

Immunoglobulin-binding Bacterial Proteins (IBP) Conjugates and their Reactivity with Immunoglobulin in Enzyme-Linked Immunosorbent Assays (ELISA)

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Abstract

The aim of this study was to create universal chimeric conjugates able to react with both avian and mammalian immunoglobulins to be used as a reagent in Enzyme-linked Immunosorbent Assays (ELISAs). The periodate method was used in the conjugation process of linking horseradish peroxidase to immunoglobulin-binding bacterial proteins. ELISAs were used to prove the efficacy of the conjugates, namely newly synthesized conjugates (NSC) in the detection of immunoglobulins. All NSC bound to mammalian immunoglobulins, but failed to bind avian immunoglobulin Y (IgY), with the exception of the SpLAG-anti-IgY-HRP that was the most versatile binding to the all panel of purified immunoglobulin and sera. In conclusion, the results of these experiments show that SpLAG-anti-IgY-HRP conjugate, a novel reagent, was the most versatile product among NSC and commercially-available conjugates.

Keywords: Immunoglobulins; ELISA; Immunoglobulin-binding bacterial proteins; Protein conjugates

Introduction

Immunoglobulin-binding bacterial proteins (IBBP) are molecules that are widely found in the cell walls of several bacteria and they have the capacity to bind to the F_c or F_{ab} regions of immunoglobulins from different mammalian species. The most well-known IBBP are: Staphylococcal protein A (SpA) [1], Streptococcal protein G (SpG) [2] and Peptostreptococcal protein L (SpL) [3]. They have been used linked to enzymes as immunodetection tools (conjugates) in Enzyme-linked Immunosorbent Assays (ELISA) for detection of antigens and antibodies [4].

Peroxidase-(HRP) labelled IBBP are commercially available conjugates. They are considered rather than specific, universal conjugates (bind too many diverse immunoglobulins), including SpA-HRP, SpG-HRP, SpL-HRP, recombinant chimeric SpL-SpA-HRP (SpLA-HRP) [5,6], and some others, such as recombinant chimeric SpA-SpG-HRP (SpAG-HRP) and SpL-SpG-HRP (SpLG-HRP). None of the commercially-available conjugates react universally to both avian and mammalian immunoglobulins. Their binding to avian immunoglobulins is weak or they are non-reactive. In this study, we create a universal chimeric conjugate able to react with both avian and mammalian immunoglobulins to be used as a reagent in Enzyme-linked Immunosorbent Assays (ELISAs): the SpLAG-anti-IgY-HRP. In addition, we prepared other chimeric conjugates to compare their affinity to mammalian IgG, including the SpLA-HRP, SpAG-HRP, SpLG-HRP, SpLAG-HRP and SpLA-LG-HRP that were produced by mixing their individual components. All homemade conjugates were named newly synthesized conjugates (NSC). As a part of the study, we compare the sensitivity of the homemade conjugates with that of commercially-prepared products.

Materials and Methods

Conjugate preparation (SpA-HRP, SpG-HRP and SpL-HRP)

All proteins, chemicals and buffers used in these protocols were

commercially-available products (Sigma-Aldrich Co. St. Louis, Missouri).

Preparation of chimeric doubled conjugates

The SpAG-HRP conjugate was prepared by mixing 100 µl of both SpA-HRP and SpL-HRP. The SpLA-HRP conjugate was prepared by mixing 100 µl of both SpL-HRP and SpA-HRP. The SpLG-HRP conjugate was prepared by mixing 100 µl of both SpL-HRP and SpG-HRP.

Preparation of chimeric tripled conjugates

The SpLAG-HRP conjugate was prepared by mixing 50 µl of each SpL-HRP, SpA-HRP and SpG-HRP. The SpLA-LG-HRP was prepared by mixing 50 µl of each SpLA-HRP, SpL-HRP and SpG-HRP.

Preparation of a chimeric quadrupled conjugate

The SpLAG-anti-IgY-HRP was prepared by mixing 50 µl of anti-chicken-IgY-HRP with 50 µl each of SpL-HRP, SpA-HRP and SpG-HRP.

Immunoglobulin-Y isolation

The IgY fraction was isolated from the egg yolks of a variety of birds, including chicken, bantam hen, guinea hen, quail, goose, duck,

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pigeon, parakeet, cattle egret, pheasant and ostrich. The IgY fraction was isolated by the chloroform-polyethylene glycol (PEG) method [7]. The eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 v/v in phosphate buffered saline (PBS), pH 7.4. To 1/3 of the egg yolk mixture, an equal volume of chloroform was added, the mixture was then shaken and centrifuged for 30 min (1000×g, RT). The supernatant was decanted and mixed with PEG 6000 (12%, w/v), stirred and incubated for 30 min at room temperature (RT). The mixture was then centrifuged, as previously described. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/6 of the original volume of the egg yolk and dialyzed against 1L of PBS (pH: 7.4 for 24 h at 4°C). The IgY was removed from the dialysis tubing. IgY concentration was determined by the Bradford method [8]. IgY samples were stored at -20°C.

ELISAs

Briefly, the microplates were coated, 25 µg/well in 50 µl carbonate-bicarbonate buffer pH 9.6 (Sigma) with different avian and mammalian immunoglobulins, 50 µl avian egg whites or 50 µl of sera from different animal species. Plates were washed 4X with 150 µl PBS-Tween 20 buffer (Sigma-Aldrich Co, St. Louis Missouri). Then, 100 µl of each NSC was added, diluted 1:1000 in PBS-non-fat milk to each well and incubated for 1 h at room temperature (RT). The plates were washed 4X with PBS-Tween. 100 µl of 3 mg/ml o-phenylenediamine solution (OPD) was added and the plates were incubated 15 minutes at RT. The reaction was stopped with 50 µl of 3M H₂SO₄ solution. The plates were visually assessed for the development of colour (indicating a positive result), and read in a microplate reader at 492 nm. A positive result was taken as equal or above the cut-off point (0.10-0.20 weakly interaction; 0.21-0.30 moderate interaction and above 0.31 strong interaction; 1+, 2+ and 3+, respectively).

Results and Discussion

Figure 1 shows the working dilutions of both homemade and commercially-prepared conjugates. They worked well in dilution between 1:1000 and 1:2000. Some of them, such as the SpLA-HRP (Sigma) and the SpLAG-anti-IgY-HRP, gave good signal in dilutions of 1:4000. In addition, as shown in Table 1, the NSC reacted with goat and sheep IgGs. The chicken IgY was detected using the SpLAG-anti-IgY-HRP conjugate only.

This paper discusses the interaction between IBBPs, which are proteins with the capacity to interact with immunoglobulins of mammalian species, and few avian IgYs. The importance of these protein interactions is that they can be useful reagents in the detection of specific antibodies. In addition, IBBPs might be useful in affinity chromatography for IgY purification from the blood or egg yolk of birds [9].

The NSC, including SpLA-HRP, SpLG-HRP, SpAG-HRP, SpLAG-HRP and SpLA-LG-HRP proved to be efficient binding reagents with the capacities to react with Ig's from a number of mammalian species. The reactivity of the NSC with avian Ig was more restricted than that of mammalian species. This limitation was not encountered when SpLAG-anti-IgY-HRP conjugate was used. This reacted with a wide range of avian and mammalian immunoglobulins. This is the first documented report of the successful preparation of a SpLAG-anti-IgY-HRP conjugate, which can be considered the most universal of these reagents. The broad reactivity of SpLAG-anti-IgY-HRP across mammalian and avian species would allow for its use in ELISAs and immunoblot analyses. The SpLAG-anti-IgY-HRP conjugate becomes even more important, since there are no commercially available conjugates that can be used for immunodetection in a wide variety of avian and mammalian species, e.g. in the study of zoonotic diseases [4].

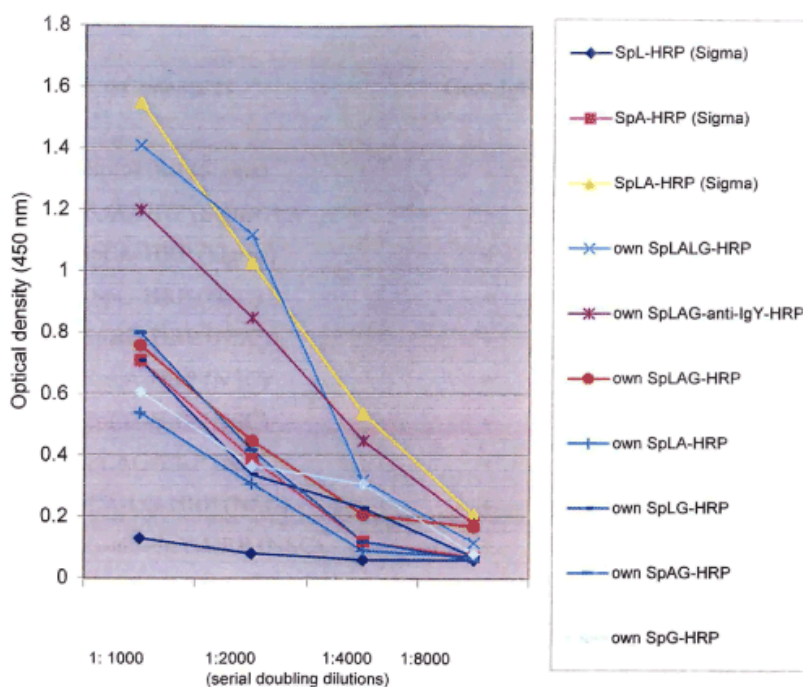


Figure 1: Dilutions 1:1000 and 1:2000 were considered as optimal dilutions of both homemade (NSC) and commercially-prepared protein conjugates.

This conjugate could be used for immunotesting of infectious diseases in zoo animals, without having the necessity of using species-specific ELISAs, which are expensive and restricted to a particular species.

Potential uses of the NSC include epidemiological surveys of infectious diseases affecting poultry, aiding in the selection of healthy animals for human consumption. For example, NSC could be used in ELISA for the diagnosis of avian diseases, including: infectious bronchitis, Newcastle disease, avian influenza virus infection, reticuloendotheliose virus infection and turkey rhinotrachetis virus infection, chicken anaemia agent, avian encephalomyelitis, avian leucosis/RSV infection and avian retroviruses.

Table 1 showed a direct ELISA that records the cross-reactivity of a commercial anti-chicken IgY-HRP conjugate with avian IgY extracted

from the egg yolk. The anti-chicken IgY-HRP conjugate did not react with avian egg whites, because those did contain IgM molecules, but not IgY proteins. Tables 1 and 2 recorded the reactivity of a SpLA-LG-HRP conjugate and a SpLAG-HRP conjugate. These homemade conjugates binding capacity was comparable to that of the commercial SpLA-HRP conjugate. In addition, the comparison of the reactivities between NSCs and commercial conjugates showed that NSCs were more versatile.

Nygren and Hansson [10] had reported that the modified periodate method allowed the preparation of conjugated protein A, which when analyzed by SDS-polyacrylamide gel electrophoresis, showed the presence of polymeric conjugates of large molecular size. Justiz Vaillant et al. [9] reported similar results. Perel'man et al. [11] reported that the periodate method was efficient in the preparation of SpA-peroxidase conjugates, while the preparation of these immunochemicals failed

Ig samples	SpLAG-HRP (NSC) Reactivity score	SpAG-HRP (NSC) Reactivity score	Anti-chicken IgY-HRP (Sigma) Reactivity score	SpLA-HRP (Sigma) Reactivity score
Ostrich IgY	1+	1+	1+	1+
Pheasant IgY	0	0	1+	0
Bantam hen IgY	0	2+	2+	1+
Duck IgY	0	1+	1+	1+
Guinea hen IgY	0	0	1+	0
Goose IgY	0	0	1+	0
Quail IgY	0	0	1+	0
Chicken IgY	0	0	2+	0
Pigeon egg white	0	0	0	1+
Pheasant egg white	0	0	0	1+
Duck egg white	0	0	0	1+
Bantam egg white	0	0	0	1+
Ostrich egg white	1+	1+	0	1+

A positive result was taken as equal or above the cut-off point (0.20-0.30 weakly interaction; 0.31-0.40 moderate interaction and above 0.41 strong interaction; 1+, 2+ and 3+, respectively). Eight replicates of each sample were tested.

Table 1: Comparison of the reactivity of avian IgY with commercial and newly synthesized conjugates.

Samples	SpLA-LG-HRP (NSC) Reactivity score	SpLAG-anti-IgY-HRP (NSC) Reactivity score	SpLA-HRP (Sigma) Reactivity score	SpA-HRP (Sigma) Reactivity score	SpL-HRP (Sigma) Reactivity score
Pig IgG	3+	3+	3+	2+	2+
Rabbit IgG	3+	3+	3+	2+	2+
Goat IgG	2+	2+	2+	1+	0
Sheep IgG	2+	2+	2+	1+	0
Human IgG	3+	3+	3+	3+	3+
Mouse IgG	3+	3+	3+	3+	3+
Cat IgG	2+	2+	2+	2+	0
Skunk sera	2+	2+	2+	2+	0
Coyote sera	2+	2+	2+	2+	0
Raccoon sera	3+	3+	2+	2+	3+
Quail IgY	0	1+	0	0	0
Goose IgY	0	1+	0	0	0
Ostrich IgY	1+	2+	+	1+	0
Duck IgY	0	1+	1+	1+	0
Pheasant IgY	0	1+	0	0	0
Chicken IgY	0	2+	0	0	0
Cattle egret IgY	0	1+	0	0	0
Guinea hen IgY	0	1+	0	0	0
Bantam hen IgY	0	2+	+	1+	0
Pigeon IgY	0	1+	+	1+	1+

A positive result was taken as equal or above the cut-off point (0.20-0.30 weakly interaction; 0.31-0.40 moderate interaction and above 0.41 strong interaction; 1+, 2+ and 3+, respectively). Eight replicates of each sample were tested.

Table 2: Comparison of the reactivity (moderate and high reactivities) of the newly synthesized conjugates and commercially available conjugates (Sigma) for the detection of purified or serum immunoglobulins.

when glutaraldehyde was used as a cross-linker. Yolken and Leister [12] synthesized and used a SpA-peroxidase conjugate in an ELISA. The results of this study suggest that the NSC were effective in their binding to immunoglobulins of different animal species. The development of conjugates, such as SpLAG-anti-IgY-HRP, is of general interest, and could be useful both as a tool in ELISA and western blot, but more importantly for studies of zoonotic diseases.

Conclusion

In conclusion, the results of these experiments show that SpLAG-anti-IgY-HRP conjugate, a novel reagent, was the most versatile product among NSC and commercially-available conjugates. Potential uses of this conjugates are immunodiagnosis and Immunoglobulin detection.

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Competing Interests

The authors declared that no competing interests exist.

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