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Purification and Characterization of Growth Hormone of Water Buffalo, *Bubalus bubalis* (Neeli Ravi) from Pakistan

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Abstract.- Somatotropin or growth hormone (GH) was isolated from pituitaries of freshly slaughtered animals in slaughter house of Lahore. Proteins were first concentrated with ammonium sulfate in a homogenate of pituitaries in saline buffer pH 9.5 and then passed through an ion-exchange column (pH 9.5), using a linear salt gradient. GH eluted as a shoulder of major protein peak, was further purified on preparative poly-acrylamide gel electrophoresis. Bubaline GH appeared as a single band on SDS-PAGE. Properties of bubaline GH were compared with the authentic bovine GH.

Key words: Growth hormone, bubaline GH, farm animals

INTRODUCTION

Growth hormone (GH) is a polypeptide, produced by the pituitary gland involved in linear growth and a number of anabolic activities. GH has proven to be of great importance in farm animals, as it's exogenous supply has a positive impact on the production of milk and meat with an increase in growth rate (Bauman, 1992). GH is composed of 121 amino acid residues with 2 disulphide bridges. Its tertiary structure shows the presence of 4 alpha helices arranged in left twisted fashion (Abdel Meguid *et al*., 1987). Amino acid sequence of a number of farm animals has been reported including sheep (Warwick *et al*., 1989), goat (Yato *et al*., 1988), pig (Vize and Wells, 1987), ox (Wallis, 1973) water buffalo (Verma *et al*., 1999, Di Mauro *et al*., 2002), yak (Qing *et al*., 2004) and camel (Maniou *et al*., 2004). It may be noted that the primary structure of GH produced by the farm animals share more than 90% homology including bovine, ovine, caprine and bubaline GH, while the protein produced by the camel shared the homology with the sea mammals (Wallis *et al*., 2006). The crystal structure of GH-receptor complex shows the binding of two receptor molecules to one GH molecule required for the biological activity of GH (de Vos *et al*., 1992; Wells and de Vos, 1993). A number of studies have been reported on the structure related function studies of the molecule, as reviewed by Bauman (1992). Application of GH to cattle has proven useful for dairy industry. Due to the availability of the molecular biology techniques, for the production of recombinant DNA-derived GH, there is a large number of reports for the production of hormone. Different strategies have been reported for obtaining the maximal levels of expression of GH gene from different farm animals. Higher levels of expression for bovine GH has been obtained by increasing bases A or T are located near the ribosomal binding site (Dalboge *et al*., 1987; Martial *et al*., 1979; Goeddel *et al*., 1979; Schoner *et al*., 1984). Studies on the effect of changes in nucleotide sequence coding for the N-terminus on the expression levels of ovine growth hormone variants in *Escherichia coli* showed that the presence of a sequence ATGACC/ATGACA at the N-terminus of the protein for the pUC based plasmid is responsible for the higher expression of recombinant DNA-derived ovine growth hormone variants (Sami *et al*., 1990). In this report the characterization and purification of GH isolated from pituitaries of *B. bubalis* is reported and the changes in the primary structure are compared to the authentic bGH using latest bioinformatics approach.

MATERIALS AND METHODS

All the chemicals used were of analytical grade until otherwise stated. Bubaline pituitaries were collected from local slaughterhouse, Bucker

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Mandi, Bund Road Lahore. Samples were collected from freshly slaughtered animals and stored at -20°C until further used. A weighed amount (50.0 gm) of pituitaries was washed with distilled water and homogenate in 0.05M sodium carbonate/sodium bicarbonate buffer pH 9.5. The crude protein was precipitated with 90% saturation of ammonium sulfate and centrifuged at 5000xg for 10 minutes. The pellet obtained was dissolved in 50ml of 0.05M bicarbonate buffer pH 9.5. The protein solution was dialyzed against the same buffer. After dialysis, precipitated proteins were removed by centrifugation at 5000xg for 10 minutes. The clear supernatant was used as a source of protein.

Proteins were purified by using a sequential procedure comprising ion-exchange chromatography and preparative PAGE. A column (1.8x15cm) was packed with pre-swollen DEAE Sepharose in 0.05M sodium carbonate/bicarbonate buffer pH 9.5. A concentrated protein solution volume of 5ml was loaded onto the column and proteins were eluted at a flow rate of 0.8ml /min, using a linear salt gradient ranging 0-0.2M NaCl in the same buffer at 4°C. A total of 100 fractions of 1ml each were collected. Protein concentration estimated in each fraction by Bradford reagent. Purification procedure was monitored by SDS-PAGE according to the method described by Laemmli (1970). Proteins were desalted by dialysis and further purified on SDS-PAGE. A 3mm thick 10% polyacrylamide gel was prepared and a sample of about 1ml containing 2 mg of protein was loaded on to the gel. The gel was run overnight at a constant current supply of 35 V at 4C^{o}. Next morning the gel was removed and sliced with a sharp razor and proteins were eluted as described previously (Sami *et al.,* 1988). Molecular weight of the protein was estimated on SDS-PAGE. The amino acid sequence for the first 5 amino acid was determined on Applied Biosystem gas-phase sequencer. Molecular model of the molecule was drawn using Swiss PDB-Viewer and energy minimization was performed with GROMOS96 implementation of Swiss PDB-Viewer.

RESULTS AND DISCUSSION

The bubaline pituitary was 2.3cm with an average weight of about 1.22 gram. About 50 gram of pituitaries were homogenized in 200ml of 0.05M buffer solution pH 9.5. The clear supernatant obtained after centrifugation, was concentrated with 90% ammonium sulfate and it was noted that about 80% of the protein was recovered after this process, as located on SDS-PAGE (Fig. 1, lanes 1 and 2). Protein fractionation on an ion-exchange column showed the presence of one major peak with a shoulder eluted with salt in the range of 0.0-0.2M NaCl (Fig. 2). Fractions from the shoulder of the major peak showed the presence of a protein band comparable to authentic bGH as appeared on SDS-PAGE (Fig. 1, lane 4). This band was further purified on preparative SDS-PAGE. The purified protein had an apparent molecular weight more than 21K. The purified bubaline GH proved equivalent to bGH on SDS-PAGE (Fig.1, lanes 4, 5 and 6). The amino acid sequence for the first five amino acid residues determined as FPAMS which was in agreement with the results reported by Verma *et al.* (2002) with UniprotKB/Swiss Prot entry 018938. There was heterogeneity at the N-terminus of the protein with methionine. At the N-terminus

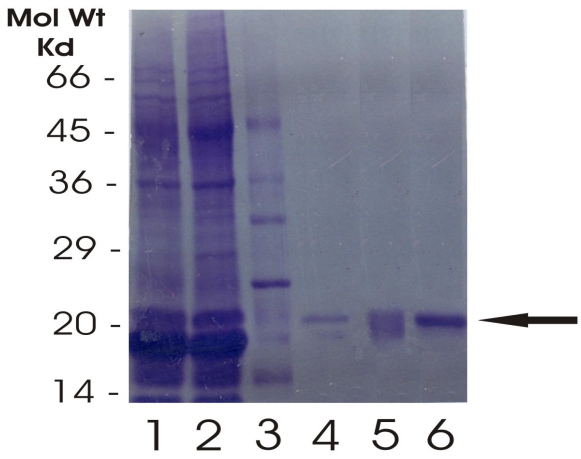

Fig. 1. Electrophoresis of proteins from bubaline pituitary on 10% polyacrylamide gel electrophoresis. Lane 1, total proteins; Lane 2, ammonium sulfate precipitated proteins; Lane 3, Mol. wt. markers; Lane 4, authentic bGH; Lane 5, protein of shoulder of major peak after ion exchange chromatography; Lane 6, purified bubaline GH.

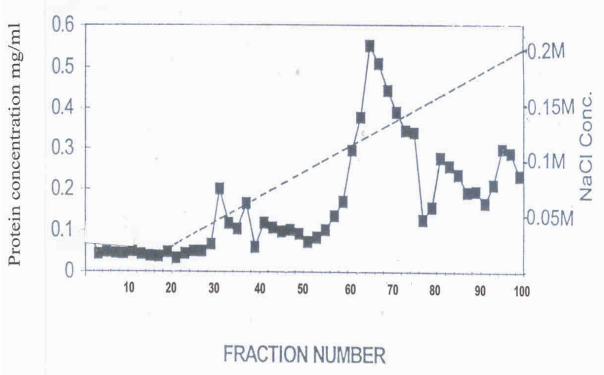

Fig. 2. Ion exchange chromatography on DEAE-Sephacel column (15x1.8 cm) of bubaline pituitary extract obtained after ammonium sulphate precipitation. Proteins were eluted at flow rate of 0.8ml per minuted at 4°C. with a linear salt gradient in 50 mM Carbonate buffer at pH 9.5.

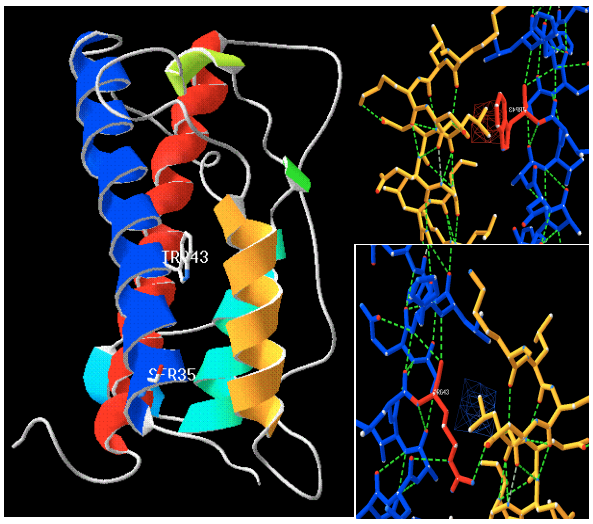

Fig. 3. a) Molecular model of bubaline GH generated on Swiss Model Server; b, in bubaline GH a close look at Trp in the helix revealing areas of hydrophobic interactions and hydrogen bonding; c, helix revealing areas of hydrophobic interactions and hydrogen bonding for Ser at position 35.

heterogeneity of the protein was noticed for bubaline GH with methionine and alanine. In nature, bovine and ovine GH at the N-terminus is normally heterogeneous as reported by Wallis (1973). The most likely explanation given was the enzymatic cleavages of the precursor GH at two different positions. Similar reason could be given for the heterogeneity of N-terminal for bubaline GH. According to the receptor-GH complex, the N-terminus and the helix 1 of GH bound to the receptor 1 in binding site (de Vos *et al*., 2002). It may be noted that the first 5 amino acid residues are not involved in the binding. Thus the heterogeneity may not have any affect on the binding characteristics of the GH molecule. There are two changes in the bubaline sequence as compared to bovine GH sequence (Verma *et al*., 1999). Both the changes were found in the helix 1 of bubaline GH sequence. At position 35 there was Gly and at position 43 it was Trp instead of Arg. A close look at the chemistry of the amino acids showed that Ser is an uncharged amino acid with polar side chain while Gly is a non polar smaller amino acid residue. Obviously there could be introduction of some weaker bonds (due to the polarity of the Ser side chain within the molecule) for further strengthening the alpha helical structure. This was confirmed by the molecular model of the molecule (Fig. 3, a, c, hydrogen bonds are shown in green dotted lines). For the second change in which Arg at position 43 (bovine GH) was replaced by Trp for bubaline GH. Arginine has a polar side chain, while Trp has a non-polar side chain. The three dimensional structure of GH showed that helix 1 (shown in blue) comes in close contact with helix 3 (shown in yellow) and in helix 3 amino acid residues Lys138-Arg151 sequence (two hydrophilic amino acid residues followed by one hydrophobic amino acid residues) the hydrophilic side chains protrude outward to interact with aqueous environment. Trp 43 is located (shown in blue) was in close contact with the helix shown in yellow (Lys138 to Arg 151). This suggests that the Arg side chain does not cause any change in the conformation or packing of helix, instead it assumes a configuration that is sterically permissible and may allow H-bond contacts with aqueous environment.

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