Full Length Research Paper

Production, purification and characterization of two recombinant DNA-derived N-terminal ovine growth hormone variants: oGH3 and oGH5

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Two recombinant DNA-derived variants of ovine growth hormone were produced, purified, characterized and compared with the authentic pituitary derived GH. The variants oGH3 and oGH5 were isolated by differential centrifugation method and were purified after refolding by ion-exchange chromatography and gel filtration. Both the proteins showed single band on SDS-PAGE and had molecular weight and iso-electric point closer to authentic pituitary GH. The variants oGH3 and oGH5 were compared with the authentic pituitary derived GH in radio immuno assays, radio receptor assays and binding with the monoclonal antibodies OA 11 and OA12.

Key words: Growth hormone, GH, oGH3, oGH5, radioimmuno assay, radio receptor assay.

INTRODUCTION

Growth hormone (GH) is a polypeptide involved in the linear growth of mammals. GH is produced by specialized cells in the pituitary gland of mammals, known as somatotrops. Structure and functions of GH has been reviewed in detail and reported (Sami, 2007; Bauman, 1992; Wallis, 1985). GH binds with two receptor molecules to exhibit its biological activity (de Vos et al., 1992). Gene for GH expression and peptide has been isolated, purified and chatecterised from different farm animals including bubaline, bovine, ovine, porcine and caprine origin (Sami, 2006; Verma et al., 1999; Schoner, 1985; Wallis, 1973; Yato, 1988; Warick et al., 1989; Vize et al., 1987). A number of reports are available on the cloning and sequencing of GH gene. Effect of different nucleotide sequences on ovine growth hormone variants and reported (Sami et al., 1990; Wallis et al., 1989). Recombinant GH has been produced as more than 20 % of the total cellular protein (Wallis and Wallis 1989, Sami et al, 1990). GH, like many other eukaryotic proteins, is produced as inclusion bodies in E.coli (Schoner et al., 1985; Langley et al., 1987). Wallis and Wallis (1990) reported the production of an ovine GH variant, oGH1, as inclusion bodies. The inclusion bodies are mainly comprised of denatured recombinant DNA-derived foreign protein. Inclusion bodies containing GH can be obtained in an insoluble pellet fraction after cell breakage and centrifugation (Schoner et al. 1985; Langley et al. 1987; Wallis and Wallis, 1990). The GH in such insoluble granules can be purified to some extent using differential centrifugation (Schoner et al. 1985). Use of DNA-derived protein is only meaningful if it shows similar biological activity in a number of assays with little or no side effects. The studies on the heterogeneity of recombinant DNA-derived GH have been widely reported. Although recombinant DNA-derived GH has been produced in mature form, failure to remove the N-terminal initiating methionine is common. Processing of methionine in *Escherichia coli* is dependent on the activity of an enzyme called methionine aminopeptidase. The activity of this enzyme is dependent on the radius of gyration of the amino acid adjacent to the initiating methionine (Tsunasawa et al., 1985; Ben-Bassat et al., 1987).

The study reported here was intended to investigate structure-function relationships in the N-terminal region of the GH molecule. This report describes the production, purification and properties of two purified N-terminal variants of oGH, named oGH3 and oGH5, produced by recombinant DNA-technology.

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MATERIALS AND METHODS

A series of plasmids carrying plasmids for the expression of oGH variants was produced with a variety of level of expressions named pOGH plasmids. Two clones carrying plasmids pOGH3 and pOGH5 were used in this study (Wallis and Wallis 1987; Sami et al., 1990).

32 g of Bacto tryptone, 20 g of yeast extract, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5g NaCl were dissolved in distilled water and the volume was adjusted to 1 l. The medium was sterilized by autoclaving at151 b/in² for 20 min. 10 g of Bacto tryptone, 5 g of Bacto yeast extract, 1 g glucose and 5 g of NaCl were dissolved in distilled water and the volume was adjusted to 1 l with distilled water. pH was adjusted to 7.5 with 5 N NaOH. The medium was sterilized by autoclaving for 20 min. Bacterial strains were grown in 2.5 ml 2 medium at 37ºC overnight on a shaker in a 20 ml culture tube. The cultures were stored in 30% dimethyl sulphoxide (DMSO) in two different batches at -20 and -80ºC.

Production of oGH variant proteins in *E.Coli*

E. coli clones carrying expression plasmids for oGH variants were grown in 25 ml of sterile medium containing ampicillin (50 or 100 µg/ml). 0.25 ml of an overnight culture was used to inoculate 25 ml of the medium. The flasks were incubated on a rotary shaker at 37ºC for about 2-3 h. When the optical density (OD) at 600 nm of the culture reached 0.6, 250 µl of a solution containing 20 mg IPTG/ml were added and incubation was continued for another 18 h.

After incubation flasks were removed from the shaker and the bacterial cells were harvested by centrifuging at 6,000 x g for 10 min at 4ºC. The pellet of bacterial cells from 500 ml of culture was washed with 100 ml of 0.1 M NaCl in 0.01 M Tris-HCl buffer, pH 8.0. The bacterial pellet was again washed with 0.01M Tris-HCl, pH containing 0.1 M NaCl. The bacterial pellet obtained after these washings was used for further studies.

Isolation and solubilisation of oGH variant granules

The bacterial pellet was suspended in 12 ml of 0.1 M Tris-HCl buffer, pH 8.0. Cells were broken by sonication for 8 x 15 s at amplitude 20 µm (MSE 150 Watt Ultrasoninc Disintegrator MK2) and were then centrifuged at 12,000 x g for 10 min at 4ºC. The supernatant was removed and the pellet fraction was washed with the same buffer. After washing, the pellet was dissolved in 24 ml of 6 M guanidinium chloride in 50 mM ethanolaminie-HCl, pH 9.5 containing 5 mM dithiothreitol, as reported by Wallis and Wallis (1990).

Purification of oGH variants

After renaturation a solution of each oGH variant (30-40 ml in ethanolamine-HCl buffer, pH 9.5 containing 20-30 mg protein) was applied to an ion-exchange column (1.6 x 18 cm) packed with DEAE-Sephacel equilibrated with 20 mM ethanolamine-HCl buffer, pH 9.5 at 4ºC. Proteins were eluted at a flow rate of 12 ml/h with a linear salt gradient (0.0-0.2 M NaCl). Protein content of the eluted fractions (2 ml) was measured by reading absorbance at 280 nm. Fractions containing protein were subjected to SDS-PAGE and were cooled together.

For further purification after ion-exchange chromatography the oGH variant (20 ml) was passed through a gel filtration column (2.5 x 96 cm) packed with Sephadex-G100 using 0.1 M Tris-HCl buffer (pH8.0) containing 0.02% sodium azide. Proteins were eluted at a flow rate of 24 ml/h at 4ºC. 1 ml fractions were collected and their absorbance was measured at 280 nm. The fractions containing pro-tein were subjected to SDS-PAGE. After purification oGH variant protein was dialysed against double distilled deionised water overnight with several changes and was freeze dried.

GH assays

The Radioimmunoassay method was based on methods previously described by Ray and Wallis (1982). Radioreceptor assays were based on the method previously described by Cadman and Wallis (1981).

RESULTS AND DISCUSSION

The two N-terminal variants were produced on a large scale by growing *E. coli* clones carrying plasmid pOGHe103 and pOGHe105 in 500 ml of rich medium containing ampicillin (50 or 100 µg/ml) and IPTG (160 µg/m) at 37ºC either shaking for 22 h. Bacterial cells were harvested by centrifugation.

Both the oGH N-terminal variants were produced in an insoluble form, presumably as inclusion bodies (Figure 1, Lane 1 and Figure 2, Lane 2). Production of GH in the form of insoluble granules has been reported previously (Schoner et al., 1985: Wallis and Wallis, 1990). In each case the oGH variant, was present as a major cellular protein in the pellet fraction, and formed more then 50% of the protein present. Washing of the inclusion bodies with 0.1 M Tris-HCl buffer, pH 8.0 did not extract any oGH- like protein (Figure 1, Lane 2,3 and Figure 2, Lane 2,4). Differential centrifugation is helpful in separating cellular debris from these inclusion bodies (Schoner et al., 1985; Langley et al., 1987), since the inclusion bodies sediment at a greater rate than the cell debris. Washing of the isolated granules was also reported by various workers. Schoner et al. (1985) reported the use of 5 M urea for solubilising most of the unwanted proteins form granules of recombinant bGH.

After solubilization it is necessary to achieve correct folding of the protein in order to obtain the biologically active molecule (Figures 1 and 2). The proteins were refolded according to the method described by Wallis and Wallis (1989). The refolded oGH variants were in solution form. (Figure 1 lane 7, Figure 2 lane 7).

For both the N-terminal variants ion-exchange chroma-tography was used for purification. The amino acid sequence deduced from the nucleotide sequence show-ed that for oGH5 the sequence was identical to that of pituitary-derived alanyl-GH except that the N-terminal alanine is replaced by threonine (Figure 3). The isoelec-tric points of the purified proteins were estimated by isoelectric focusing. It was found that both oGH3 and oGH5 had an isoelectric point of 7.8, which is similar to that of authentic pituitary-derived bGH. The sequence analysis of the two proteins showed that oGH5 has an extension of 2 amino acids, Met- Thr-, followed by the complete sequence of phenylalanyl oGH. This extension of two amino acids has an overall neutral effect on the
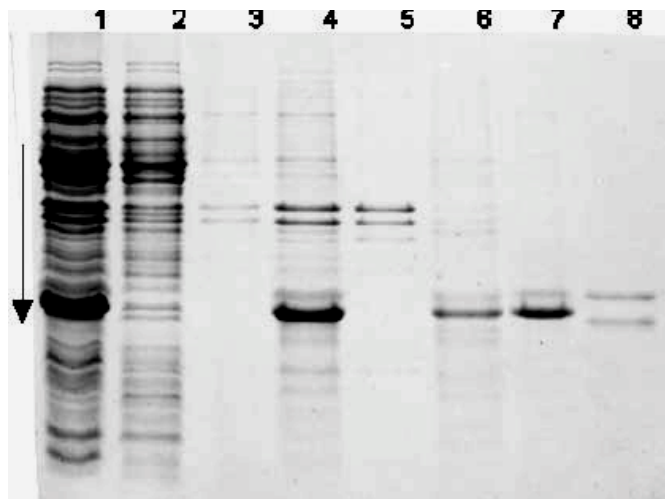
Figure 1. SDS-PAGE gel illustrating the production, solubilisation and refolding of OGH3 from inclusion bodies in *E.coli* cells. Gel shows electrophoresis of samples obtained at each stage of the production, isolation, solubilisation and renaturation of oGH3. 1: Total sonicate. 2: Supernatant obtained after centrifugation of total sonicate. 3: Supernatant obtained after centrifuging the suspended pellet (P1). 4: Suspended pellet (P1) obtained after centrifuging the total sonicate. 5: Pellet (P2) obtained after centrifuging the diluted (2 m) guanidinium-Cl (pellet P1 was solubilised in 6 M guanidinium-Cl and 5 mM DTT). 6: Pellet (P3) obtained after dialysis (oGH3 in 2 M guanidinium-Cl was dialysed against 20 mM ethanolamine-HCl buffer pH 9.5). 7: Supernatant after final dialysis (renatured OGH3). 8: bGH and Oprl.
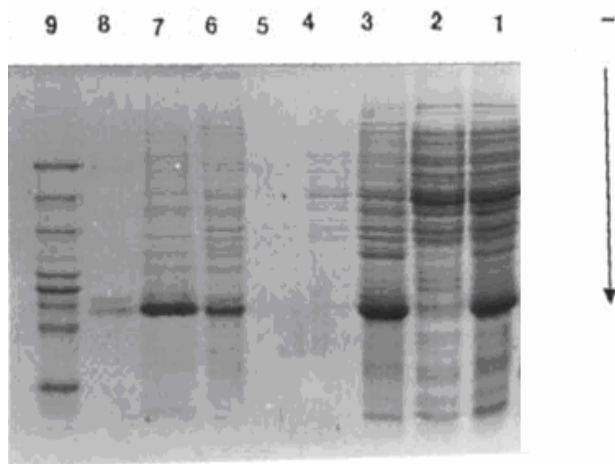

Figure 2. SDS-PAGE gel illustrating the production, solubilisation and refolding of oGH3 from inclusion bodies in E.coli cells. Gel shows electrophoresis of samples obtained at each stage of the production, isolation, solubilisation and renaturation of oGH3. 1: Total sonicate. 2: Supernatant obtained after centrifugation of total sonicate. 3: Suspended pellet (P1) obtained after centrifuging the sonicate. 4: Wash fraction from pellet P1. 5: Pellet (P2) obtained after centrifuging the diluted quanidinium-Cl solution of pellet P1. 6: Pellet (P3) obtained after centrifuging the dialysed oGH3 solution. 7: Supernatant after final dialysis (renatured oGH5). 8: Pituitary – derived bGH and ovine prolactin. 9: Molecular weight markers.

Met Thr Met Ile Thr Asn Ser **Ala Met** ----   **pOGHe 103**

Met Thr Phe Pro **Ala Met**---------------   **pOGHe 105**

Figure 3. Amino acid aequence for the N-terminus of oGH3 and oGH5 deduced from the DNA sequence as reported, previously (Sami et al., 1990).
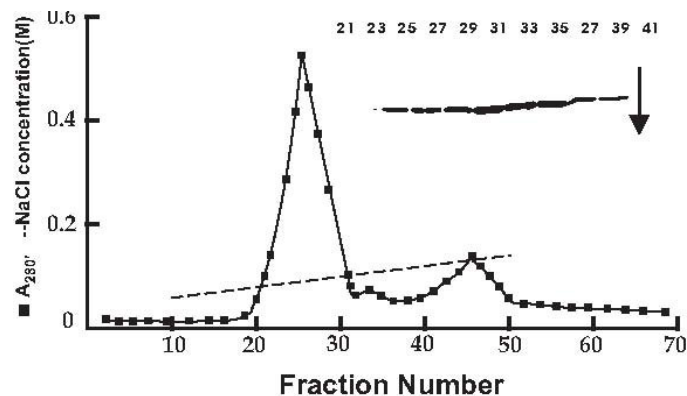

Figure 4. Ion-exchange chromatography of renatured oGH3 on a DEAE-Sephacel column (1.6 x 18 cm). Proteins were eluted with a linear salt gradient (0.0-0.2 M NaCl) in 20 mM ethanolamine-HCl buffer, pH 9.5 at 4°C at a flow rate of 12 ml/h (fraction size 2 ml). Proteins were estimated by measuring absorbance at 280 nm for the eluted fractions. NaCl concentration was estimated by measuring conductance of the eluted fractions on a conductivity meter. 20 µl of each of the fractions indicated was subjected to SDS-PAGE as shown in the photograph.

isoelectric point of the molecule. For oGH3, 3 amino acids at the N-terminus of oGH were replaced by an extension of 6-7 amino acids. This change also has a neutral effect on the charge of the molecule, leaving the isoelectric point unchanged. Thus, pH 9.5 was selected for ion-exchange chromatography because it is more that one point higher than the pI and was helpful in purifying oGH5 in preliminary experiments. For oGH3, three amino acid residues at the N-terminus of oGH were replaced by six amino acids from ß-galactosidase and the linker region of the plasmid pOGHe103. The chemistry of these amino acids shows that they have an overall neutral effect on the charge of the molecule. This procedure was helpful in purifying these two proteins to homogeneity i.e., a single band on SDS-PAGE (Figures 4 and 5).

After ion-exchange chromatography oGH3 was further purified on a gel filtration column on Sephade x -G100. For oGH5 gel, filtration was not carried out as it appeared pure after the ion-exchange chromatography (Figure 6). The molecular weights of both the proteins were estimated on SDS-PAGE oGH3 and oGH5 had a molecular weight, closer to 21,500.

The variants oGH3 and oGH5 were tested in radio-immunoassay and compared with authentic pituitary-derived bGH. The results are shown in Figure 7. Each of
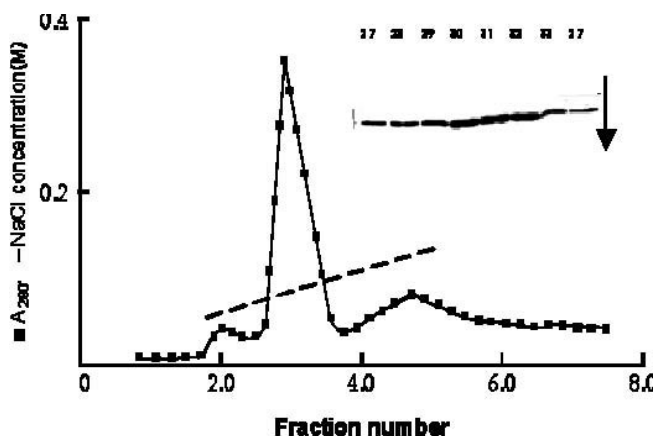
Figure 5. Ion-exchange chromatography of renatured oGH5 on a DEAE-Sephacel column (1.6 x 18 cm). Proteins were eluted with a linear salt gradient (0.0-0.2 M NaCl) in 20 mM ethanolamine-HCl buffer, pH 9.5 at 4℃ at a flow rate of 13ml/h (fraction size 3 ml). Proteins were estimated by measuring absorbance at 280 nm for the eluted fractions. NaCl concentration was estimated by measuring conductance of the eluted fractions on a conductivity meter. 20 µl of each of the fractions indicated was subjected to SDS-PAGE as shown in the photograph.
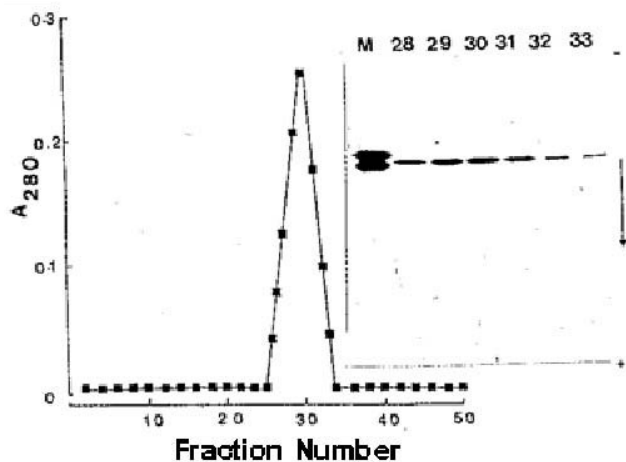

Figure 7. Comparison of oGH3, oGH5 and pituitary-derived bGH in an immunoassay. Assays were carried out using ^{125}I-labelled bGH and a polyclonal antibody reised against bGH in rabbit.

Figure 6. Gel filtration of oGH3 (after ion-exchange chromatography) on a Sephade x-100 column (2.5 x 96 cm). Proteins were eluted at a flow rate of 20 ml/h at 4℃ (fraction size 7 ml). Proteins were estimated in eluted fractions by measuring absorbance at 280 nm. 20 µl of fractions indicated were subjected to SDS-PAGE. M: Pituitary bGH.

the variants behaved similarly to authentic pituitary-derived bGH. These results suggest that all these three molecules have s similar shape and structure and anti-genic regions since all three (pituitary-derived bGH, oGH3 and oGH5) were equivalent to bGH in terms of recognizing the polyclonal antibody preparation. It is likely that at least some of the antibody binding is conformation specific rather than sequence specific, so these results further suggest that recombinant DNA-derived oGH3 and
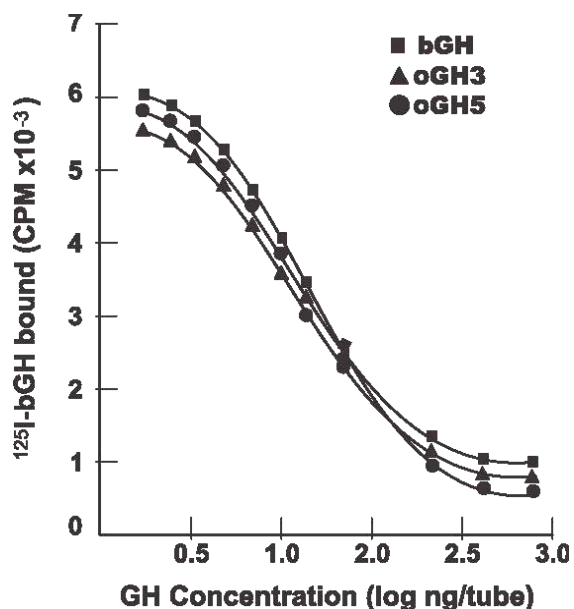
oGH5 were correctly folded. Changes introduced at the N-terminus of oGH therefore did not affect the binding to the polyclonal antibody, suggesting that this is not a major epitope.

The binding characteristics of oGH3 and oGH5 were compared with pituitary-derived bGH in radioreceptor assay. Receptors were prepared from pregnant rabbit liver. The results showed (Figure 8) that both the variants behaved similarly to authentic pituitary-derived bGH. Therefore oGH3 and oGH5 are very similar to bG in the regions which are required for receptor binding. These data also confirm that the molecules were folded correctly.

Ashkenazi et al. (1987) reported that the N-terminal sequence of GH is important for receptor binding. The results shown here demonstrate that changes close to the N-terminus (including extensions) did not have any effect on the receptor-binding characteristics of the molecules.

Binding studies with monoclonal antibodies

A panel of monoclonal antibodies was raised against bGH by Aston et al. (1987). These were designated OA11-OA17. This panel of monoclonal antibodies has been tested for the potentiation of the actions of GH on growth and elevation of somatomedin C levels in hypo-physectomized rats. One of the monoclonal antibodies, OA11, has been studied for potentiation of GH actions in sheep (Pell et al., 1989).

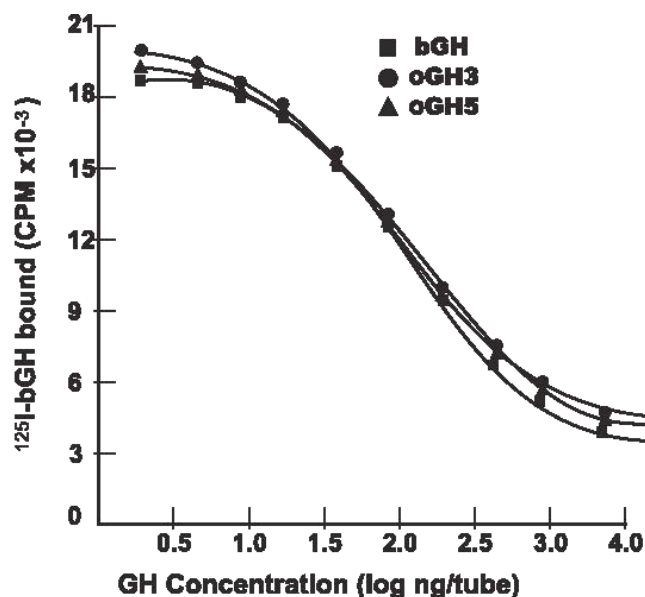Two of the monoclonal antibodies from this panel,

Figure 8. Comparison of oGH3, oGH5 and pituitary-derived bGH in a radioreceptor assay. Displacement of ^{125}I-labelled human GH (hGH) from rabbit liver microsomal receptors by unlabelled oGH3, oGH5 or pituitary-derived bGH is shown.

OA11 and OA12, were used to study the immunological characteristics of three N-terminal variants, oGH1, oGH3 and oGH5. The results of this immunological study are shown in Figures 9 and 10. oGH1 and oGH3 behaved similarly to the authentic pituitary-derived bGH. The same amount of bGH, oGH1 and oGH3 was required to give 50% displacement of ^{125}I-bGH. This indicates that bGH, oGH1 and oGH3 have the same affinity for OA11 and for OA12.

Thus, oGH1 and oGH3 were equivalent to pituitary-derived bGH in recognition of binding sites on OA11 and OA12.

For oGH5 different results were obtained in the binding studies. Both OA11 and OA12 were able to differentiate between bGH and oGH5. The determinants defined by monoclonal antibodies OA11 and OA12 were expressed to a lesser degree on Met-Thr- oGH than on bGH. The two monoclonal antibodies OA11 and OA12 were thus able to reveal changes in the antigenic structure of the oGH5 molecule which presumably derive from its N-terminal sequence.

The amino acid sequence analysis of these three oGH variants shows that oGH1 and oGH5 have the complete sequence of phenylalanyl oGH. oGH5 has a 2-3 residue N-terminal extension (Met-Thr-Phe-) while oGH1 has a longer extension and the sequence –Gly-Asp-Phe- in the equivalent positions. oGH3 does not have the first 3 amino acid residues of oGH and has the sequence –Ile-Thr-Asn- in equivalent positions. It is possible that the lowered binding of oGH5 to OA11 and OA12 is due to the presence of Met- close to the normal N-terminus, though in many of the molecules Met- was not retained.
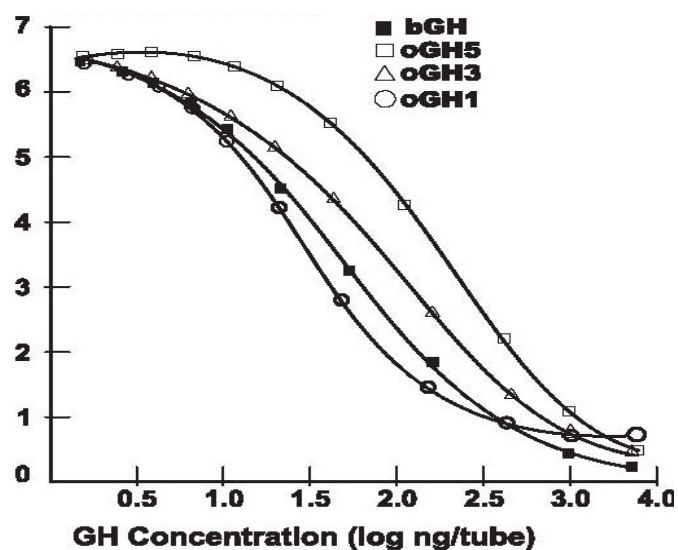

Figure 9. Comparison of oGH1, oGH3, oGH5 and pituitary-derived bGH in an immunoassay. Assays were carried out using ^{125}I-labelled bGH and a monoclonal antibody (OA11) raised against bGH.
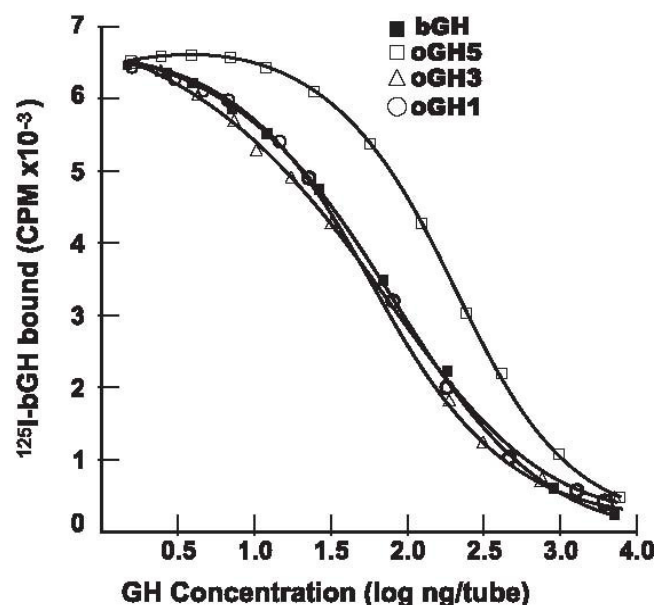

Figure 10. Comparison of oGH1, oGH3, oGH5 and pituitary-derived bGH in an immunoassay. Assays were carried out using ^{125}I-labelled bGH and a monoclonal antibody (OA12) raised against bGH.

Work described here confirms that recombinant DNA-derived GH analogues with altered N-terminal sequences could be use as a model for studying structure-function relationships. This could be extended by introducing different types of mutations at the N-terminus of the molecule. A variety of assay systems using bioassays, immunoassay and receptor assays could be used. Use of

other monoclonal antibodies will be useful in probing the antigenic determinants. This work provides a basis for further studies on N-terminal variants of GH designed to completely understand the role of the N-terminus in the function of the molecule.

ACKNOWLEDGEMENT

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