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Preparation of Universal Peroxidase-Labelled Bacterial Protein Conjugates and Separation Properties

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Abstract

The aim of this study was to produce chimeric bacterial protein conjugates, to separate them by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and study their reactivities with human serum immunoglobulins by immunoblot analysis. Universal conjugates: protein LA-peroxidase (SpLA-HRP), protein LG-peroxidase (SpLG-HRP), protein AG-peroxidase (SpAG-HRP), protein LAG-peroxidase (SpLAG-HRP) and others were prepared by the periodate method. The conjugates were polymeric, with high molecular weights (MW) ranging from between 80-90 kDa to greater than 220 kDa. All conjugates have similar numbers of protein bands. However SpLA-LG-HRP and SpLAG-anti-IgY-HRP were less polymeric. Immunoblot blot analysis showed that all conjugates reacted with human serum Igs. These conjugates have a potential use in epidemiological surveys of zoonotic infections affecting avian and mammalian species.The periodate method was effective to producing chimeric conjugates of peroxidase-labelled immunoglobulin-binding proteins and the immunoblot analysis showed that they were funtional and effective in reacting with human serum immunoglobulins.

Keywords: Peroxidase-labelled immunoglobulin-binding protein; Staphylococcal protein-A (SpA); Streptococcal protein-G (SpG); Peptostreptococcal protein L (SpL); Commercially-prepared recombinant protein LA (SpLA); SDS-PAGE; Immunoblot analysis

Introduction

The reactivity of immunoglobulin-binding proteins for immunoglobulins (Igs) of mammalian species is well-known. These proteins are Staphylococcal protein-A (SpA), Streptococcal protein-G (SpG), Peptostreptococcal protein L (SpL) and a commercially-prepared recombinant protein LA (SpLA) [1-4]. Perel'man et al. reported that the periodate method was efficient in the preparation of SpA-peroxidase conjugates, while the preparation of bacterial protein conjugates failed when glutaraldehyde was used as a cross-linker [5]. Yolken and Leister synthesised and used a SpA-peroxidase conjugate in an ELISA [6] and Nygren and Hansson had reported that the modified periodate method allowed the preparation of conjugated protein A, which when analysed by SDS-polyacrylamide gel electrophoresis showed the presence of polymeric conjugates of large molecular size [7]. This study aimed to produce chimeric bacterial protein conjugates, to separate them by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and record their reactivities with human serum immunoglobulins by immunoblot analysis.

Materials and Methods

Preparation of universal conjugates

Protein LA-peroxidase (SpLA-HRP), protein LG-peroxidase (SpLG-HRP), protein AG-peroxidase (SpAG-HRP) and protein LAG-peroxidase (SpLAG-HRP) by the periodate method.Horseradish peroxidase (HRP) labelled SpA, SpG and/or SpL conjugates were prepared using the periodate method described by Nakane and Kawoi [8]. Horseradish peroxidase (500 µg in 50 µl $NaCO_3$, pH 9.6) was mixed with freshly made sodium periodate solution (1.71 mg/ml) followed by incubation in the dark for 2 h. In the preparation of SpLA-HRP, SpLG-HRP, SpAG-HRP and SpLAG-HRP conjugates the corresponding mixtures containing SpA and SpL (250 µg respectively); SpL and SpG (250 µg respectively); SpA and SpG (250 µg respectively); and equal amounts of SpA, SpG, and SpL giving a total weight 500 µg were added to the horseradish peroxidase-sodium periodate. The mixtures were incubated for 3 hours at 4°C with gentle agitation. Forty µl of freshly prepared NaBH4 solution (5 mg $NaBH_4$/ml 0.1 mM NaOH) was then added to each of the preparations, which were incubated for 90 min at 4°C in the dark with gentle agitation. Cold 50% saturated ammonium sulphate solution (pH 7.4) was added drop by drop in the ratio 1:1 (v/v). The mixtures were then centrifuged for 25 min at 4°C. The pellets were resuspended in 200 µl of PBS pH=7.4 and dialysed against 1L of PBS for 24 h with 3 buffer changes. An equal volume of glycerol was added to the dialysates followed by 100 µl of bovine serum albumin, BSA (20 mg/ml). The conjugates were then stored at -20°C until used. Preparation of both horseradish peroxidase labelled rabbit anti-chicken IgY-HRP, proteins LA [9], L and G (SpLAG-anti-IgY-HRP) and horseradish peroxidase labelled proteins LA,L and G (SpLA-LG-HRP).

To 100 µl of anti-chicken-IgY-HRP (Sigma-Aldrich Co.) conjugate was added 100 µl of a commercial 0.1 M carbonate-bicarbonate buffer pH: 9.6, 200 µl of freshly prepared sodium periodate (1.7 mg/ml) was added and the mixture centrifuged (13,000×g; 10 min; RT). A mixture containing 30 µg each of SpLA [9] and SpL (Sigma) and 50 µg SpG (Sigma) was then added; before centrifugation (30 min; RT). Sodium borohydride (38 µl of a 5 mg $NaHB_4$/ml plus 0.1 mM NaOH solution) was added and the mixture centrifuged (13,000×g; 10 min; RT in the dark). One volume of cold saturated ammonium sulphate solution was added and the mixture again centrifuged (13, 000×g, 25 min, 4°C). The pellet was resuspended in 200 µl of PBS pH 7.4 and dialysed against 1 L of the same buffer for 24 h with mild agitation and occasional buffer changes. An equal volume of glycerol was added to the dialysate

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followed by 100 µl of BSA (20 mg/ml). The conjugate was then stored at -20°C until used. A similar procedure was followed for the preparation of SpLA-LG-HRP conjugate except that an unlabelled HRP was used in the oxidation step (sodium periodate treatment). Analysis of conjugates by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.The conjugates were analysed by SDS-PAGE as described by Neville [10] and Justiz Vaillant et al. [11] using 10% acrylamide gels. Aliquots of 5-7 µg/µl of each protein conjugate and aliquots of 2-4 µg/µl of HRP, SpA or SpL (boiled for 5 min at 100°C in sample buffer containing 0.125 M TrisCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8, 10 ml) were electrophoresed at 150 V for 1 h. A MW marker (10-220 kDa, Sigma-Aldrich Co, St. Louis, Missouri) was included with each SDS-PAGE. Gels were stained with Coomassie brilliant blue.

Following electrophoresis the conjugates were blotted onto nitrocellulose membranes (approximately 75 minutes at 40 mAmps using running buffer: 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, 0.5% SDS). The membranes were blocked by incubating 2 h in 10% non-fat dry milk in PBS (with 0.05% Tween 20, pH 7.4). The membranes were washed 4×10 min with PBS-Tween 20 and incubated overnight at 4°C in human serum (diluted 1:100). The following day membranes were again washed 4×10 min with PBS-Tween 20. SpLA-HRP (homemade) diluted 1:5000 was added to the membranes, which were incubated for 2 h at RT and the washing procedure was repeated. Tetramethylbenzidine (TMB) solution (Sigma) was added to the membranes, which were then incubated in the dark at RT for 5 min after which the membranes were shaken gently, rinsed thoroughly in de-ionised water, dried and photographed.

IgG purification

A commercially prepared protein-A antibody purification kit (Sigma-Aldrich Co, St. Louis Missouri) based on affinity chromatography was used to purified IgG molecules from different species of sera including horse, donkey, mule, dog, skunk, coyote and raccoon. For the IgG purification were followed the manufacturer's instructions [11].

Results and Discusion

As shown in Figure 1 the conjugates are high MW compounds ranging from between 80-90 kDa to greater than 220 kDa. Similar numbers of protein bands were found for all the conjugates. However SpLA-LG-HRP and SpLAG-anti-IgY-HRP were less polymeric than

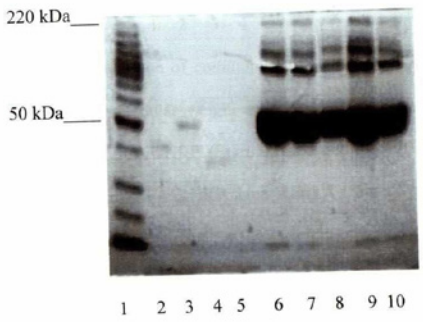

Figure 1: 10% Denaturing SDS-PAGE of chimeric conjugates: lane 1 molecular marker, lane 2 HRP, lane 3 SpA, land 4 SpL, lane 5 buffer, lane 6 SpLA-HRP, lane 7 SpG-HRP, lane 8 SpLAG-HRP, lane 9 SpLG-HRP and lane 10 SpAG. The conjugates are high molecular weight (MW) compounds ranging from between 80-90 kDa to greater than 220 kDa. Similar numbers of protein bands were found for all the conjugates.

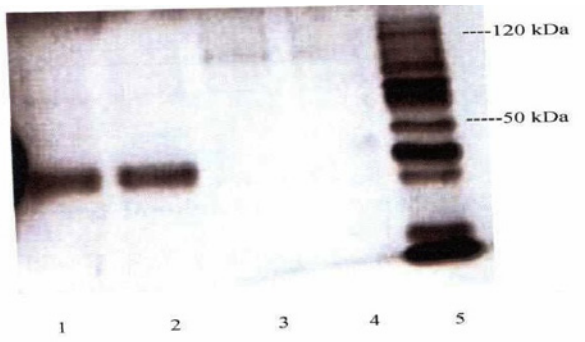

Figure 2: 10% Denaturing SDS-PAGE of SpLA-LG-HRP conjugate (lanes 1-2) about 30 kDa and SpLAG-anti-IgY-HRP conjugate (lanes 3-4) that was denatured by heat and protein bands are very faint. Lane 5 is the MW marker.

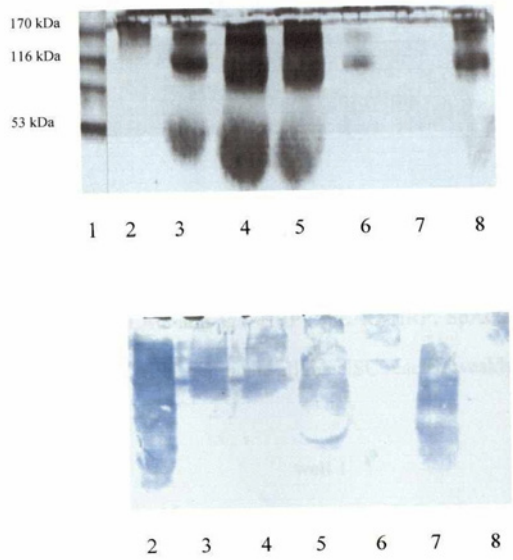

Figure 3: Denaturing SDS-PAGE and immunoblot analysis of chimeric conjugates: lane 1 MW marker, lane 2 SpLA-HRP (Sigma), lane 3 SpLA-HRP (homemade), lane 4 SpLG-HRP (homemade), lane 5 SpG-HRP (home made), lane 6 SpAG-HRP (homemade), lane 7 SpLA-LG-HRP and lane 8 SpLAG-HRP (homemade). Immunoblot analysis showed that all chimeric conjugates reacted with human serum immunoglobulins and it also confirmed their polymeric nature.

the other conjugates (Figure 2), which did not contain the commercial unlabelled SpLA. Immunoblot blot analysis (Figure 3) showed that all conjugates reacted with human serum IgG. The optimal working dilutions of conjugates range from 1:1000 to 1:5000. Figure 4 shows the 10% non-denaturing SDS-PAGE of sera and purified immunoglobulins (IgGs) by affinity chromatography using a commercial kit (Sigma-Aldrich Co St. Louis Missouri). The purified mammalian IgG had a MW of approximately 150 kDa.

Potential uses of the conjugates include epidemiological surveys of infectious diseases affecting poultry and mammals aiding in the selection of healthy animals for human consumption and enhancement of this industry. For example, conjugates used as a tool in ELISAs could be used in the diagnosis of avian and mammalian diseases or zoonotic diseases affecting both animals and humans.

Conjugates including SpLA-HRP, SpLG-HRP, SpAG-HRP, SpLAG-

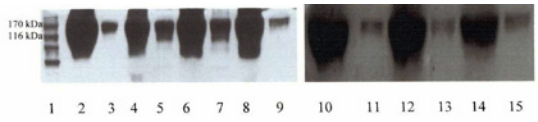

Figure 4: The 10% non-denaturing SDS-PAGE of sera and purified immunoglobulins (IgGs). Lane 1 MW marker, lane 2 mule serum, lane 3 mule IgG, lane 4 donkey serum, lane 5 donkey IgG, lane 6 horse serum, lane 7 horse IgG, lane 8 dog serum, lane 9 dog IgG, lane 10 skunk serum, lane 11 skunk IgG, lane 12 coyote serum, lane 13 coyote IgG, lane 14 raccoon serum and lane 15 raccoon IgG. A commercially prepared protein-A antibody purification kit (Sigma-Aldrich Co, St. Louis Missouri) based on affinity chromatography was used to purified IgG molecules from the above different mammalian sera. The purified mammalian IgG had a MW of approximately 150 kDa.

HRP, SpLA-LG-HRP and SpLAG-anti-IgY-HRP proved to be efficient binding reagents having the capacities to react with human serum immunoglobulins. The periodate method was efficient in the successful preparation of the peroxidase-bacterial protein conjugates. Although the SDS-PAGE analyses of the conjugates showed the presence of horseradish peroxidase excess, it did not affect the sensitivity of the immunoblot analysis as the HRP is washed away in the washing procedures, where conjugates were tested. In future work for optimizing the preparation of these products, different molar concentrations of the enzyme and the bacterial Ig-receptors should be tested. The enzyme-protein ratio of 1:1 used here allowed the formation of stable compounds and did not affect the binding of the different conjugates to antibodies and substrates.

Conclusions

I conclude that the periodate method was effective to producing chimeric conjugates of peroxidase-labelled immunoglobulin-binding proteins and the immunoblot analysis showed that they were funtional and effective in reacting with human serum immunoglobulins.

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