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Hormonotoxins: the role of positive charge of lysine residue on the immunological, biological and cytotoxic properties of ovine lutropin-S-S-gelonin conjugates

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Abstract

Since the positive charge on the lysine residues plays an important role in the receptor recognition ability of oLH, the hormonotoxin has been synthesised with the use of 2-iminothiolane HCl (2IT) and N-Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP). The oLH activated with 2IT (oLH-10) was then mixed with SPDP activated gelonin (gelonin-30) in order to obtain a oLH-S-S-gelonin hormonotoxin. The conjugation mixture containing hormonotoxin was purified by gel-filtration chromatography according to the molecular weight and a complete physico-chemical, immunochemical and biochemical analysis were performed. The linkage occured through the ε-NH_2 groups of α-subunit of oLH as judged from RP-HPLC analysis. A 1 : 1 (oLH : gelonin) molar ratio was obtained when determined with the use of several techniques. The hormonotoxins retained substantial receptor binding, steroidogenic activity and immunoreactivity. The competitive displacement analysis indicate that the binding occurs via the hormone part leaving the gelonin free which was probed with the gelonin antibodies. The presently described (C150A-02, C160A-02 and C170A-02) hormonotoxins exhibited higher receptor binding and toxicity to the target cells than the hormonotoxins prepared with the use of SPDP only. Therefore it is concluded that higher receptor binding and cytotoxicity may be due to the retention of positive charge on the lysine residues of oLH which was preserved during the conjugation process. (Mol Cell Biochem **130:** 91–101, 1994)

Key words: gonadotropin, gelonin, immunoreactivity, receptor binding, cytotoxicity, steroidogenesis, protein synthesis

Abbreviations: BSA – Bovine Serum Albumin, CMC – Carboxy methyl Cellulose, DTT – Dithiothreitol, DMEM – Dulbeco's Modified Eagle's Medium, DTNB – Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)], EDTA – Ethylenediaminetetraacetic acid, FPLC – Fast Protein Liquid Chromatography, FCA – Freund's Complete Adjuvant, FCS – Fetal Calf Serum, Gelonin-30 – Gelonin modified by SPDP, GnRH – Gonadotropin-Releasing Hormone,

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Gelonin-SPDP – SPDP modified derivative of gelonin, HEPES – (N-[2-hydroxyethyl] piperazine-N′-[-2-ethanesulphonic acid]), IFA – Incomplete Freund's Adjuvant, 2IT – 2-Iminothiolane, IODOGEN – 1,3,4,6 -tetrachloro 3α,6α-diphenylglycouril, oLH – Ovine Luteinizing Hormone, oLH-SPDP – SPDP modified derivative of oLH, oLH-10 – oLH modified by 2IT, oLH : 2IT – Molar ratio of oLH and 2IT, PDP – 2-Pyridyl-dithiopropionate, PAP – Pokeweed Antiviral Protein, RIP – Ribosome Inactivating Protein, RP-HPLC – Reverse-Phase High Performance Liquid Chromatography, RPMI – Roswell Park Memorial Institute, RIA – Radioimmunoassay, RRA – Radioreceptor Assay, SPDP – N-Succinimidyl-3(2-pyridyldithio)propionate, SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, TCA – Trichloroacetic acid, TFA – Trifluroacetic acid

Introduction

The selective killing of tumor cells by using specific hybrid molecules possessing both functions of specific recognition and cytotoxicity has been under active investigation during last several years [for review see 1–7]. Such hybrid molecules were synthesized by covalently cross-linking of tumor cells specific monoclonal antibodies to highly cytotoxic agents and termed as 'IMMUNOTOXINS' [1–7]. RIPs which catalytically inactivate 60S ribosomal unit have also been used in the design of immunotoxin [8–10]. One group of RIPs is composed of two polypeptide chains, the chain-B that binds to galactose residues on the cell surface and A-chain translocates in the cell and arrests protein synthesis machinery by enzymatically inactivating the ribosomes [2, 8–11]. The non-specific toxicity due to B-chain was avoided either using immunotoxins in the presence of excess galactose or by completely depleting B-chain from the toxin. Due to the non-specific cytotoxicity of intact ricin, either ricin-A-chain alone representing catalytic unit of the toxin or a recombinant version of the whole toxin have been used in the design of immunotoxins [1–7]. In order to avoid non-specific cytotoxicity, single chain RIPs (type 1) have covalently been linked to monoclonal antibodies to synthesize immunotoxins [1–7]. These are innocuous to the cell in the free form but can inactivate ribosomes once provided entry into the cell by a receptor mediated process. Gelonin, isolated from an Indian plant *Gelonium multiflorum* of *Euphorbiaceae* family has been used to prepare specific cytotoxic hybrid molecules to target to the tumor cells [10] and the extent of cytotoxicity was comparable to that of ricin-A chain based immunotoxins [for review see 1–7].

Since, hormones are quite specific in their interaction with their receptors, therefore, analogous to immunotoxins, hormonotoxins (so called because the hormone component of the construct provided specificity for the toxin action) were constructed by conjugating gonadotropin hormones [such as oLH or human chorionic gonadotropin, hCG] with the RIP, gelonin to target hybrid complex to selected cells in the gonads [12–16]. The ovine luteinizing hormone, was conjugated to gelonin with the use of heterobifunctional cross-linking agent, SPDP to obtain oLH-S-S-gelonin hormonotoxin [12–16]. The present paper is an attempt to synthesize and bioeffectivity evaluation of hormonotoxins in which the positive charge on the lysine residue is preserved. This is based on the observation that the receptor binding of the gonadotropin hormone is best maintained if positive charge on the lysine residues are preserved [17].

Materials and methods

Materials

oLH was isolated from the lyophilized sheep pituitary powder according to a procedure published earlier [18, 19] and stored as a lyophilized powder at 4° C. The dry seeds of *Gelonium multiflorum* were obtained from United Chemicals and Allied Products, 10, Clive Row, Calcutta-1, India. IODOGEN, SPDP and DTT were obtained from Pierce Chemicals Co. (Rockford, IL, USA). Sephadex G-25 (medium), G-100 and CMC-52 were purchased from Pharmacia, Fine Chemicals, Sweden. BSA (Cohn fraction IV), FCA and IFA were purchased from Sigma Chemicals, St. Louis, MO, USA. Radioactive iodine ($Na^{125}I$, IMS-30) was purchased from Amersham Corporation. L-(3,4,5-^{3}H) leucine of specific activity 143 Ci/mmol was obtained from DuPont-NEN. All other chemicals used in the investigation were of highest purity available commercially.

Antibodies

Polyclonal antibodies against oLH and gelonin were raised in the rabbits by immunizing them following the protocol described earlier [12, 13, 15] using FCA followed by IFA. Sera were collected after appropriate booster injection and the immunoglobulin fraction was purified by ammonium sulfate precipitation as described [12, 13, 15]. The immunoglobulins were extensively dialysed, lyophilized and stored as powder at 4° C.

Leydig tumor cells

The murine leydig tumor cell line, MLTC-1 used in the present study were obtained from Dr. R. Victor Rebois of the National Institute of Health, Bethesda. The cells were propagated and maintained in culture according to the published procedure [20]. The cells grown in the 24 multiwell plates were used to determine steroidogenic and cytotoxic activity of the conjugates.

Purification of oLH and gelonin

oLH purified from the ovine pituitary powder was subjected to physico-chemical and biological investigation especially for receptor binding and steroidogenic activity prior to use for covalently coupling to gelonin. Gelonin isolated from the seeds of *Gelonium multiflorum* using aqueous extraction [21], ammonium sulfate fractionation, cation-exchange and gel-filtration chromatography. The extracted protein was characterized for its purity, homogeneity and molecular weight by RP-HPLC and SDS-PAGE analysis and judged to be over 98% pure [22]. Recently, a comparative study [22] of three different methods of purification of gelonin suggested that gelonin obtained by this method was devoid of the non-proteineous material that absorbs at 280 nm as described [21].

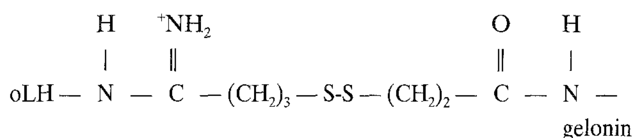
Conjugation of oLH to gelonin

The oLH-gelonin hormonotoxin was prepared with the use of 2IT, the Traut's reagent [23] by the following method as described earlier for immunotoxin synthesis [24, 25]. The oLH was dissolved in 50 mM triethanolamine-HCl containing 100 mM NaCl, 1 mM EDTA and the pH was adjusted to 8.0 with 0.2 M NaH_2PO_4. Appropriate concentration of freshly prepared solution of 2IT was added in the oLH solution. To obtain an average of one -SH group/molecule, the oLH was incubated with the reagent in a molar ratio of 1 : 5 (oLH : 2IT) for 1 h at 25° C and the reaction was stopped by adding 100 µl of borate buffer-saline containing 2.2 M glycine. An aliquot of the derivatized oLH was then treated for 1 h at room temperature with 2 mM DTNB, the Ellman's reagent [26]. Both DTNB treated and untreated fractions were then applied to a Sephadex G-25 column equilibrated with nitrogen flushed buffer consisting of 0.1 M sodium phosphate (pH 7.4), 0.1 M NaCl and 1 mM EDTA. The number of activated disulfide groups introduced into the oLH was determined by reducing the DTNB treated sample with dithiothreitol and measuring the absorption of the released 3-carboxylate 4-nitrothiophenolate ion which has molar absorptivity of $1.36 \times 10^4\ M^{-1}cm^{-1}$ at 412 nm [26]. To initiate conjugation with gelonin, the SPDP-modified gelonin (as described below) in 0.1 M phosphate buffer pH 7.4 containing 0.1 M NaCl was added in oLH-2IT derivatized solution. The conjugation mixture was left at 25° C for 24 hr. The mixture was concentrated with Centriprep-10 (Amicon Danvers, MA, USA) before fractionation on a Sephadex G-100 column at 4° C in 50 mM NH_4HCO_3. The protein eluting before the ingredients was due to the increase in molecular weight and therefore assumed to be the oLH-gelonin conjugate. These fractions were concentrated again and rerun on the same column under the similar conditions. The purified conjugate fraction was lyophilized and stored at 4° C.

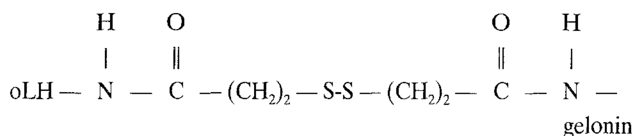
In order to compare the immunological and biological properties of the above synthesized oLH-gelonin conjugate with the conjugates prepared with the use of SPDP only, the synthesis of oLH-S-S gelonin conjugate was also carried out using SPDP following the procedure reported earlier [12–16]. Both oLH and gelonin were thiolated separately. The thiolated gelonin (gelonin-SPDP) was subjected to DTT treatment to generate -SH group. Later on both oLH-SPDP and gelonin-SH were mixed to initiate conjugation [12–16]. Briefly, oLH/gelonin dissolved in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.1 M NaCl, was reacted with SPDP in the minimal volume of absolute ethanol. The reaction mixture incubated at 25° C for 30 min was passed through G-25 (medium) column equilibrated and eluted with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl. Gelonin-SPDP was treated with DTT to generate free thiol group. The increase in absorption at 343 nm after DTT reduction was used to determine the number

(s) of amino groups modified by SPDP. This and our earlier studies on oLH [18] and gelonin [18, 27] indicate that a 1 : 2 (hormone/gelonin : SPDP) molar ratio incorporated average number of thiol groups of 1.20 ± 0.10 (SD). The SPDP activated gelonin was dialysed against 0.1 M sodium phosphate buffer (pH 6.0) containing 0.15 M NaCl and reduced with DTT to generate -SH groups for conjugation.

After removing small molecular weight reduction products, the protein was immediately mixed with oLH-SPDP in 0.1 M sodium phosphate buffer, (pH 7.5) containing 0.15 NaCl. The conjugation reaction mixture was left at 25° C for 24 hr and then concentrated by Centriprep-10. The reaction mixture was then fractionated on G-100 column at 4° C in 0.05 M ammonium bicarbonate. Prior to G-100 chromatography, the UV-VISIBLE spectra was recorded on a Shimadzu UV-VISIBLE Spectrophotometer model UV-160A to determine the liberation of pyridine-2-thione during the conjugation reaction (see results and discussion section). The contribution of pyridine-2-thione absorption at 280 nm was corrected in calculating the molar concentration of pyridine-2-thione librated and the hormone-gelonin molar ratio was determined. Appropriate conjugate fractions as described in Results were pooled and lyophilized directly. The structures of the hormonotoxins prepared with 2-IT and SPDP are shown below:



[when oLH is modified by 2-IT and gelonin by SPDP]



[when both oLH and gelonin are modified by SPDP]

Characterization of hormonotoxins

The hormonotoxins prepared as described above were subjected for extensive physico-chemical, immunochemical and biological characterization. The conjugates were also analysed on SDS-PAGE in order to establish purity and molecular weight of the conjugates.

SDA-PAGE analysis

The conjugates of oLH to gelonin as described above were analysed by SDA-PAGE in the slabs gel cast with acrylamide gradient (5–15% w/v), according to Laemmli [28], under both reducing and non-reducing condition as reported earlier [12, 13]. The gels were fixed and stained with Coomassie Brilliant Blue R-250 dye. Low and high molecular weight reference standards used were from Bio-Rad, Richmond, CA.

RP-HPLC analysis

RP-HPLC was used to analyse the ingredients and hormonotoxins prepared by 2-IT and SPDP methods. RP-HPLC was performed on a Waters (Milford, MA) μBondapak phenyl column (4 mm × 250 mm) using a continuous gradient [12–16]. The solvent used were of HPLC grade purity. The samples dissolved in H_2O containing 0.1% TFA (Solvent A) were run on the above column pre-equilibrated in solvent A + 25% CH_3CN containing 0.1% TFA. A gradient of 25–50% was run over 30 min at a flow rate of 2 ml/min in a Dupont's HPLC and data analysed by a computer [12–16].

Immunoreactivity determination

The immunoreactivity of oLH, SPDP-oLH/gelonin and hormonotoxins was determined by radioimmunoassay in a competitive displacement technique as described [12–16, 18, 21, 22, 27]. The radioiodinated oLH required for RIA was labelled by using IODOGEN method essentially following the procedure described for GnRH [29–31]. Free $Na^{125}I$ and ^{125}I-oLH were separated by gel-filtration chromatography on a Sephadex G-75 (1.0 × 50 cm). The specific activity of ^{125}I-oLH was in range of 50–80 μCi/μg as determined by autodisplacement method [29–31]. The labelled hormone was stored in the aliquots at − 70° C and was used within a month of the preparation.

The competitive displacement assay was performed essentially following the procedure described earlier [12–16, 18, 21, 22, 27] with minor modification. The assay was performed in disposable polyethylene tubes (12 × 75 mm). The anti-oLH antibody, test samples unlabelled oLH and labelled oLH were diluted in 0.05 M sodium phosphate buffer pH 7.5 containing 0.15 M NaCl and 0.1% each BSA and sodium azide. One hundred mi-

crolitre of a dilution of antibody giving 30–50% binding in the same buffer was incubated with 0.1 ml of different concentrations of test samples (competitors). After 12 hr incubation at 4° C, 0.1 ml of ^{125}I-oLH was added and reaction mixture was further incubated for 12 hr at 4° C. Bound and unbound ^{125}I-oLH were separated by addition 0.2 ml of 1 : 5 dilution of Pansorbin (Calbiochem). The tubes were further incubated at room temperature for 20–30 min and centrifuged at 3000 × g for 15 min. The supernatant containing unbound ^{125}I-oLH was removed by aspiration and pellet was counted in a LKB-Rack Mini Gamma Counter (Wallac Oy, Turku, Finland). The inhibition lines were obtained by plotting (B/Bo) × 100 against the logarithm of the dose of competitor (where Bo represent the binding of ^{125}I-oLH in absence of cold oLH and B in the presence of competitor). The extent of cross-reactivity (as percentage) was expressed as: $C = (L_{50}/U_{50}) \times 100$, where L_{50} is the dose of competitor which shows 50% inhibition and U_{50} is the dose of oLH which shows 50% inhibition [13, 15, 22, 27, 29–31].

Receptor binding determination

The receptor binding of oLH, oLH-SPDP, oLH-2-IT and oLH-gelonin conjugates was determined by performing RRA using rat testicular homogenate as a source of gonadotropin receptor as described earlier [12–16, 18]. Briefly, 0.1 ml each of the labelled hormone (~ 70,000 CPM/tube, test samples, unlabelled oLH and assay buffer (25 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mg/ml BSA) were incubated at 22° C for 16–20 hr. The reaction was terminated by addition of 2 ml of assay buffer and centrifuged in cold at 3000 × g for 30 min. The supernatant was removed by aspiration and pellet was counted as described above for immunoreactivity determination.

Steroidogenic ability determination

The steroidogenic activity of oLH, oLH-SPDP, oLH-2IT and oLH-gelonin conjugates was evaluated by incubation with ovarian granulosa cells from immature female rats. Immature female rats were primed with equine chorionic gonadotropin (eCG). After 48 hr of stimulation, the granulosa cells were collected and incubated with samples in DMEM (Gibco), containing 20 mM HEPES, pH 7.5 and 0.1% BSA at 37° C under 95% oxygen and 5% CO_2 for 4 hr. Progesterone secreted in the medium was estimated by a specific RIA as described [12–16, 18, 32, 33].

Binding and cytotoxicity to Leydig tumor cells

The binding and cytotoxicity experiments were carried out as described earlier [12–16]. Presently Leydig tumor cells MLTC-1, obtained from Dr. Victor Rebois, NIH, Bethesda, USA were used for the binding experiments. The MLTC-1 cells (~ 1,50,000 cells/well) were plated in 24 wells (flat bottom, Flow Laboratory) and grown for 24–48 hr. When the cells occupied most of the space, the plate was washed two times with the serum free RPMI-1640 medium. The cells received different concentrations of hormonotoxins and appropriate concentrations of gelonin as control. The plates were incubated for 2–4 hr at 37° C in a humidified chamber containing 95% oxygen and 5% CO_2. After incubation, the wells were washed 3 times with serum free medium and each well received 50 μg of gelonin antibody (gamma globulin fraction) purified by 50% ammonium sulfate fractionation. After 2 hr incubation, the wells were again washed with serum free medium and incubated with ^{125}I-protein-A (~ 200,000 CPM/well) for 2 hr. The wells were washed extensively and the bound radioactivity was counted after solubilizing cells with 0.1 N NaOH. In a competition experiments, 10 μg/well concentration of conjugate was used. The concentration was determined after a preliminary binding experiments. Fifty micrograms per well of cold oLH was added together with conjugate. After 2 hr incubation in the presence of native oLH, the wells were again washed with serum free medium and the conjugate bound to the cells was estimated by addition of ^{125}I-protein-A as described above.

The cytotoxic ability of the conjugate was determined on Leydig tumor cells, MLTC-1 following the procedure described above. Briefly, the cells were plated in RPMI-1640 medium supplemented with 10% FCS in a 24 multiwell plates and after 24 hr culture, the medium was replaced with the fresh medium and different concentrations of test samples were added. The plate was further left at 37° C for 18–24 hr in a humidified chamber containing 5% CO_2 and 95% O_2. The wells washed after 24 hr with serum free RPMI-1640 medium were pulsed for 2 hr with ^{3}H-Leucine (0.5 μCi/well) in the RPMI-1640 medium (depleted with cold leucine). After 4 hr incubation, the cells were washed with 5% TCA to remove non-specifically bound radioactivity. The cells

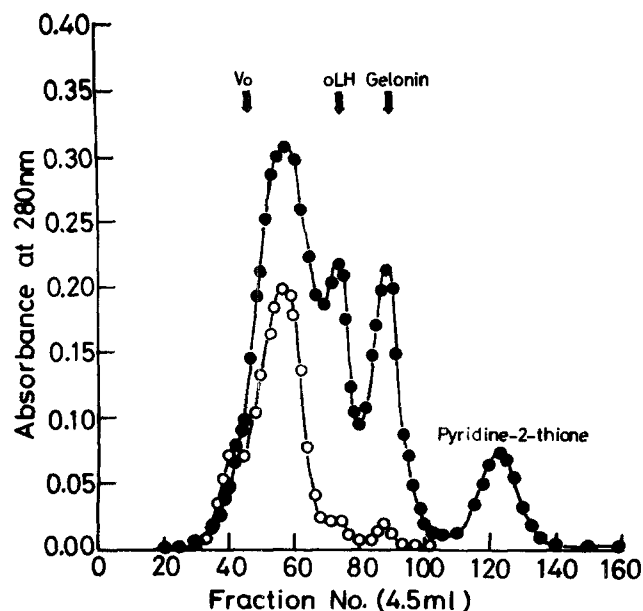


Fig. 1. A representative figure of purification of a conjugate by gel-filtration chromatography. Gel-filtration chromatography of oLH-gelonin conjugate C150 (●–●). The conjugation mixture was separated on a Sephadex G-100 (3.4 × 110 cm) column and eluted with 0.05 M ammonium bicarbonate at a flow rate of 20 ml/hr. Three peaks coded as C150A, C150B and C150C were the pool fractions of 45–65, 72–78 and 84–95 respectively. The pool fraction of C150A (45–65) was rerun on the same column. The rechromatography of C150A peak (as shown by ○–○) on the same column yielded four fractions, C150A-01 (35–48), C150A-02 (50–62), C150A-03 (70–79) C150A-04 (82–94). The different fractions were directly lyophilized for further analysis. The elution position of native oLH and gelonin and the void volume of the column are shown in the upper portion of the figure. The other conjugates, C160 and C170 also showed similar elution profiles.

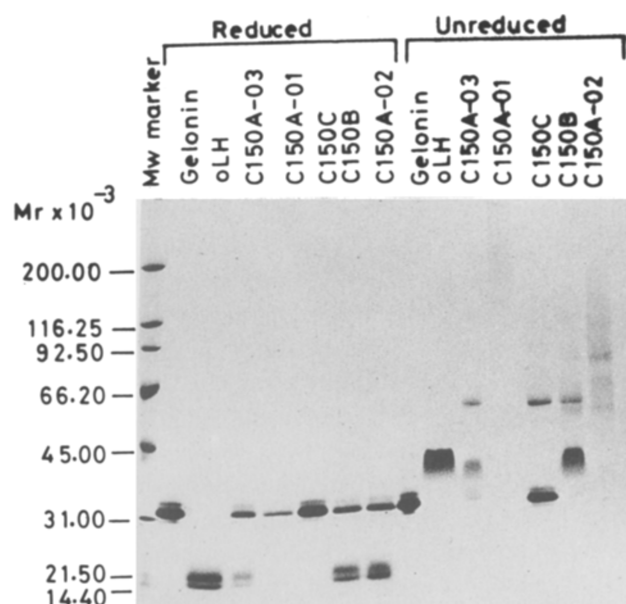


Fig. 2. A representative SDS-PAGE analysis of C150 conjugate. The electrophoretic behaviour of different fractions of C150. A 30 μg each of the sample of the different fractions were run under the unreduced (right panel) and reduced (left panel) conditions. A gel consisting of 5–15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained with Comassie blue. As shown, the molecular weight markers, myosin (200 kDa), β-galactosidase (116.25 kDa), phosphoamylase (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa) carbonic anhydrase (31 kDa), soyabean trypsin inhibitor (21.5 kDa) and lysozyme were used. Densitometric scan was carried out on the reducing gel in order to quantitate the gelonin and hormone in the conjugate for the determination of the molar ratio.

were solubilized with warm 0.5 ml of 0.1 N NaOH and the solubilized protein mixed with 5 ml of scintillation fluid (toluene/triton X-100-Umniflour cocktail, 66 : 33) and counted in a LKB-Liquid scintillation counter.

Results and discussion

A careful titration study of oLH : 2-IT was carried out to obtain an average of one -SH group/molecule of oLH. A 1 : 5 (oLH : 2-IT) molar ratio was required to modify one ε-NH_2 to generate -SH group (Singh *et al.*, Unpublished result). Similarly, present and earlier experience [21, 22] revealed that a molar ratio of 1 : 2 gelonin : SPDP thiolated 1.2 ± 0.2 amino groups per molecule. The conjugation of oLH to gelonin was initiated by the method described in the Materials and methods. The conjugation mixture was fractionated on a Sephadex G-100 column and a representative elution profile (of C150) is shown in Fig. 1. The figure shows the elution positions of oLH, gelonin and the void volume of the column. A small peak appearing between 110–140th fractions was due to the release of pyridine-2-thione which apart from its absorption at 343 nm also absorbs at 280 nm. Therefore, in order to estimate molar concentration using molar extinction coefficient of pyridine-2-thione a correction for its contribution at 280 nm was applied [34]. Both ingredients, oLH and gelonin eluted in a relatively sharp peak while their conjugate eluted prior to oLH and gelonin. Three peaks coded as A, B, C were pooled (as described in legend of Fig. 1). Pool A (coded as C150A) was the conjugate which was rerun on the same column. Typical rechromatographic elution profile of C150A pool on the same column is shown in Fig. 1. This yielded four fractions C150A-01, C150A-02, C150A-03 and C150A-04. The pooled fractions were further subjected to gel-filtration chromatography under pressure using HPLC and ensured that the fractions were devoid of ingredient contamination (Figure not shown). The conjugates were subjected to SDS-PAGE analysis to ascertain the appar-

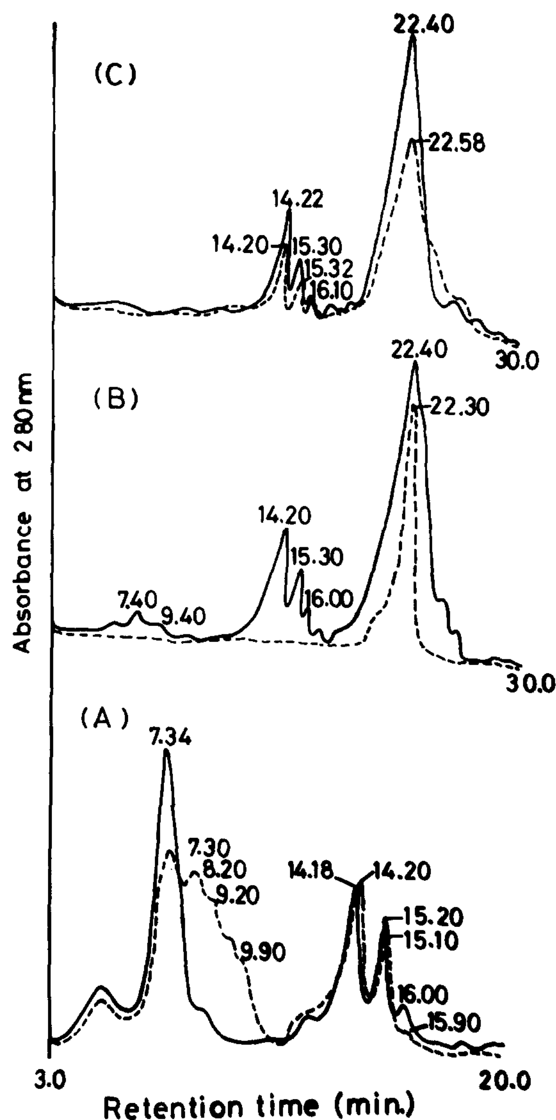


Fig. 3. A representative RP-HPLC analysis of oLH, oLH-2IT (oLH-10) and oLH-gelonin conjugates C150. The test samples were dissolved in H_2O containing 0.1% (solvant A) and the analysis was performed on a Water's μBondapak column (4 x 250 mm) equilibrated in Solvant A + 25% CH_3CN and 0.1% TFA. A gradient of 25–50% was run over 30 min at a flow rate of 2 ml/min in a HPLC system as described in Materials and methods section. The RP-HPLC chromatograms of native oLH (——) and oLH-10 (---) of panel (A) were analysed between 3 and 20 min. Panels B and C chromatograms were analysed between 3 and 30 min. The RP-HPLC chromatograms shown in panel B by (——) and (---) indicate the profile of C150A and gelonin-SPDP respectively. The chromatograms shown in panel C by (---) and (——) indicate the profile of C150A-02 and C150A-01 respectively. The oLH was separated in both α- and β-subunits (panel A). Peak at $t_{7.34}$ was due to α-subunit which was affected after modification (---) with iminothiolane. Few new peaks at $t_{8.20}$, $t_{9.20}$, and $t_{9.90}$ appeared. However β subunit remained unaffected. SPDP modified gelonin (--- of panel B) peak appeared at $t_{22.30}$ which was broadened after conjugation to oLH (—— of panel B & C, --- of panel C). Note the disappearence of peak in α-subunit region but β-subunit dimer peaks were dissociated from the conjugate under the acidic condition. This is further suggesting that the conjugation occurs via the α-subunit of the hormone.

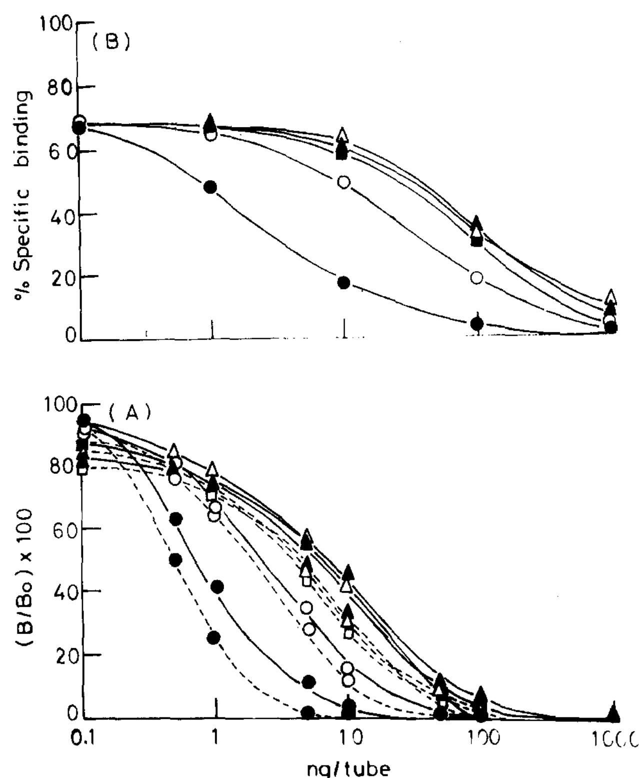


Fig. 4. (A) Immunological reactivity of oLH, oLH-2IT, gelonin, gelonin-SPDP and their conjugates in a competitive RIA system. The cross-reactivity was determined by using competitive displacement method by using ^{125}I-oLH/^{125}I-gelonin and rabbit anti-oLH/anti-gelonin antibodies. The solid lines show the oLH immunoreactivity and dashed line, the gelonin immunoreactivity. Competitive displacement curves of oLH (•–•), oLH-IT (○–○), C150A (△–△), C150A-01 (▲–▲) and C150A-02 (□–□) conjugates. Competitive displacement curves of gelonin (•--•), gelonin-SPDP (○--○), C150A (▲--▲), C150A-01 (△--△) and C150A-02 (■–■). The quantitative cross-reactivity data calculated according to the method described in the text are recorded in Table 1. (B) Receptor binding activity oLH, oLH-2IT and conjugates of gelonin. The rat testicular homogenate was used as a source of gonadotropin receptors and ^{125}I-oLH to determine receptor binding activity of native oLH (•–•), oLH-2IT (○–○), C150A (△–△), C150A-01 (▲–▲) and C150A-02 (■–■) in a competitive displacement analysis. The binding activity calculated as percentage are recorded in Table 2.

ent molecular weight and purity. A representative analysis of C150 conjugate under unreduced and reduced conditions are shown in Fig. 2. The pattern of ingredients is also shown in Fig. 2. The oLH band appears in between 31–41 kDa and gelonin appears as a sharp band at ~ 30 kDa under the conditions used in the present analysis. Under the unreduced conditions, the conjugate did not show the contamination of ingredients (Fig. 2 right). Since the conjugate was linked by a S-S bridge, upon reduction both the ingredients were shown (Fig. 2 left, lane A).

The unreduced SDS-PAGE clearly indicated the conjugates were pure but of variable molecular weight species and devoid of unreacted oLH or gelonin (Fig. 2, left panel). Since, conjugation occured via S-S bond, the bond was reduced by DTT-mercaptoethanol and run on a SDS-PAGE (Fig. 2, left panel). This caused a separation of oLH and gelonin from the conjugates. The reduced gel shows the presence of both the ingredients (Fig. 2, left panel). Gelonin after reduction did not alter its position. oLH is a dimer of α- and β-subunits which are held through a electrostatic attraction [17]. Upon reduction, both subunits are separated and appear at $\sim$ 21.5 kDa. On a densitometric scan and quantitation on the gel, the concentration of oLH and gelonin was estimated using a standard curve pre-constructed by running the different concentrations of oLH and gelonin as described [16]. The molar ratio of oLH : gelonin in the conjugates was determined by SDS-PAGE analysis and by using oLH and the gelonin immunoreactivity data. The immunoreactivity of oLH and gelonin was determined by RIA by taking oLH-2IT or gelonin-SPDP as 100% (assuming 1 : 1 conjugation), the total activity was estimated and oLH : gelonin molar ratio was calculated as oLH (immunoreactivity)/gelonin (immunoreactivity). Based on these methods, the molar ratio data was comparable and found to be nearly in a 1 : 1 ratio.

Table 1. Immunoreactivity of oLH, oLH-10, gelonin, gelonin-30 and their conjugates. oLH and gelonin reactivity was determined by using specific antibodies in a competitive RIA. The activities are expressed as percentage with respect to native oLH which was taken as 100%. The method of determining % activity is described in Materials and methods. ED_{50} is the dose causing 50% displacement

Code	Immunoreactivity (%)			
	oLH		Gelonin	
	ED_{50} (ng)	% Activity	ED_{50} (ng)	% Activity
oLH	0.70	100	–	–
oLH-10	2.45	28.50	–	–
Gelonin	–	–	0.50	100
Gelonin-30	–	–	1.90	46.00
oLH-S-S-Gelonin conjugates				
C150A-02	5.55	12.60	4.20	11.90
		25.20[a]		23.80[a]
		(44.15)		(45.20)
		(88.30)[a]		(90.40)[b]
C160A-02	5.90	11.90	4.10	12.20
		23.80[a]		24.40[b]
		(41.50)		(46.30)
		(83.00)[a]		(92.60)[b]
C170A-02	5.40	13.00	3.96	12.60
		26.00[a]		25.20[b]
		(45.40)		(47.98)
		(90.80)[a]		(95.90)[b]

Values in parentheses indicate the immunoreactivity when oLH-10/gelonin-30 was taken as 100%. [a] Values are corrected for contribution of gelonin in 1 : 1 conjugate. [b] Values are corrected for contribution of oLH in 1 : 1 conjugate. The experiments were carried out in triplicate. Three separate experiments have shown 3–7% deviation in both oLH and gelonin immunoreactivity. Molar ratio determined using percentage immunoreactivity data of oLH and gelonin as described in Materials and methods was found to be close to 1 : 1 which was in agreement with the ratio determined by Spectrophotometric and SDS-PAGE analysis. oLH-10 is the code of oLH modified by 2-iminothiolane. Gelonin-30 is the code of gelonin modified by SPDP.

The conjugates were subjected to RP-HPLC analysis to further assess purity and the site of linkage. Figure 3A shows a comparative RP-HPLC chromatograms of native oLH and oLH modified by 2-iminothiolane. It is clear that the modification primarily occurs in the $-NH_2$ groups of α-subunit which is in agreement with our earlier observations made for SPDP [18]. Since, the elution zone at 12–16 min remains essentially unaltered, it may be concluded that the ε-NH_2 groups of beta subunit are resistant to such modification at the concentration used in the present investigation. Figure 3B shows RP-HPLC chromatograms of gelonin-30 and C150A, oLH-gelonin

Table 2. Receptor binding and steroidogenic activity of oLH, oLH-10 and their gelonin conjugates. The activities were determined as mentioned in Table 1

Code	Receptor binding		Steroidogenic activity	
	ED_{50}	% Activity	ED_{50}	% Activity
oLH	0.95	100	1.60	100
oLH-10	9.30	10.20	1.77	90
oLH-S-S-Gelonin conjugates				
C150A-02	36.50	2.60	13.00	12.30
		5.20[a]		24.60[b]
		(25.50)		(13.60)
		(51.00)[a]		(27.20)[b]
C160A-02	41.30	2.30	13.60	11.80
		4.60[a]		23.60[b]
		(22.50)		(13.00)
		(45.00)[a]		(26.00)[b]
C170A-02	28.80	3.30	12.40	12.90
		6.60[a]		25.80[b]
		(32.30)		(14.28)
		(64.60)[a]		(28.50)[b]

[a] Values are corrected for the contribution of gelonin in 1 : 1 conjugate.
[b] Values are corrected for the contribution of oLH in 1 : 1 conjugate. The experiments were carried out in triplicate and 5–12% deviation was observed in receptor binding and 10–15% in steroidogenic activity.

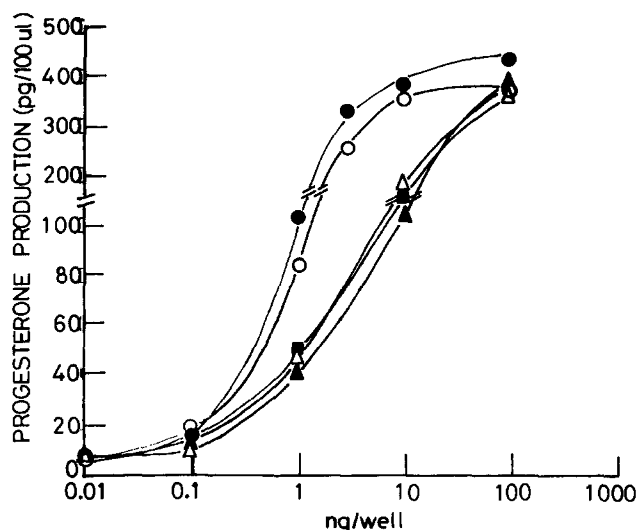


Fig. 5. Steroidogenic potential of oLH (•–•), oLH-2IT (o–o) and oLH-gelonin conjugates, C150A (△–△), C150-01 (▲–▲) and C150A-02 (■–■). The progesterone produced in 4 hr incubation was quantitated by specific RIA as described in Materials and methods section. The quantitative data are recorded in Table 2.

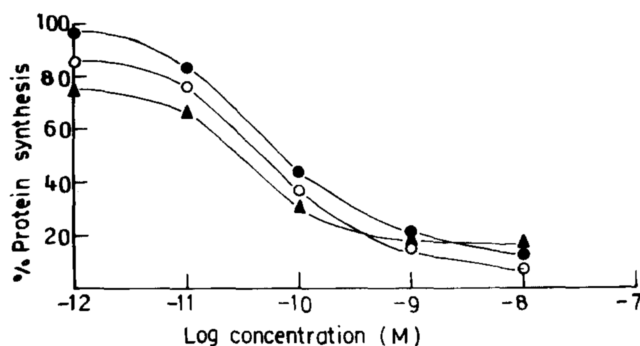


Fig. 7. Cytotoxic evaluation of hormonotoxins in Leydig tumor cells. Inhibition of protein synthesis in mouse leydig tumor cells by hormonotoxins, C150A (o–o), C150A-01 (•–•) and C150A-02 (▲–▲). ^{3}H-Leucine (2,50,000 ± 40,000 CPM/well) incorporated in untreated (control) wells was taken as 100% protein synthesis. The percentage inhibition in treated wells are expressed relative to the controls. Data shown mean ± SE (n = 3), the latter being within the symbol and hence now shown.

conjugate. Gelonin-30 eluted at $t_{22.30}$ as a sharp peak, which after conjugation to oLH broadened (Fig. 3B and C). The oLH peak eluted in t_{12-16} zone. The β-oLH dissociated from the conjugate under the acidic conditions and can be seen in Fig. 3B and C (t_{14-16}). Therefore, it is concluded that the α-subunit was cross-linked to gelonin in the conjugates which further confirms our earlier observation where SPDP was used as a cross-linking agent [12–16]. Although, we attempted to correlate the broadening of gelonin peak with the molar ratio of the conjugate, but no conclusion could be made. RP-HPLC data further confirm that the conjugates were devoid of ingredient contamination as observed in SDS-PAGE analysis (Fig. 2).

Immunoreactivity of oLH and gelonin in their conjugates was determined by competitive RIA as described in Materials and methods. The competitive displacement curves of oLH and gelonin in the conjugates are shown in the Fig. 4A and B respectively and the quantitative immunoreactivity data are recorded in Table 1. The immunoreactivity of oLH was reduced upto 72% after a single ε-NH_2 group modification with 2IT (Table 1, Fig. 4A). However, after conjugation, the oLH immunoreactivity was not further reduced drastically (Table 1). When oLH-10, (2IT-modified oLH) activity was taken as 100%, the activity varied from 40–46%. Assuming 1 : 1 oLH-gelonin conjugation, further molar ratio was determined in terms of immunoreactivity. A ratio of percentage oLH and gelonin activity was used to determine molar ratio (A 1 : 1 molar ratio was found to be comparable with that of SDS-PAGE analysis data.

The primary requirement of biological activity of hormonotoxin was its binding to the receptor which was determined by using rat testicular homogenate as a source of gonadotropin receptors. Like immunoreactivity, in receptor binding, a single ε-NH_2 group modification reduces the receptor recognition to ~ 10% which was substantially affected (reduced to 2–4%) after conjugation (Fig. 4B, Table 2). When values were corrected for the increase in the molecular weight of the conjugate, the activity was accordingly increased. When oLH-10 activity was taken as 100%, the calculated activity was reduced to half (Table 2). The steroidogenic activity was relatively unaffected after a single ε-NH_2 group modification (Fig. 5, Table 2). As observed in receptor binding studies, the steroidogenic activity was also decreased after conjugation (Table 2).

The binding of the conjugates to leydig tumor cells was demonstrated by using gelonin antibody and ^{125}I-Protein-A as a probe. Figure 6 shows the binding of hormonotoxin, C150A, C150A-01 and C150A-02 to the tumor cells. The non-specific binding was determined by incubation of appropriate quantity of gelonin. Since, the conjugate binds to gonadotropin receptor via gonadotropin to the receptor and leaves gelonin free for its binding to anti-gelonin antibodies, the extent of binding of gelonin antibodies to cell reflected the binding efficiency of the hormonotoxins. The binding of anti-gelonin antibodies was assayed by ^{125}I-Protein-A. A 5–10 μg/well conjugate saturated the receptors (Fig. 6A). That this binding indeed occurred via oLH, a competitive dis-

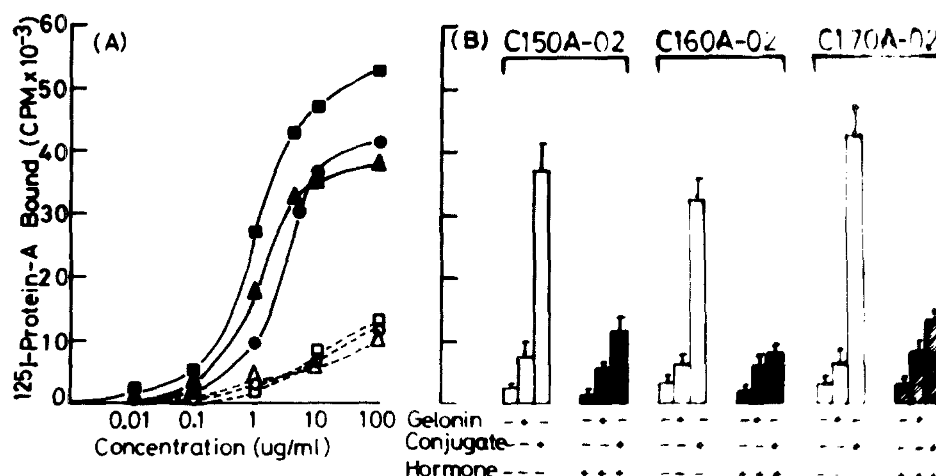


Fig. 6. Saturation binding and competition ability of hormonotoxin of oLH to the mouse Leydig tumor cells. Figure 6A shows the representative saturation analysis of the oLH-gelonin C150A (▲▲), C150A-01 (•–•) and C150A-02 (■–■) conjugates. Cells (~ 100,000/well) grown in RPMI-1640 media supplemented by 15% foetal calf serum for 48 hr and subsequently washed with serum free medium. The cells were then incubated with the test samples. Following incubation for 2 hr and washing to remove the unbound proteins, the cells were then challenged with anti-gelonin antibodies. The amount of antibody bound to cells (via the gelonin in the conjugate) was quantitated in a subsequent reaction with ^{125}I-labelled protein-A. The number of counts (cpm) observed in each instance is a direct reflection of hormonotoxin binding via the LH receptor binding sites in the conjugate and detected by the antibody directed against gelonin. The non-specific binding (NSB) is shown by the broken lines and open symbols obtained by using the gelonin fraction in each fractionation. Figure 6B shows the competition ability of native oLH to hormonotoxins for binding to leydig tumor cells. The assay was performed according to the procedure described in Materials and methods. The cells were grown in RPMI-1640 medium in the presence of 15% FCS for 24 hr and later washed with serum free medium. The last samples were incubated for 2 hr. After incubation, the cells were washed and then incubated with gelonin antibodies. The bound antibody was quantitated in the subsequent reaction with ^{125}I-Protein-A. The radioactivity incorporated was the direct reflection of hormonotoxin bound to the cell. Note in the presence of the competitor (i.e., oLH), the bound radioactivity was significantly inhibited (as shown by the hatched bar).

placement study was carried out. Figure 6B shows the binding of hormonotoxins to the leydig tumor cells (open bar) and competition with native oLH (hatched bar) (Fig. 6B). As shown, the binding of ^{125}I-Protein A was drastically inhibited (Fig. 6B, closed bar) which clearly revealed that the binding occurs via oLH. These data further support our earlier observations when SPDP mediated oLH-S-S-gelonin conjugates were used for the binding and competition experiments [13, 15, 16].

After having established that the binding of hormonotoxins occurs via the hormone, the cytotoxicity experiments were carried out. Figure 7 shows the cytotoxicity to the cells and their toxicity was essentially higher than that of the conjugates described earlier [12–16]. In a parallel set, the conjugates incubated in the presence of the native oLH did not show cytotoxicity (data not shown). The present investigation clearly demonstrated that the synthesis of hormonotoxins preserving the positive charge on their lysine residue through which the coupling occurs, was approximately one log higher than that of the earlier hormonotoxins [12–16] cytotoxicity to the leydig tumor cells. The site of disulfide cross-linking was the α-subunit which is reported to be very sensitive to biological activity. The reduced receptor binding may be due to the site of conjugation. The ε-NH_2 groups of β-subunit being insensitive to chemical modification were the ideal site for the conjugation. These ε-NH_2 groups could not be exploited for the preparation of hormonotoxin as β-oLH-gelonin conjugate failed to recombine with the native α-oLH and thus the bioeffectivity could not be recovered [32].

The positive charge on αoLH plays an important role in the receptor recognition. The SPDP conjugation lead to reduction of a positive charge. The hormonotoxins presented in the present paper retained their positive charge which may be the cause of higher receptor recognition and cytotoxicity of the hormonotoxins.

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