I J R B A T, Issue (X), Vol. I, Jan 2022: 111-120
A Double-Blind Peer Reviewed & Refereed Journal

OPEN ACCESS

e-ISSN 2347 – 517X
Original Article

INTERNATIONAL JOURNAL OF RESEARCHES IN BIOSCIENCES, AGRICULTURE AND TECHNOLOGY

 www.ijrbat.in

PARTIAL CHARACTERIZATION OF SEX HORMONE-BINDING GLOBULIN (SHBG) IN *LABEO ROHITA*

DVNS Suresh* and R S Bagade
Department of Zoology, Dr Ambedkar College, Deekshabhoomi, Nagpur 440010, India.
*Corresponding author: sureshdvns@gmail.in

Communicated: 22.10.21 | Revision : 16.11.21 & 31.12.2021 Accepted: 20.01.2022 | Published: 30.01.2022

ABSTRACT:
Although much of the aspects of its biology have been well studied, there is a lack of knowledge on the reproductive endocrinology of this Indian major carp. *Labeo rohita* is the most cultivable and relished fish of India which needs an attention on its high yield production with healthy and robust fish and an exclusive knowledge about its reproductive regulations and the steroidal involvements. The serum and tissue samples were subjected to sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) for the separation of proteins to isolate the SHBG in the gel and then transferred to a poly vinyledene difluride (PVDF) membrane. The transferred protein band can be analyzed for the immuno confirmation of SHBG with help of anti-SHBG (DSL, Texas, USA). With increased serum graded samples, immuno-confirmation was positively observed in incremental reactions against anti-SHBG. Although albumin has not been found in fish, proteins with some of the characteristics of albumin are present. Interference of high concentrations of vitellogenins, however, may generate small changes in kDa of SHBG. The binding characteristics of SHBG remain unchanged at these reproductive stages. The possible molecular weight of the *Labeo rohita* SHBG (lrSHBG) is approximately 30254 Da. There is a poor evolutionary conservation of SHBG among species of same class.
Key words: - SHBG; SDS PAGE; Immunoblotting; *Labeo rohita*

INTRODUCTION:

The sex-hormone binding protein (SHBG) has been characterized in plasma from several freshwater and saltwater fish species including rainbow trout (Fostier and Breton, 1975), Atlantic salmon (Lazier et al., 1985), spotted seatrout (Laidley and Thomas, 1994), goldfish (Pasmanik and Callard, 1986), Japanese eel (Chang et al., 1994), and carp (Chang and Lee, 1992; Suresh *et al.,* 2008). Recent work has also demonstrated that anadromous Arctic charr (*Salvelinus alpinus L.*) from the Svalbard Islands (Norway) exhibits a plasma protein that binds estrogens and androgens with high affinity and moderate capacity (Øvrevik et al., 2001).

Interestingly, it has been demonstrated that natural and synthetic chemicals are able to interact with fish SHBG and modulate their sex steroid binding properties (Milligan et al., 1998; Kloas et al., 2000; Tollefsen, 2002; Tollefsen et al., 2002). Recent studies also indicate that presence of chemicals in industrial effluents may interact with and modulate the properties of SHBG in fish (Hewitt et al., 2000; Pryce-Hobby et al., 2003) and thus potentially contribute to disrupt normal endocrine function (endocrine disruption) in feral fish species. Among the persistent organic pollutants found in Arctic regions, several are known or suspected endocrine disrupters.

At present, plasma SHBGs are identified in most vertebrates except birds and some mammalian species (Westphal, 1986). Best characterized is the human SHBG (hSHBG), a homodimeric glycoprotein of

90–100 kDa with a single sex steroid binding site. The hSHBG shares the same primary structure as the human testicular androgen binding protein (ABP) which differs only with respect to the attached oligosaccharides (Hammond and Bocchinfuso, 1995). Both proteins are products of a single gene (*SHBG*), which is expressed in several tissues including liver, testis, placenta, brain, and endometrium. In fish as in humans, liver seems to be the main organ for SHBG synthesis (Foucher *et al.,* 1991; Hammond and Bocchinfuso, 1995).

As pointed out by Laidley and Thomas (1994) plasma SHBGs display considerable species variation in both affinity and specificity and clear phylogenetic patterns are not obvious. Species variation is also evident from molecular weight estimates, which for teleost SHBG alone range from 64 kDa in eel (Chang *et al.,* 1994) to 194 kDa in carp (Chang and Lee, 1992). Immunoreactive studies suggest poor evolutionary conservation of these proteins, even within classes of animals (Renoir *et al.,* 1980), which may account for some of the observed species variation. Considering this lack of uniformity, phylogenetic interpretations on SHBG distribution or characteristics would be highly uncertain even within classes of vertebrates. This work focuses on the separation of *Labeo rohita* SHBG from its blood by SDS-PAGE and confirmation thorugh immunoblotting.

Although much of the aspects of its biology have been well studied, there is a lack of knowledge on the reproductive endocrinology of this Indian major carp. *Labeo rohita* is the most cultivable and relished fish of India which needs an attention on its high yield production with healthy and robust fish and an exclusive knowledge about its reproductive regulations and the steroidal involvements.

MATERIALS AND METHODS

SDS-PAGElectrophoresis

Sodium dodecyl sulfate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Assemble two glass plates (one notched) with two side spacers, clamps, grease, etc. as shown by demonstrators. Stand assembly upright using clamps as supports, on glass plate. Pour some pre-heated 1% agarose onto glass plate, place assembly in pool of agarose this seals the bottom of the assembly. *Gel concentration of 12.5% in 0.25 M Tris-HCl pH 8.8 is resolving gel Gel concentration of 4.5% in 0.125 M Tris-HCl pH 6.8 is stacking gel.* Grind a little leaf material (eg. 2 grams) in a mortar, centrifuge for 3 min. Take supernatant and mix 100ul 1:1 (v:v) with SDS-PAGE disruption mix: this is 125mM Tris-HCl pH

6.8 / 10% 2-mercaptoethanol / 10% SDS / 10% glycerol, containing a little bromophenol blue. Layer samples under buffer on stacking gels. Connect up apparatus and electrophorese. Make up stain: 0.2% *Coomassie Brilliant Blue* (CBB) in 45:45:10 % methanol: water: acetic acid. Cover gel with staining solution, seal in plastic box and leave overnight on shaker (RT) or for 2 to 3 hours at 37 degree Celsius. Destain with 25% 65% 10% methanol water acetic acid mix. Rinse gel in distilled water and seal in a plastic.

Immunobloting

To study proteins that are expressed at very low levels, it is recommended that immunoprecipitation be followed by immunoblotting for more sensitive detection. After the above SDS-PAGElectrophoresis of protein separation samples are now subjected to the tranfere to PVDF membrane (Millipore Immobion-P #IPVH 000 10). Assemble "sandwich" for Tranfer Instrument for 1 hr at 1 amp at 4°C on a stir plate. Bigger proteins might take longer to transfer. For Transfer Instrument it's 100 V for 1 hr with cold pack and pre-chilled buffer. Incubate with primary antibody diluted in Blocking buffer for 60 min at room temperature and followed by Incubation with secondary antibody and detect with Sigma AEC kit

RESULT & DISCUSSION:

The serum and tissue samples were subjected to sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) for the separation of proteins to isolate the SHBG in the gel and then transferred to a polyvinyledene difluride (PVDF) membrane. The transferred protein band can be analyzed for the immuno confirmation of SHBG with help of anti-SHBG (DSL, Texas, USA).

Earlier all the tissue samples, like gonads, liver and kidney, were tried for the separation of SHBG, but later only the serum samples were tried as the quantity of protein separated from these tissue samples was insufficient for further analyses of immuno confirmation.

Plate 1 shows the SDS-PAGElectrophoresis and its computer generated image of bands separated of serum, liver, ovary and kidney at 10% running gel and 4% stacking gel combination.

Then the same electrophoresis was adopted to run the serum samples of different phases of reproductive cycle, preparatory, prespawning and spawning phases and the banding pattern shows a clear indication of increase in protein content as the fish approached the spawning phase. The suspected proteins around the 40 kDa increased significantly as the albumin-like proteins and the globulins increase during he same phase (plate 2).

Plate 3 shows the native–PAGE, which is the same electrophoretic procedure with no SDS in any of the solutions and reagents; and gels to separate the proteins in their structural existence rather than getting straightened up (primary structure) as in SDS_PAGE. These banding patterns were used usually for the transfer on PVDF membranes for immuno confirmation through Western Blot.

Blot results were displayed in plate 4 where in the upper image, the portion of the gel transferred to membrane and the immuno-analyzed PVDF membrane were arranged. The analyzed gel bands with documentation software reveal the suspected band as of 30254 Da molecular weight which gives distinct reaction with anti-SHBG on membrane (right scanned image of membrane). Lower image of scanned membrane displays graded samples loaded in gel electrophoresis and then blotted for immuno-confirmation giving positive incremental reactions against anti-SHBG with increased serum samples. Thus SHBG in the major carp, *Labeo rohita* might be of molecular weight 30254 Da.

The consistent and rapid synthesis of silver nanoparticles was achieved using banana peels which is a waste material. As an alternative to conventional microbiological, physical, or chemical methods, this green synthesis method appears to be a cheaper, non-toxic, eco-friendly solution. It is also suitable for developing a biological process for large-scale production.

Crystalline, uniform, spherical, monodisperse silver nanoparticles were synthesized from banana peel extract which had an average particle size of 23.7 nm. Antimicrobial properties were demonstrated for human pathogenic bacteria tested with the synthesized nanoparticles. Additionally, they demonstrated a synergistic effect of Tetracycline on the antimicrobial activity against the Gram-positive and Gram-negative bacteria.

The biochemical properties of SHBG have already been reviewed in great detail (Petra, 1979; Petra et al., 1983; 1986). In essence, SHBGs from a variety of mammalian species appear to exist as dimeric glycoproteins of approximatetly 90 kDa (see review Hammond, 1990). Under denaturing conditions, human SHBG usually dissociates into two subunits of approximately 52 and approximately 48 kDa, and these are present in approximately a 10 :1 ratio respectively (Cheng et al., 1983). In some individuals, an additional subunit with an molecular weight of approximately 56kDa has also been identified in the same relative amount as the 52kDa subunit (Hammond and Robinson, 1984, Khan et al., 1985), similar differences in subunit size have also been observed in other species, but their relative amounts vary (Suzuki and Sinohara, 1984).

Despite variations in subunit size and electrophoretic mobility (Hammond and Robinson, 1984; Cheng et al., 1983; Khan et al., 1985; Suzuki and Sinohara, 1984; Luckock and Cavalli-Sforza, 1983), amino-terminal sequence analyze of human SHBG (Hammond and Robinson, 1984; Petra et al., 1986; Hammond et al., 1986) have detected the presence of only a single polypeptide. These studies, together with immunochemical evidence for two identical epitopes per dimer (Hammond et al., 1986), support the assumption that human SHBG is a homodimer comprised of a complex mixture of variously sized subunits, with two 52 kDa subunits forming the

prominent species (Cheng et al., 1983). This therefore raises the interestmg question of whether various combinations of subunits are functionally important.

The subunit size heterogeneity of SHBG can be eliminated by complete removal of carboydrates (Denzo et al., 1989), which account for 11 to 12% of each subunit (Hammond et al., 1986). Furthermore, the presence of variable amounts of sialic acid probably contributes to the series of bands observed when SHBG is examined by isoelectrofocusing.

The binding characteristic studies in fishes of other workers reveal that the two peaks are relatively distinct suggesting that the smaller peak may consist of an albumin-like protein responsible for the low-affinity, high-capacity binding to E_2, as shown by albumin in mammals (Westphal, 1986). Although albumin has not been found in fish, proteins with some of the characteristics of albumin are present (Davidson *et al.,* 1989; Maillou and Nimmo, 1993a,b). In contrast, the E_2 binding profiles from the E_2-treated fish did not show two distinct peaks. A possible explanation for this is interference of the very high levels of vitellogenin with the later elution of smaller-molecular weight proteins including SBP and albumin off the gel filtration column. This could result in less separation between peaks of lower-molecular weight proteins.

The molecular weight of SHBG has been reported for only three teleost species to date and ranges from 64 kDa for the eel *Anguilla japonica* (Chang *et al.,* 1994) to 194 kDa for the carp (Chang and Lee, 1992). The molecular weight of rainbow trout SBP from the present study was estimated to be around 65 kDa by gel filtration after partial purification. Molecular weights of the same molecule can differ when estimated using different methods. The molecular weight of carp and eel SHBGs were both determined by HPLC after purification of the molecule, suggesting that substantial differences in the size of the SHBG of these two species are real. Estimates of spotted seatrout SHBG differed when measured by gel filtration (around 150 kDa) and native PAGE (approximately 135 kDa) in the same study (Laidley and Thomas, 1994). Gel filtration after partial purification is not an optimal method for determining molecular weight, but does provide an initial estimate for rough comparative purposes. An estimate of 65 kDa suggests that rainbow trout SBP may be more similar to eel than to common carp SHBG. In conclusion, *B*max is higher in vitellogenic than in nonreproductive female rainbow trout and black bream. This difference in trout does not appear to result directly from elevated plasma E_2 and vitellogenin levels experienced by the fish at this time. Interference of high concentrations of vitellogenin, however, may generate small changes in kDa. In greenback flounder, the binding characteristics of SHBG remain unchanged at these reproductive stages, suggesting a lesser role for SHBG in maintaining elevated plasma levels of steroids in this species

Studies to conclude that the possible molecular weight of the *Labeo rohita* SHBG (lrSHBG) is approximately 30254 Da confirmed with the help of SDS-PAGE, Native-PAGE, Western Blot and anti-SHBG. Results from present studies tell about the molecular weight of the monomer of preexisting dimeric molecule of SHBG in major carp plasma as it is well known that under denaturing conditions (Cheng et al., 1983) in the procedural steps of SDS- or Native-PAGE alter the dimeric-SHBG into monomer.

The discussion on molecular weights of SHBG in different fish species compel to infer that there is a poor evolutionary conservation of it among the species of same class.

CONCLUSION:

Very recent molecular studies on fish SHBG in seabass (Miguel-Queralt_et al., 2005) and zebrafish (Miguel-Queralt_ et al., 2004) further revealed the exact molecular weight of the corresponding SHBGs and sequenced the gene responsible for its translation. The 361-residue seabass SHBG is 39894 Da and 356-residue zebrafish SHBG is 39243 Da molecular weights.

Although albumin has not been found in fish, proteins with some of the characteristics of albumin are present. Interference of high concentrations of vitellogenin, however, may generate small changes in kDa of SHBG. The binding characteristics of SHBG remain unchanged at these reproductive stages. The possible molecular weight of the *Labeo rohita* SHBG (lrSHBG) is approximately 30254 Da. There is a poor evolutionary conservation of SHBG among species of same class.

ACKNOWLEDGEMENTS

Sincere thanks to Dr Shivkumar Chauhan, PreGen Preventive Genetics Diagnostic Centre, Nagpur, India for helping in performing immunoblotting.

REFERENCES

Chang, C.-F., Lee, Y.-H., (1992): Purification of the sex steroid-binding protein from common carp (Cyprinus carpio) plasma. Comp. Biochem. Physiol., B 101, 587–590.

Chang, C.-F., Lee, Y.-H., Yoshida, T., Sun, L.-T., (1994): Characterization of the plasma sex steroid-binding protein in eel (Anguilla japonica). Comp. Biochem. Physiol., B 108, 189– 197.

Cheng C Y,-Musto N A, Gunsalus G L, Bardin C W (1983): Demonstration of heavy and 'light protomers of human testosterone , esterdiol-binding globulin. J Steroid Biochem 19:1379.

Davidson, W. S., Bartlett, S. E., Birt, T. P., Birt, V. L., and Green, J. M. (1989): Identification and purification of serum albumin from rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* 93B, 5–9.

Denzo B J, Bell B W., Black J H. (1989): human testosterone binding globulin is a dimmer composed of two identical protomers that are differently glycosylated. Endocrinology, 124:2809

Fostier, A., Breton, B., 1975. Binding of steroids by plasma of a teleost: the rainbow trout, Salmo gairdnerii. J. Steroid Biochem. 6, 345–351.

Foucher, J. L., Niu, P. D., Mourot, B., Vaillant, C., and Le Gac, F. (1991: In vivo and in vitro studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): Influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Mol. Biol.* 39, 975–986.

Hammond GL, Robinson P A (1984): Characterization of a monoclonal antibody to human sex hormone binding globulin. FEBS Lett 168:307 .

Hammond G. L, Robinson PA, Sugino H, Ward DN, Finne J (1986): Physiochemical characteristics of human sex hormone binding globulin: evidence for two identical subunits. J Steroid Biochem 24:815.

Hammond, G. L., and Bocchinfuso, W. P. (1995). Sex hormonebinding globulin/adrogen-binding protein: Steroid-binding and dimerization domains. *J. Steroid Biochem. Mol. Biol.* 53, 543–552.

Hewitt, L.M., Parrott, J.L., Wells, K.L., Calp, M.K., Biddiscombe, S., McMaster, M.E., Munkittrick, K.R., Van Der Kraak, G.J., (2000): Characteristics of ligands for the Ah receptor and sex steroid receptors in hepatic tissues of fish exposed to bleached kraft mill effluent. Environ. Sci. Technol. 34, 4327– 4334.

Khan MS, Ehrlich P, Birken, S, Rosner W (1985): Size isomers of testosterone-estradiol-binding globulin exist in the plasma of individual men and women. Steroids 45:463.

Kloas, W., Schrag, B., Ehnes, C., Segner, H., (2000): Binding of xenobiotics to hepatic estrogen receptor and plasma sex steroid binding protein in the teleost fish, the common carp (Cyprinus carpio). Gen. Comp. Endocrinol. 119, 287–299.

Laidley, C.W., Thomas, P., (1994): Partial characterization of a sex-steroid binding protein in the spotted seatrout (Cynoscion nebulosus). Biol. Reprod. 51, 982–992.

Luckock A, Cavalli-Sforza LL (1983): Detection of genetic\ariation with: radioactive'"Ugands.' V. Genetic variants of testosterone , binding globulin in human serum Am J Hum Genet 35:49.

Maillou, J., and Nimmo, I. A. (1993b): Identification and some properties of an albumin-like protein in the serum of pre-spawning Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol.* 104B, 401–405.

Miguel-Queralt S, Avvakumov G V, Blazquez M, Piferrer F, Hammond GL.(2005): Sea bass (Dicentrarchus labrax) sex

hormone binding globulin: molecular and biochemical properties and phylogenetic comparison of its orthologues in multiple fish species. Mol Cell Endocrinol. 2005 Jan 14;229(1-2):21-9.

Milligan, S.R., Khan, O., Nash, M., (1998): Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. Gen. Comp. Endocrinol. 112, 89–95.

Øvrevik, J., Stenersen, J., Nilssen, K., Tollefsen, K.-E., (2001): Partial ch aracterization of a sex steroid-binding protein in plasma from Arctic charr (Salvelinus alpinus L.). Gen. Comp. Endocrinol. 121, 31–39.

Pasmanik, M., Callard, G., (1986): Characteristics of a testosterone-estradiol binding globulin (TEBG) in goldfish serum. Biol. Reprod. 35, 838–845.

Petra, P.H. (1979): The serum sex steroid-binding protein. Purification, characterization and immunological properties of the human and rabbit proteins. *J Steroid Biochem* 11:245

Petra, P.H, Stanczyk FZ, Senear DF, Namkung PC, Novy MJ, Ross JBA, Turner E, 'Brown JA (1983): Current status of the molecular structure and function of the plasma sex steroid-binding protein (SBP). J Steroid Biochem 19:699 .

Petra:, P.H, Kumar S, Hayes R, Ericsson LH,' Titani K (1986): Molecular organization of the se:\ steroid-binding protein (SBP) of human plasma. J' $,teroid Biochem 24:45.

Pryce-Hobby, A.C., McMaster, M.E., Hewitt, L.M., Van Der Kraak, G., (2003): The effects of pulp mill effluent on the sex steroid binding protein in white sucker (Catostomus commersoni) and longnose sucker (C. catostomus). Comp. Biochem. Physiol., C 134, 241– 250.

Renoir, J.-C., Mercier-Bodard, C., and Baulieu, E.-E. (1980): Hormonal and immunological aspects of the phylogeny of sex steroid binding plasma protein. *Proc. Natl. Acad. Sci. USA* 77, 4578–4582.

Suresh, DVNS, Baile, V.V., Prasada Rao P.D. (2008): Annual reproductive phase-related profile of sex steroids and their carrier, SHBG, in the Indian major carp, Labeo rohita.*Gen Comp Endocrinol* 159 (2), 143-149.

Suzuki Y, Sinohara' H (1984): Subunit structure of sex-steroid binding plasma proteins from ,inan, cattle, dog,.l!nd rabbit. J Biochem 96:751.

Tollefsen, K.-E., (2002): Interaction of estrogen mimics, singly and in combination, with plasma sex steroid-binding proteins in rainbow

trout (Oncorhynchus mykiss). Aquat. Toxicol. 56, 215– 222.

Tollefsen, K.E., Meyes, J.F.A., Frydenlund, J., Stenersen, J., (2002): Environmental estrogens interact with and modulate the properties of plasma sex steroid-binding proteins in Atlantic salmon (Salmo salar).Mar. Environ. Res. 54, 697–701

Westphal, U. (1986): Sex steroid-binding protein (SBP). *In* "Steroid–Protein Interactions II," Monographs on Endocrinology, pp. 198–264. Springer-Verlag, New York.

EXPLANATION OF FIGURES

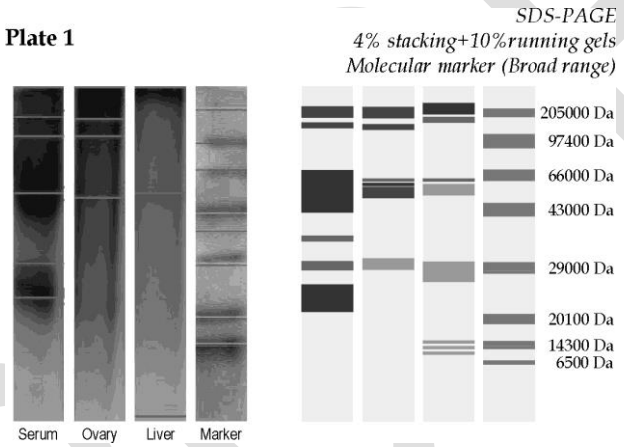

Plate 1. SDS-PAGElectrophoretic banding patterns of the samples of serum and the extract samples of ovary and liver of L rohita. Image besides is the computer generated electrophoregram showing bands more clear to visualize

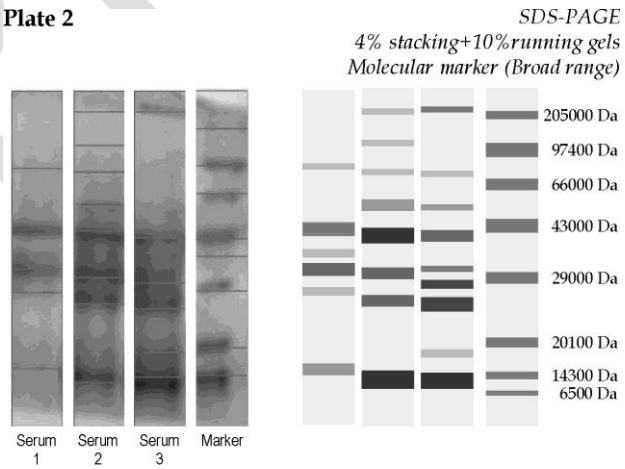

Plate 2. SDS-PAGElectrophoretic banding patterns of the samples of serum of preparatory, prespawning and spawning phases of reproductive cycle of L rohita. Image besides is the computer generated electrophoregram.

e-ISSN 2347 – 517X
Original Article

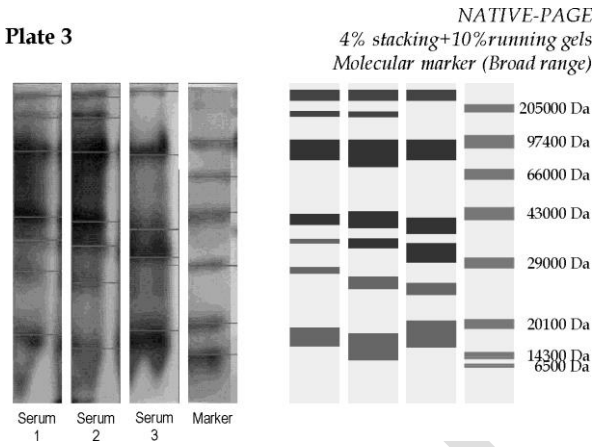

Plate 3. NATIVE--PAGElectrophoretic banding patterns of serum samples of preparatory, prespawning & spawning phases of reproductive cycle of L rohita. Image besides is computer generated electrophoregram of the same.

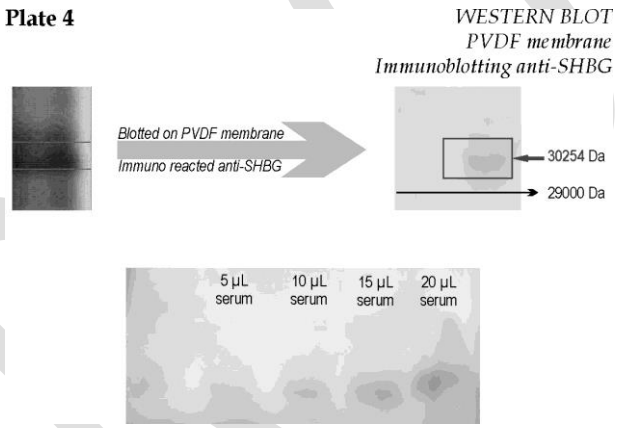

Plate 4. Right side image is the positive reacted blot PVDF membrane against anti-SHBG and confirming the band as SHBG band. Below scanned PVDF membrane image showing the graded serum samples with SHBG immunoconfirmation.