Preparation of Anti-Buffalo- IgG Alkaline Phosphates as Specific Conjugate for Serodiagnosis of Buffalo Species Using Protein-A-Sepharose Affinity Chromatography.

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Abstract: Anti-buffalo IgG conjugated with calf intestinal alkaline phosphates were prepared. Buffalo serum was precipitated with 50% saturated ammonium sulfate (SAS), dialyzed and concentrated with polyethylene glycol (PEG) . Affinity chromatography utilizing protein A covalently bound to sepharose beads was applied to separate pure immunoglobulin-G from buffalo serum. Anti-buffalo IgG were prepared by immunization of goats with purified buffalo IgG then goat anti- buffalo IgG were prepared. Goat anti- buffalo IgG was conjugated with alkaline phosphates enzyme. [Hanan M.El-Hewa**iry. Preparation of Anti-Buffalo- IgG Alkaline Phosphates as Specific Conjugate for Serodiagnosis of Buffalo Species Using Protein-A-Sepharose Affinity Chromatography.** Journal of American Science 2012; 8(4): 43-47].(ISSN: 1545-1003). http://www.americanscience.org. 6

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1. Introduction

Immunoglobulin-G- is the main immunoglobulin of serum (Reynolds and Johnson, 1970; Heddle and Rowely, 1975). Besides its protective functions, IgG plays an important role in conferring specificity to serological tests used in the diagnosis of infectious diseases.

Several methods including gel filtration, ion exchange chromatography and affinity chromatography are available for the purification of immunoglobulin.

Immunoaffinity chromatography is a powerful method of purification **(**Chan, 1996) and **(**El-Hewairy et al., 2003).

Protein-A is a major cell wall component of most strains of *Staphylococcus aureus.* It was characterized and molecule is a single polypeptide chain of no carbohydrate. It is covalently linked to the peptidoglycan portion of the cell wall and is secreted during the exponential growth phase of the organism (Goding, 1976).

The major feature of protein A is its extraordinary affinity for immunoglobulin, in Particular IgG. Protein-A was found to bind different immunoglobulin classes of several mammalian species including mice, rabbits, horses, pigs, cats, goats, sheep and bovines (Goding, 1976; Goudswaard *et al*., 1988). Affinity chromatography on protein-A- Covalently bound to sepharose leads has been utilized to pure immunoglobulin isotypes from mammalian sera (Warr and Hart, 1979 & El-Said, 1995) reported that chromatography on protein- A sepharose proved to be an uncomplicated procedure that does not require. Sophisticated equipment and can be performed under our laboratory conditions.

Serum proteins of buffalo have received relatively less attention than those of other domesticated animals. Because the diagnosis of diseases is often based on serology, we considered it pertinent to define buffalo immunoglobulin and prepare anti-buffalo-IgG Labeled with alkaline phosphates. The present study was directed to produce anti-buffalo IgG conjugated with alkaline phosphates.

Materials and Methods

Samples

Serum was separated according to (Goers, 1993) from about 1 liter of pooled blood collected by jugular puncture immediately before slaughtering from 8 normal healthy male buffalo slaughtered in the Draw abattoir at Aswan governorate, the separated serum was further clarified in a refrigerated centrifuge at 3000 r.p.m for 10 minutes and stored at -20^{o}c until used.

Purification of Buffalo' immunoglobulin G (IgG)

Buffalo immunoglobulin's (Igs) were precipitated with 50% saturated ammonium sulfate (SAS) according to (feteanu,1978), (Ungur – waron et al., (1987) and (El-Hewairy *et al*., (2004) by adding 100 ml of SAS to 200 ml of serum, the procedure was repeated twice and finally the precipitate was dissolved in borate buffered saline to a final volume or less than half that of the original volume sample and dialyzed against several changes of phosphate buffer saline (1x PBS. pH 7.2) overnight at about 4^{o}C till no ammonium sulfate was detected.

Immunoglobulin (Igs) were concentrated approximated ten times by polyethylene glycol (PEG) according to (Inghum, 1990).

Affinity chromatography was performed using protein–A covalently bound to sepharose, using modification of the technique of Warr and Hart, (1979). Protein-A sepharose Cl-4B (Pharmacia, Sweden) was rehydrated, washed and equilibrated in

0.1m Phosphate buffer pH 7.5 according to the manufacture instructions.

The bed material was packed in 1x15cm chromatographic column, 5mg of whole Igs were Loaded to over saturate the column was washed with 0.01m PBS pH 7.2, The column put on the shaker for 2-3 hours, the column was washed thoroughly (ten times the bed volume) to remove the unbound proteins using 0.1m phosphate buffer PH 7.2. Elution of the IgG was achieved by running of 5ml of 0.1 glycine-HCl buffer pH 2.4. Three ml fractions were collected manually and neutralized to PH 7.0 with 4 N NaoH. Protein content of the eluted fractions was mentored by UV spectrophotometer at 280 nm. Dialysis of elutes, concentrated with PEG – 600 and finally protein content were determined by Lowery's ***et al.,*** (1951), then it was stored at -20^{o}c until further use.

Preparation of anti-buffalo' IgG

It was prepared by goat immunization. Ten mg of the prepared buffalo IgG was thoroughly mixed with equal volume of freund's complete adjuvant (FCA) was injected sub/cut in goat. Four booster doses mixed with equal volume of fraund's incomplete adjuvant (FICA) were injected subcutaneously in the goat; each booster dose was injected one week interval. Serum samples were collected before the immunization, after 1st booster, 2nd booster then weekly. The samples were taken to detect the protein content on spectrophotometer and agar gel precipitation test (AGPT).

Conjugation of the prepared anti-buffalo IgG with alkaline phosphatase

The anti-buffalo IgG was conjugated with alkaline phosphates enzyme according to **O'sultivan and Marks (1981)** as following, 3mg of alkaline phosphates enzyme were added to 1.5mg of goat anti-buffalo IgG in PBS, gluteraldehyde was added to 0.20% with gentle vortex, the mixture was incubated for 2 hours at room temperature, desalted by chromatography on sephadex G-25 and finally stored at 4^{o}c after sterile filtration or addition of sodium azide.

The prepared anti-buffalo IgG labeled with alkaline phosphatase was evaluated by Enzyme Linked Immuno-Sorbent Assay (ELIZA) using eleven blood sample (seven collected from brucellosis buffalo and four collected from buffalo proved to be free from brucellosis).

Evaluation of prepared anti-buffalo IgG labeled with alkaline phosphatase were subjected to Enzyme Linked Immuno-Sorbent Assay (ELIZA).By using of eleven blood sample, seven of them were collected from brucellosis buffalo (examined by bacteriology) and four samples were collected from buffalo proved to be free from brucellosis are negative control.

3. Results

The yield of whole Igs after 50% saturated ammonium sulfate solution (SAS) precipitation, dialysis and concentrated with polyethylene glycol (PEG) was about (30) ml.

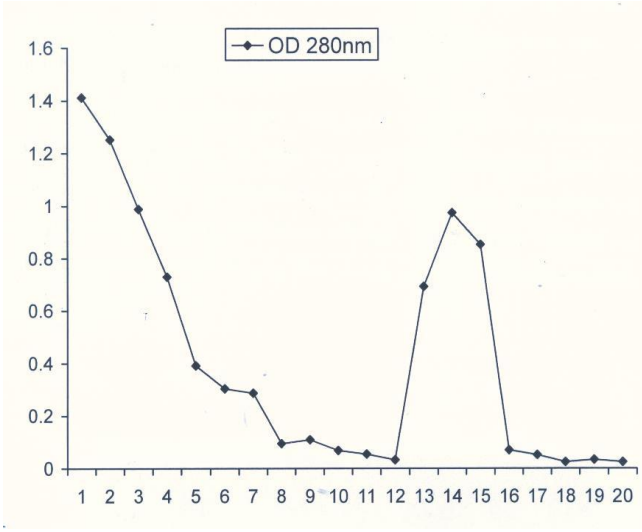
It was found that the protein content of Igs was 3.2mg/ml. The yield of obtained buffalo immunoglobulin's (Igs) was (96) mg.

Serum buffalo globulins were subjected to protein-A- sepharose affinity chromatography, Elutes were collected and absorbance value was recorded at 280 nm to generate elution profile. The collected fractions of peak I were globulins other than IgG, this peak was discarded, the fractions of peak II were collected, dialyzed and concentrated by PEG-600 and stored a t-20^{o}c till used, the yield of obtained IgG was about 10ml, the protein content was 5.4 mg/ml while the total amount of IgG was 54 mg.

***Table (1): Showing the optical density value of the first step purification of buffalo IgG on protein-A sepharose affinity chromatography.**

Tubes number	OD 280nm	Tubes number	OD 280nm
1	1.412	11	0.054
2	1.251	12	0.032
3	0.988	*13	0.692
4	0.729	*14	0.974
5	0.391	*15	0.852
6	0.304	16	0.067
7	0.287	17	0.051
8	0.094	18	0.024
9	0.109	19	0.033
10	0.068	20	0.024
Tubes representing peak 1		Tubes representing peak 2	

*Elutes were collected in these tubes after addition of 0.1M glycine HCL, pH2.4.



***Figure(1): Showing the OD value of the purification of buffalo IgG on protein-A-sepharose affinity chromatography.**

Blood sample were taken from goats at the zero day, at the 1st booster, at the 2nd booster dose and weekly until the time of bleeding. Total globulins of goat were increased about 8.9 times from zero day until the slaughtering time.

The goat anti-buffalo sera were tested by AGPT. The precipitation lines were cleared after 21 days, the reaction (Precipitation line) was continued till the end of experiments at 38 days. Serum were collected, precipitated by saturated ammonium sulphate, dialyzed, concentrated and purified as described previously. The protein content of goat anti-buffalo IgG was 4mg/ml.

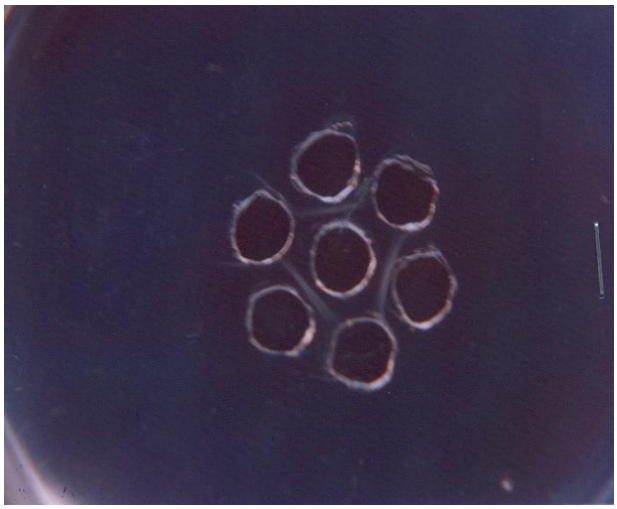


***Photo (1) Showing precipitation line of identity between buffalo IgG and goat anti-buffalo IgG.**

The goat anti-buffalo IgG conjugated alkaline phosphates were desalt by sephadex G-25 chromatography after 2 hours of incubated time.

Results of evaluation of prepared conjugated anti-buffalo IgG alkaline phosphatase by ELISA cleared that, the prepared anti-buffalo IgG alkaline phosphatase till 1:200 dilution in PBS for capture the antibody in positive buffalo sera. The result of stability prepared anti-buffalo IgG alkaline phosphatase by ELISA cleared that the conjugate is still active and able to capture the antibody in the positive buffalo sera when stored at 4°C till six months. Application of prepared anti-buffalo IgG alkaline phosphatase in comparison with protein-A conjugate for examination of buffalo sera, the result cleared that , the prepared conjugate gave 100% sensitivity while the application of protein-A-alkaline phosphatase gave 78% sensitivity.

4. Discussion

Many conjugated anti-IgG of different animals are available commercially but anti-buffalo-IgG conjugated with enzyme still represent a deficient diagnostic reagent.

The purity of immunoglobulin is essentially the backbone of enzyme immunoassay since they are coupled to immunoreactants.

Immunoglobulin-G is the main immunoglobulin of serum (Reynolds and Johnson,1970), (Heddle and Rowely,1975). Besides its protective function, IgG plays an important role in conferring specificity to serological tests used in the diagnosis of infectious diseases.

Several methods including gel filtration, ion exchange chromatography and affinity chromatography are available for the purification of immunoglobulin.

In the present study, buffalo Igs were precipitated by using 50% SAS. Many authors used ammonium sulphate for the precipitation of Igs **(James, 1983; Kewick,1990; Logan *et al*., 1990; Hannant *et al*., 1992; Azwai *et al*., 1993; Svendsen *et al*., 1995).** Ammonium sulphate is inexpensive and used for purification of Igs in large scale, **Baick-Seungchun *et al*., (1995)** separated bovine Igs by 33% ammonium sulphate. **Ungur-Waron *et al.* (1987), Reddy and Giridhar (1990), Ramesh-Babu and Reddy (1992)** and **El-Hewairy *et al*. (2004)** used 50% SAS for precipitation of Igs. While **Steinbuch and Andran (1969), Russ *et al.* (1983), Mckinney and Parkinson (1987) and Kataria and Sharma (2000)** used caprylic acid for precipitation of Igs, and **Joshi *et al*. (1997)** precipitated buffalo IgG by anhydrous sodium sulphate.

The precipitated buffalo Igs were extensively dialyzed against 1 x PBS pH 7.2 for several days to

remove ammonium sulphate and the end of dialysis was detected by absence of whole precipitate which appear in dialyzate containing ammonium sulphate tested with few drops of 10%-barium chloride **James, (1983).** Concentration of Igs was performed according to **Ingham, (1990).**

Different methods have been used to isolate IgG. Some workers produced IgG by gel filtration chromatography on sephadex G-200 or sepharose 6-B **(Heddle and Rowely, 1975).** However, this method is time-consuming, Laborious and contamination of IgG with IgA is Likely **(Fey *et al.*, 1976).** Anion-exchange chromatography is simpler method for IgG isolation, **(Butler and Maxwell, 1972).** Both gel filtration and anion-exchange chromatography require expensive equipment for chromatography that may not be available in most of our Laboratories, **(El-Said, 1995).**

In the present study, buffalo IgG was separated by affinity chromatography which is considered one of the most powerful procedures that can be applied to protein purification. Affinity chromatography utilizing protein A covalently bound to sepharose beads was applied to isolate pure immunoglobulin-G from buffalo serum.

Some authors separated IgG by affinity chromatography over protein-A-Sepharose, (**Goding, 1976; Joshi *et al*., 1997; El-Said, 1995)** utilized affinity chromatography on protein-A to separate canine serum IgG. **Chan (1996)** recorded that the affinity chromatography is simple, one step, highly efficient method for IgG purification, **Kakker and Goel (1993)** used *S.aureus* protein A to remove IgG_2 from buffalo IgG1. Chromatography on protein A sepharose proved to be an uncomplicated procedure that does not require sophisticated equipment, **(El-Said, 1995).**

It can be concluded that purification of anti-species IgG by affinity chromatography is more efficient but is more expensive, this result was in agreement with **Chan (1996**) who recorded that the affinity chromatography is simple, one step, and highly efficient method for IgG purification. After purification of buffalo IgG, goats were inoculated to produce anti-buffalo IgG. Goats are widely used for production of specific antibodies, (**Rantamoki and Mutler, 1995; Kataria and Sharma, 2000).** The scheme 20 mg of buffalo IgG mixed with equal volume of Freund's complete adjuvant subcutaneously, after 7 days, 14 days, 21 days and 28 days the goat were inoculated 20 mg of IgG mixed with equal volume of Freund's incomplete adjuvant. In the present study, **Kataria and Sharma (2000)** immunized goats(aging 4-5 years and weighing about 35 Kg) to raise anti-buffalo serum. The goats were bled after one week of last inoculation. Detection of anti-buffalo IgG in sera of immunized animals was performed by AGPT according to **Ouchterlony (1962).**The specific line of precipitation appeared only in sera collected after 21 days from the first dose (Photo 1) . This result was agreement with **Reddy and Giridhar (1990)** who recorded that the test of bleeding showed a single precipitating line by Ouchterlony techniques.

Goat anti-buffalo IgG were prepared as previously described and the protein content was determined by lowery's method for goat anti-buffalo IgG, the protein content was 3mg/ml.

As the labeled anti-buffalo IgG with enzymes for Enzyme Linked Immuno Sorbent Assay (ELISA) is not available in commercial level. The present investigation was directed for producing Labeling anti-buffalo IgG with alkaline phosphates enzyme. In the present work, anti-buffalo IgG were labeled by alkaline phosphates enzyme bind by gluteraldehyde using affinity chromatography technique by sephadex-G- 25, 2 ml of anti-buffalo IgG.

Indirect method of ELISA was done to evaluate and measure the optimum dilution of the prepared conjugate, The result cleared that the highest dilution of alkaline phosphatase labeled anti-buffalo IgG that gave OD 3 fold the mean of negative sera was at 1/200.This dilution which can be used for using the prepared conjugate in ELISA. Also stability of the prepared conjugate was detected by investigation of preserved conjugate at 4°C by testing of sera collected from brucellosis buffaloes. Also the result revealed that the prepared conjugate conjugate has sensitivity of 100% all positive and negative buffalo sera were tested with protein A conjugate.

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