed with Freund's adjuvant and inoculated several times intramuscularly in a Leghorn chicken until a high level of antibodies against GST was detectable. The chicken was used as an anti-GST antibody source since avian IgY is the functional equivalent and evolutionary ancestor of mammalian immunoglobulin G. IgY derived from chickens has the advantage of not cross-reacting with mammalian antibodies, hence eliminating interference in immunoassays. Most importantly, it is not recognized by protein G (data not shown). Chickens are easy to keep, and IgY constitutes 70% of total immunoglobulins (Warr and Higgins, 1995). Delipidized chicken serum was bound to the bottom of a microwell plate, and kept at 4°C until use. Bacteria transformed with the recombinant plasmid pGEX/p24 were induced to produce the fusion protein. Several assays were designed to compare the capability of anti p24 monoclonal antibody to recognize the antigen. Recombinant and purified p24 and p50 were bound separately to the bottom of a microtitre plate. On the other hand, purified anti-GST chicken IgY, and the bacterial lysate, without any further purification step, were also loaded separately into the microtitre well. The binding of the chicken antibody to the plate can be considered an important step of purification (Table I).

The recombinant p24, fused to the GST and exposed by means of the chicken IgY, could be detected efficiently with a monoclonal antibody against p24 (Figure 2).

This double-layer detection method constitutes a very reliable and fast ELISA procedure that is applicable for the rapid screening of different bacterial colonies expressing fusion proteins with a GST residue. It can be also used to quantify the different levels of expression of a given fused protein, once the correct colony has been identified. A calibration curve has been designed for this purpose, with increasing amounts of the fusion protein bound to the IgY in the bottom of the microwell (Figure 3).

We conclude that this ELISA methodology is useful for detecting and quantifying the presence of any foreign protein or peptide expressed in the pGEX system by its

TABLE I
Different assays designed to check the ability of the anti-p24 monoclonal antibody to recognize the antigen

ON incubation at 4°C	1 h incubation at 37°C	Results	
		Positive control	Negative control
–	p50[d]	−	−
IgY[a]	p50[d]	+ + ++	−
IgY[a]	PMET	−	−
p50[b]	PMET	++	−
p24[c]	PMET	+	−

[a]Chicken IgY diluted 1:4000 in sodium bicarbonate
[b]Purified p50 diluted 1:100 in sodium bicarbonate
[c]Purified p24 diluted 1:100 in sodium bicarbonate
[d]Bacterial lysate diluted 1:100 in PMET
−, negative ($OD_{450} < 200$); +, weakly positive (OD_{450} 300–600); ++, positive (OD_{450} 600–1000); + + ++, strongly positive ($OD_{450} > 1800$)

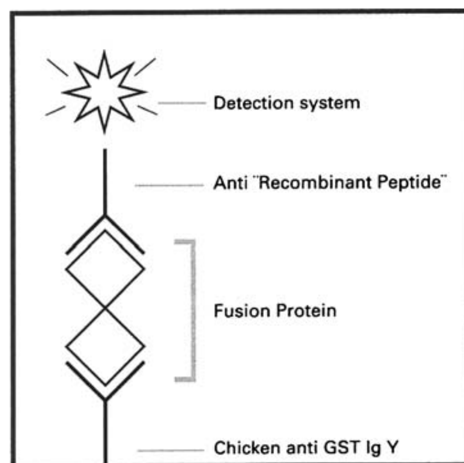

Figure 2. Scheme of the ELISA. Chicken anti-GST IgY is bound to the bottom of the plate. The fusion protein is bound to IgY, with its antigenic moiety available to bind the reacting antibodies. Any available detection system can be used

corresponding specific antibody. After the induction and lysis of the transformed bacteria, the fusion protein in the lysate is purified in one step, retained on a solid phase by the chicken anti-GST, and available for identification by its specific antibody. This ELISA-system is highly sensitive and specific, and has the potential to be readily adapted for a

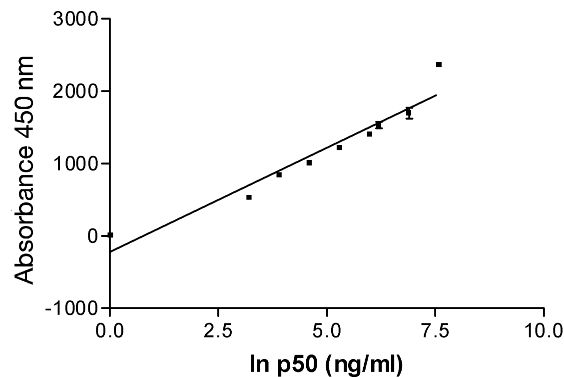

Figure 3. Calibration curve. Increasing amounts of fusion protein p50 bound to chicken IgY in the bottom of the microwell (abscissa) vs OD at 450 nm (ordinate)

large variety of proteins antigens. Most importantly, the use of an avian capturing antibody eliminates the possibility of cross-reaction with mammalian secondary antibodies.

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