PRODUCTION OF ANTI-BOVINE SOMATOTROPIN (B-ST) RABBIT POLYCLONAL ANTIBODIES

N. Nazir, F. Rehman[*], M.A. Shaheen[*], A. Riaz, A. I. Batool[**], M. Sher and M. Aslam[*]

Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore,
[*]Department of Chemistry and [**]Department of Biological Sciences, University of Sargodha, Sargodha
Corresponding author**:** fayyaz9@gmail.com & mashaheen@msn.com

ABSTRACT: Bovine somatotropin is a growth hormone found in cattle. A commercial preparation of this hormone (bGH) was used as an antigen. Antibodies were raised in rabbit. Primary immunization was done using Freund's complete adjuvant followed by successive immunizations using Freund's incomplete adjuvant. The immune response of rabbit was checked by carrying out immune blot assay and qualitative ELISA. Microtiter wells coated with the antigen (bGH) were exposed to primary antibody (rabbit antiserum). The antigen-antibody complex formation was detected by using secondary antibody (sheep anti-rabbit IgG HRP conjugate). The ability of these antibodies to detect bovine growth hormone was also checked by western blotting. Antibodies raised in this study were able to detect bGH in a western blot.

Keyword: Polyclonal antibody, antigrowth hormone Ab, *Oryctulagus cuniculus*, ELISA.

INTRODUCTION

Bovine growth hormone (bGH) or Somatotropin, a peptide hormone of anterior pituitary gland, increases rate of protein synthesis and mobilization of fatty acids; and decreases the rate of glucose utilization (Bauman, 1992). Several variants of GH exist in many higher animals due to growth hormone gene duplication, differential mRNA splicing and differential GH protein processing (Wood *et al*., 1989).

Polyclonal antibodies (pAB) specific for bovine growth hormone (bGH) can be used to detect the different preparation of the growth hormones. The purified growth hormone Antibodies against bovine prolactin (PRL) and ovine prolactin (oPRL) immunization are usually used in quantitative estimation of levels of prolactin (Carey *et al*., 1995). The chicken anti-bGH antibodies have shown promising results in combination with rabbit anti PRL antibodies. In this study, polyclonal antibodies against bovine growth hormone are produced. The antibodies so produced can be used as solid phase capture antibodies in quantitative estimation of bovine somatotropin and in the development of an in house ELISA for estimation of recombinant bovine (Berrini *et al*., 1994; Brambilla *et al*., 1993), equine (Gobbi *et al*., 1990) ovine and caprine somatotropins. Recombinant bGH can be characterized for its immunoreactive properties using these pAB.

MATERIALS AND METHODS

Primary immunization: Commercial recombinant bovine somatotropin preparation (LG South Korea) was used as immunogen. Ten (10) white Rabbits (*Oryctulagus cuniculus*) were purchased for production of polyclonal antibodies. One ml blood sample was drawn from each rabbit and serum was separated as negative control. 20µl of bovine somatotropin preparation containing 2 mg of the hormone was dissolved in phosphate buffer saline (PBS) pH 7.0. One ml of Freunds complete adjuvant (Sigma–Aldrich) was added to 1ml of immunogen formed a white milky emulsion. The eight rabbits were immunized with 2ml of this emulsion (Stills, 1994) and two of them were kept as negative controls.

Immuno dot blot analysis: After 14 days of immunization, 1ml blood was drawn; serum was separated and stored at -20°C. Nitrocellulose membrane was used for dot blot analysis. 3 µl of b-GH was poured on the membrane and dried. Membrane was soaked in blocking solution (5 % BSA) and incubated at 37°C for 45 minutes. Washing was done four times with the PBS pH 7.0 for ten minute each. Diluted primary antibody in the PBS (1:5) was poured on the membrane and incubated at 37°C for 45 minutes. After four washings with the PBS, diluted secondary antibody (anti-rabbit HRP conjugated) in PBS (1: 5000) was added and incubated at 37°C for 45 minutes. Substrate (20µl of TMB) was added on to the nitrocellulose membrane (Victor *et al.*, 1986).

First booster immunization: After 4 weeks of primary immunization, booster immunization was given to all eight rabbits under study. Incomplete Freund's Adjuvant (IFA) was used instead of the Freund's complete Adjuvant (FCA) (Huang *et al*., 1994). After fourteen days of the first booster immunization 1 cc blood was drawn from the ear vein of the rabbit. Serum was separated and stored.

Enzyme linked immunosorbent assay (ELISA): Microtiter wells were coated with of 200µl bGH (10µg/ml) in coating buffer (0.05M Na_2CO_3 & 0.05M $NaHCO_3$ pH 9.6) and incubated at 37°C for 24 hours. 300µl skimmed milk was used as blocking solution in each well and incubated at 37°C for 24 hours. After aspiring blocking solution 25µl primary antibody (rabbit anti-bovine growth hormone serum) was dispensed into one of the wells. As negative control 25µl of rabbit serum, taken prior to immunization, was added and incubated at 37°C for 1 hour. 25µl secondary antibody (sheep anti rabbit HRP conjugate) dilution with blocking solution (1:5000) was added in each well and incubated for 37°C for 1 hour. After washing three times with wash buffer, 25µl of substrate (TMB) was added in each well and color development was observed. Absorbance at 450/630 nm of positive and negative wells was taken using ELISA reader for qualitative determination of rabbit's immune response.

Western blotting: Electrophoresis was performed as described by and western blot analysis was performed as described by Laemmli (1970). After electrophoresis, antibodies were evaluated on the basis of the Western blot method of Wang *et al.* (1992). Primary antibody (rabbit anti-bovine growth hormone serum) diluted (1:50) was poured on membrane followed by incubation and washing. Secondary antibody (sheep anti rabbit AP conjugate) with 1:5000 dilutions was used and incubated at 37°C for 45 minutes. As incubation period was over, washing was carried out and 2.5ml of substrate 5-bromo, 4-chloro,3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) was added for color development Experiment was repeated three time to confirm the results.

RESULTS AND DISCUSSION

Immuno dot Blot analysis showed the development of antibody antigen complex. Development of blue color on the nitrocellulose strip used for positive sample qualitative test proves the development of antibodies against the immunogen (bGH) in rabbit. Color development was not observed in negative control strip. This indicated that no cross reacting antibodies were present in rabbit serum and nonspecific binding was also efficiently prevented by the protocol followed in this dot blot assay (Figure. 1).

HRP (substrate TMB) added in wells conjugated to antibody oxidized this substrate into a blue color complex (benzidine blue) in the positive well. Absorbance showed that no any complex was formed in negative control well (0.004+0.001) The development of blue color complex was observed in the two wells used for positive sample analysis. This indicates the formation of antigen antibody complex in both these wells (Table 1).

Electrophorised antigen (bGH) showed the molecular weight of 22 kDa on SDS-PAGE (Figure 2). Bands transferred to the nitrocellulose membrane were detected using primary and secondary antibody by formation of antigen antibody complex. Western Blot analysis showed that antigen was bound successfully to primary antibodies (Figure 3). Commercial bovine somatotropin is available in the emulsified slow releasing form and also contains vitamin E. Initially it was thought that this emulsified form might interfere with the immune response of the rabbit and antibodies would not be produced against the hormone as it is reported in some cases of polyclonal antibody production (Cartledge *et al.*, 1992). But Immuno dot blot, ELISA and Western blotting confirmed that rabbit had responded to the immunogen in a reliable manner.

Figure. 1 Comparison of Negative control (a) and sample (b) by using immune dot blot analysis.

Table.1 ELISA results confirmed the antigen antibody complex

Negative control $A_{450/630}$ nm	Sample $A_{450/630}$ nm
0.004 ±0.001	1.237 ±0.005

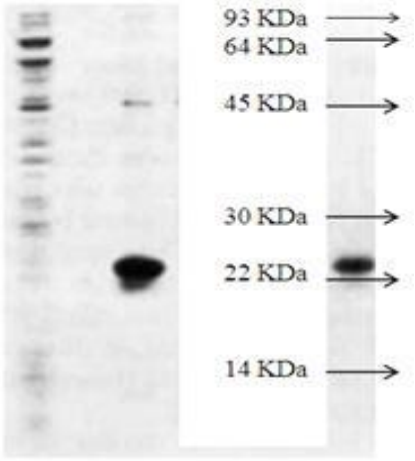

Figure. 2 SDS gel electrophoresis, molecular weight marker is in the first lane and the next four lanes show the growth hormone sample MW is 22 KDa.

Growth hormone sample showed the molecular weight of 22kDa on SDS-PAGE (Figure 2). It was same as described by Banfi *et al.,* (1992). Western blot analysis showed that primary antibody strongly bound to growth hormone (Figure 3). This showed the successful production of antibodies against the growth hormone.

The main goal of any antibody production protocol was to get high titer of specific antibodies. The antibodies could be isolated and partially purified to be used as solid phase capture antibodies in a growth hormone ELISA. These polyclonal antibodies can be used for the radioimmunoassay of growth hormone by further modifying them (Boulanger *et al.,* 1999)

Figure. 3 Western blot analysis the detection of GH band by primary antibody (rabbit anti-bGH serum).

REFERENCES

Banfi G., M. Marinelli, M. Pontillo, and P. Bonini, Standardization with Synthetic 22-kDa Monomer Human Growth Hormone Reduces Discrepancies between Two Monoclonal Immunoradiometric Assay Kits Clin. Chem. 38(10):2107-2110(1992).

Bauman, D. E. Bovine Somatotropin: review of emerging animal technology. J. Dairy Sci. 75:3432-3451 (1992).

Boulanger L., P. H. Andersen, and P. Gaudreau Development of a site-directed polyclonal antibody against the pituitary growth hormone-releasing hormone receptor and Its use to estimate GHRH receptor concentration in normal and hypothyroid rats, Neuroendocrinology, 70:117-127 (1999).

Berrini A., V. Borromeo, C. and Secchi. Monoclonal Antibodies Can Reveal Immunoreactivity Differences Between Pituitary and Recombinant Bovine Growth Hormone. Hybridoma. 13(6): 485-489. (1994).

Brambilla G. F., A. Berrini, V. Borromeo, C. Secchi, Detection of growth hormones in the plasma of cows treated with recombinant bovine growth hormone. Anal. Chim. Acta. 275(1):173-176 (1993).

Carey, W. H., E. A. James and B. Taylor Review of polyclonal Antibody production Procedures in Mammals and Poultry. ILAR J. 37:3 (1995).

Cartledge, C., C. McLean and J. Landon, Production of polyclonal antibodies in ascitic fluid of mice: Time and dose relationships. J. Immunoass 13:3339-353 (1992).

Gobbi M. B., L. A. Retegui, and C. Pena, Equine growth hormone. Detection of immunoreactive sequences using poly- and monoclonal antibodies. Int. J. Pept. Protein Res. 35(2):105-10 (1990).

Huang R. J., T. K. Chen, B. L. Miao, S. C. Chang, M. Y. Liau, and J. H. Lin, A simplified emulsification method for antigens with oil adjuvants. J. Microbiol. Immunol. 27(2):94-7(1994).

Laemmli, U. K. Cleavage of structure proteins during assembly of the head of bacteriophage T4. Nature (London) 277,680-685 (1970).

Stills, H. F. Polyclonal antibody production. in The Biology of Laboratory Rabbit, 2nd Ed, Academic Press pp.435-448 (1994).

Victor H., V. Cleave and D. W. Metzger, Heating of immunoglobulins for immunoblot analysis destroys variable region antigenicity. J. Immunol. Methods, 93(2):237-240 (1986).

Wang, C. H., M.M. Abouzied,. J. J. Pestka, and D. M. Smith, Antibody development and enzyme-linked immunosorbent assay for the protein marker lactate dehydrogenase to determine safe cooking endpoint temperatures of turkey rolls. J. Agr. Food Chem. 40:1671-1676 (1992).

Wood, D. C., W. J. Salsgiver, T. R. Kasser, G. W. Lange, E. Rowold, B. N. Violand, A. Johnson, R. M. Leimgruber, G. R. Parr, N. R. Siegel, Kimack, N. M. Smith, C. E. Zobel J. F. Ganguli, S. M. Garbow, J. R. Bild, and G. G. Krivi, Purification and characterization of pituitary bovine somatotropin. J. Biol. Chem. 264:14741-14747 (1989).