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Disulfide linked αoLH-gelonin conjugate failed to recombine with βoLH subunit to generate bioeffective hormonotoxin

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Abstract

Since, linking of ovine luteinizing hormone (oLH) to ribosome inactivating protein gelonin (in oLH-gelonin conjugate) occur via the alpha-subunit, αoLH, an attempt has been made to develop a 'universal' hormonotoxin for selective targeting to specific cells in the gonads. Four different molar ratios of oLH and N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) were used to activate the epsilon amino (φ-NH_2) groups of αoLH. The αoLH-SPDP derivatives recombine to native beta subunit of oLH (βoLH) and the purified recombinants retained substantial receptor binding, steroidogenic activity and immunoreactivity to native oLH. The disulfide linked αoLH-S-S-gelonin conjugates prepared by SPDP method were purified by gel filtration chromatography and analysed by reverse-phase high performance liquid chromatography (RP-HPLC). In order to obtain specificity and bioeffectivity, the αoLH-S-S-gelonin conjugates were allowed to recombine to native βoLH and the recombination mixture was further purified by gel-filtration chromatography. The RP-HPLC analysis of these recombinants indicated that αoLH-S-S-gelonin did not recombine to βoLH. The failure of recombination may be due to the reasons. (i) The site of ε-NH_2 activation by SPDP may be different in the αoLH than the native oLH. (ii) The activation site may be in close proximity to the annealing site which facilitates the recombination of β-subunit but failured to reassociate to αoLH-S-S-gelonin conjugate. (iii) The introduction of gelonin (30 kDa basic protein) might have induced some steric hinderence for βoLH to recombine to the αoLH site which might have been masked in αoLH-S-S-gelonin conjugates. (Mol Cell Biochem **120:** 95–102, 1993)

Key words: gonadotropin, immunoreactivity, receptor binding, steroidogenesis

Abbreviations: oLH – ovine Luteinizing Hormone, αoLH – alpha subunit of oLH, βoLH – beta subunit of oLH, BSA – Bovine Serum Albumin, DTT – Dithiothreitol, RP-HPLC – Reverse Phase High Performance Liquid Chromatography, TSH – Thyroid Stimulating Hormone, FSH – Follicle Stimulating Hormone, LH – Luteinizing Hormone, eCG – equine Chorionic Gonadotropin, DMEM – Dulbecco's Modified Eagles Medium, HEPES – 4-(2-Hydroxyethyl)-1 Piperazine Ethane Sulfonic acid, PAP – Pokeweed Antiviral Protein, RIA – Radioimmunoassay, hCG – human Chorionic Gonadotropin, TRH – Thyrotropin Releasing Hormone, CRF – Corticotropin Releasing Factor, hPL – human Placental Lactogen, TFA – Trifluroacetic Acid, αoLH-SPDP – SPDP activated derivative of αoLH

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Introduction

The use of monoclonal antibodies which recognise determinants on the surface of the tumor cells as vehicle for delivery of toxic agents, has been the subject of intense investigation in the last decade [1–5]. These cell type specific agents called 'immunotoxins' have been synthesised in many laboratories by covalent cross-linking antibody molecule to highly potent plant and bacterial toxins [1–5]. With the increasing success of *in vitro* and *in vivo* use of immunoconjugates hoped that potential immunotoxins will be made specific and less toxic than the treatments currently available for human neoplasia. Several immunotoxins have been prepared with the use of protein toxin such as ricin-A chain, abrin-A chain, gelonin, PAP, saponin or bacterial toxins, e.g., pseudomonas exotoxin, diphtheria toxin or small drugs such as daunomycin, chlorambucil and vindesin and have shown efficacy in a variety of animals [1–5]. Recently, like immunoglobulins several hormones or growth factors such as human placental lactogen (hPL) [6], insulin [7], human chorionic gonadotropin (hCG) [8, 9], thyrotropin releasing hormone (TRH) [10] and corticotropin releasing factor (CRF) [11] have also been conjugated to different toxins. These are referred as hormonotoxins. We recently prepared and characterized hormonotoxin based on gonadotropins by conjugating oLH/hCG to gelonin and such hormonotoxins could selectively inhibit protein synthesis in the leydig tumor cells (MA10) which possess receptors for gonadotropins [12–17]. The gelonin was linked to oLH by a disulfide bond using SPDP, a heterobifunctional reagent [12–17]. The analysis of this hormonotoxins revealed that the coupling occurs through α-subunit of the oLH [12–17]. This observation indirectly supported the view of developing a 'universal' conjugate by linking the alpha-subunit to the toxin (since the α-subunit could be hybridized with the β-subunit of other glycoprotein hormones of the same species such as LH, TSH and FSH) which could be recombined with the desired β-subunit of the hormone to dictate the specificity of the hormonotoxin to the specific cells. This communication is an attempt to synthesise a 'universal' hormonotoxin in which the α-subunit is cross-linked by a -S-S-bond to the gelonin and the αoLH-S-S-gelonin conjugate is allowed to recombine to the β-subunit to generate bioeffective hormonotoxin. During such studies some interesting observations were made describing the intersubunit interaction of oLH.

Materials and methods

Materials

Ovine LH was isolated from the frozen pituitary glands and lyophilized powders according the procedure [12] and stored at 4° C in the powder form. The α and β subunits of oLH were isolated and characterized as reported earlier [18]. Prior to the use of these subunits for coupling and recombination experiments, the subunits were subjected to RP-HPLC analysis in order to determine the contamination of the respective subunits. Gelonin, a single chain RIP was isolated from the seeds of *Gelonium multiflorum,* a plant of Indian origin following the procedure described earlier [19] with minor modifications [20, 21]. The protein was further characterized for its molecular weight, electrophoretic behaviour, amino acid analysis and RP-HPLC for purity. The gelonin used in the present investigation was over 98% pure as judged by RP-HPLC and retained its ability to inhibit protein synthesis in a cell free translation system [21, 22].

Heterobifunctional cross-linker, SPDP and DTT were purchased from Pierce chemicals (Rockford, IL). Chromatography materials, Sephadex G-25 (medium) and G-100 were obtained from Pharmacia Fine Chemicals, Sweden. Na ^{125}I (IMS30) used was purchased from Amersham, Inc. (Chicago, IL). Other laboratory chemicals used were of highest purity available commercially.

Methods

Modification of oLH and gelonin

The modification of αoLH [23] and gelonin [20, 21] with SPDP was carried out following the procedure described earlier. Proteins to be thiolated were dissolved in sodium phosphate buffer (0.1 M, pH 7.5 containing 0.15 M NaCl) and reacted with varying molar ratio of SPDP in the initial reaction. After 30 min standing at room temperature with occasional stirring, the reaction mixture was separated on a G-25 (medium) column. The protein peak was concentrated by ultrafiltration on a YM10 filter (Amicon). The extent of amino groups modified by SPDP was calculated by measuring the liberation of pyridine-2-thione group following the treatment with DTT. In order to avoid the intra-disulfide bond reduction, the DTT treatment was carried

out with 50 molar excess DTT in sodium acetate buffer, pH 5.5, containing 0.15 M NaCl. The reaction was carried out in the cuvette of the Spectrophotometer for the UV-VISIBLE spectra recording. From the absorption of pyridine-2-thione at 343 nm, molar concentration of the liberated group was calculated by using extinction coefficient ($\varepsilon_{343} = 8.08 \times 10^3 M^{-1}$) [24]. The absorption at 280 nm was corrected for pyridine-2-thione contribution.

Conjugation of αoLH to gelonin

The conjugation of αoLH-SPDP with gelonin-SPDP was initiated by reducing gelonin-SPDP with DTT at acidic pH in the sodium phosphate buffer pH 5.5 and then mixing with αoLH-SPDP. Typically the gelonin-SPDP was dialysed against 0.1 M sodium phosphate buffer, pH 5.5 containing 0.15 M NaCl and 50 times excess of DTT was added. After 15 min incubation at room temperature, the mixture was passed over G-25 (medium) column to separate gelonin-SH with free DTT and pyridine-2-thione. The protein peak was immediately mixed with αoLH-SPDP. The coupling reaction was carried out at room temperature for 18–24 hr. Prior to G-100 chromatography, the mixture was concentrated by ultrafiltration on YM-10 filter (Amicon). The column was pre-equilibrated with 50 mM ammonium bicarbonate.

Recombination experiments

The hybridization of native β-subunit of oLH with SPDP modified αoLH or αoLH-S-S-gelonin was carried out in 0.05 M sodium phosphate buffer pH 7.2 containing 0,1 M NaCl. Both reactants were mixed in the same buffer and left at room temperature for 18–24 hr. The recombination mixture was passed over G-100 column (as described in the result section) to separate the combinants from reactants.

RP-HPLC analysis

Alpha oLH, αoLH-SPDP, αLH-S-S-gelonin and their recombinants with native βoLH were subjected to RP-HPLC analysis for further characterization. RP-HPLC analysis was carried out on a Water's μBondapak phenyl column using a continuous gradient as described earlier [12, 13, 18, 23]. (see legend to Fig. 1 for details).

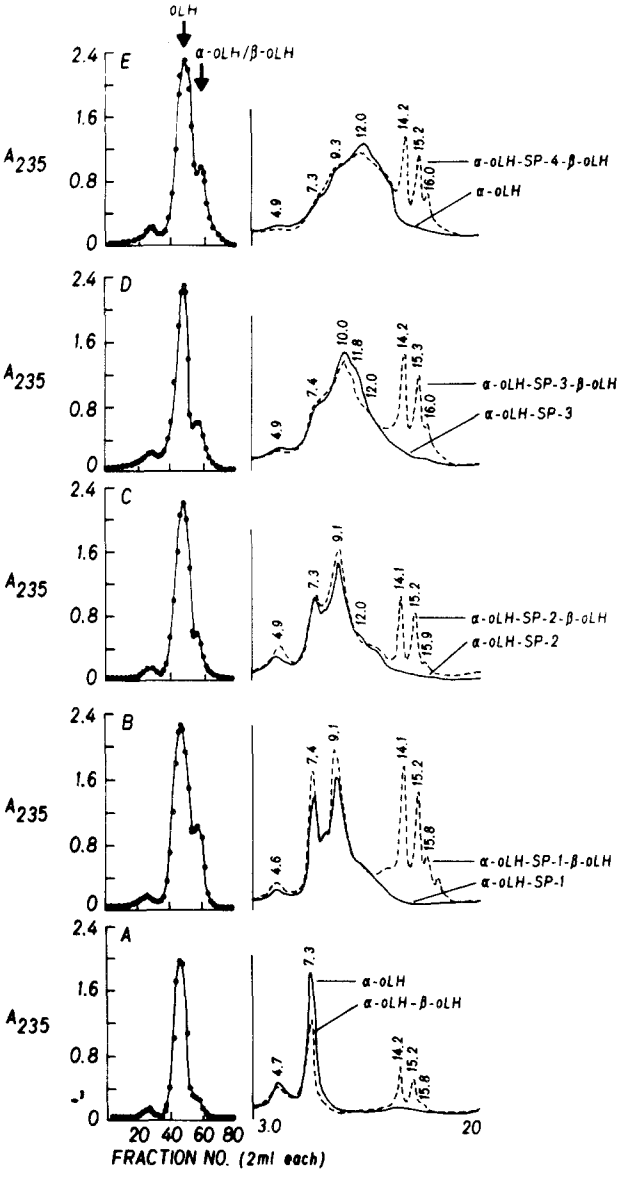

Fig. 1. Gel-filtration chromatography of recombination mixture (●——●) of native βoLH with native αoLH (A), αoLH-SP-1 (B), αoLH-SP-2 (C), αoLH-SP-3 (D), and αoLH-SP-4 (E). The arrows on the upper portions of the chromatograms show the elution positions of αLH and its α and β subunits. The column was pre-equilibrated with 0.05 M ammonium bicarbonate and the protein was eluted in the same buffer at a flow rate of 20 ml/hr. (Right) RP-HPLC analysis of native αoLH, its SPDP modified derivatives and their recombinants with βoLH. The samples were dissolved in water containing TFA 0.1% (solvent A) and the analysis was performed on a Water's μBondapak column (4 × 250 mm) equilibrated in the solvent 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 and monitored at 280 nm. The RP-HPLC chromatograms was analysed between 3–30 min. The RP-HPLC chromatograms shown (———) in panels A–E, indicate the profile of native oLH, αoLH-SP-1, αoLH-SP-2, αoLH-SP-3 and αoLH-SP-4 respectively. The chromatograms shown (by ---) are the recombined products of βoLH with αoLH (panel A), αoLH-SP-1 (panel B), αoLH-SP-2 (panel C), αoLH-SP-3 (panel D) and αoLH-SP-4 (panel E). The recombinant chromatograms analysed here represent the peak tube of gel-filtration profile (left panel top).

Immunoreactivity and radioreceptor binding studies

Radioiodination of oLH and gelonin

The radioiodination of highly purified oLH or gelonin was carried out by lactoperoxidase method as described [12, 13]. ^{125}I-oLH and ^{125}I-gelonin were separated from free radioactive iodine by gel-filtration chromatography. The specific activity of the labelled hormone was in the range of 50–80 μCi/μg. The radioiodinated oLH and gelonin were stored at − 70° C and used within four weeks of preparation.

Competitive binding assay of oLH

The immunoreactivity of native oLH and the recombinants of αoLH, αoLH-SPDP with the native βoLH was studied by competitive displacement method. Briefly, all reagents 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. The antibodies were appropriately diluted to give 30–50% binding in the absence of respective antigen. The assay was performed in polystyrene tubes (12 × 75 mm). To the diluted antibodies (0.1 ml), 0.1 ml of each of ^{125}I-oLH and competitors were added. the tubes were incubated for 24 hr at 4° C. The bound and unbound ^{125}I-oLH were separated by the addition of 0.20 ml of 1 : 5 Pansorbin (Calibiochem, LaJolla, CA). The tubes were further incubated at 37° C for 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 of 70% efficiency.

Radioreceptor assay

RRA was performed using rat testicular homogenate as a source of receptor following the procedure essentially described earlier [12, 18, 23]. In brief, ^{125}I-oLH [0.5–1.0 ng ~ 70,000 CPM), test samples 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 x g. The supernatant was removed by aspiration and pellet was counted as described above.

Steroidogensis assay

The steroidogenic activity of native oLH, recombinants of αoLH and its SPDP modified derivatives to βoLH was evaluated by incubation with ovarian granulosa cells from immature rats [12–16]. The immature female rats (23 days old) were primed with 25 IU of eCG. After 48 hr stimulation, the granulosa cells were collected and incubated with samples in DMEM (Gibco, NY), containing 20 mM HEPES, pH 7.5 and 0.1% BSA at 37° C under 95% O_2/5% CO_2 for 4 hr. The progesterone secreated into the medium was estimated by specific RIA and statistically analysed on the LKB-2144 Rackbeta liquid Scintillation counter equipped with RIA-CALC programme.

Results and discussion

The αoLH used in the present investigation was first separated from its β-subunit by using conventional salt-precipitation and counter current distribution methods. The salt precipitation method was found to be less comburssive and yielded relatively pure α-subunit. Further the anticipated β-subunit contamination was determined by RP-HPLC analysis. The RP-HPLC purified α and β subunits were used in subsequent studies. It may be recalled that HPLC pure subunits recombine fully and retain full biological activity. Four different molar ratios of SPDP was used in modification of α-subunit and the number of ε-NH_2 groups modified are recorded in Table 1. A 1 : 1, 1 : 2, 1 : 3 and 1 : 4 molar ratios of αoLH-SPDP yielded modification of 1 : 0.9, 1 : 1.90, 1 : 2.80 and 1 : 3.65 respectively. A relatively higher modification was observed in isolated α-subunit than the native oLH [23] which indicated that the ε-NH_2 groups of the isolated α-subunit were more reactive than the native oLH. This is in consistent with many previous reports showing differences between the isolated subunits and the native hormone in their conformation [25–27], digestibility by proteolytic enzymes [28], accessability of the tyrosine residues, receptor binding and immunoreactivity. The RP-HPLC chromatograms of αoLH, αoLH-SP-1, αoLH-SP-2, αoLH-SP-3, αoLH-SP-4, (as indicated by ——— in the right panel) and their recombinants with βoLH as depicted by --- in the right panel) are shown in Fig. 1. The αoLH elutes as a major peak at $t_{7.3}$ and a minor peak at $t_{4.7}$. A 1 : 1 oLH : SPDP molar ratio resulted into 0.9 NH_2 group thiolation. Due to this a new peak emerged at $t_{9.1}$ (panel B). A gradual shift of $t_{7.4}$ towards $t_{9.1}$, $t_{10.0}$, $t_{11.8}$ was observed in 1 : 2 and 1 : 3 molar ratio modification, the peak was broadened and eluted at the $t_{9.3}$, $t_{12.0}$. A shift of $t_{7.4}$ towards the higher retention time may be due to the introduction of hydrophobic groups which also abolishes the positive charges on the subunit. Under the similar conditions, thiolation of the native oLH exhibited dif-

ferent RP-HPLC chromatograms [23]. At this stage it is difficult to state whether the NH_2 groups of native oLH involved in thiolation are the same as in the α-subunit.

In a typical recombination experiment, an equal amount of βoLH (generally 3.0 mg) was mixed with equal weight of SPDP-modified αoLH in the 0.05 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Due to the hydrophobic nature of βoLH, it does not dissolve freely in the buffer. However, when left undissolved in contact with αoLH, the annealing process begins and upon completion, most of the β-subunit recombines with the αoLH and the solution becomes clear. The recombination mixture was fractionated on a pre-calibrated Sephadex G-100 column. In order to determine the elution volume/position of native oLH, αoLH/ βoLH and gelonin, these proteins were run as a control of the same column at a flow rate of 18 ml/hr. The left panel of Fig. 1 shows the gel filtration chromatography of the recombination mixture of βoLH with αoLH (A), αoLH-SP-1 (B), αoLH-SP-2 (C), αoLH-SP-3 (D), αoLH-SP-4 (E). The top tube of this peak was run on the reverse-phase column and the chromatograms are shown in the right panel of Fig. 1. The protein eluting as dublet at $t_{14.2}$ and $t_{15.2}$ was due to the β subunit found to be present in all recombinants (Fig. 1, right panel). This also demonstrate that SPDP modified α-subunit (at least upto 1 : 1–1 : 4) could hybridize to β-subunit. It may be concluded that ε-NH_2 groups that underwent modification were not directly participating in the subunit recognition or that the introduction of SPDP hydrophobic groups did not hinder reassociation.

In order to determine the anti-oLH antibody reactivity, receptor binding and steroidogenic activity of the above described recombinants, the immunological and biological characterization was carried out. The immunoreactivity was determined by using the conformation directed rabbit anti-oLH antibody and receptor recognition with the rat testicular membranes. In both the cases, the isolated α and β subunit have very low activity (data not shown). Figure 2A shows the competitive displacement curves of the recombinants of βoLH with αoLH and its SPDP modified derivatives. All the curves were parallel to the native oLH (Fig. 2A). The quantitative data are recorded in Table 1. Interestingly, the immunoreactivity and receptor binding properties of the recombinants of HPLC purified αoLH with βoLH was relatively higher than the native oLH (Table 1). This may be due to the contamination of some non-proteineous substance in the native oLH which was removed after RP-HPLC purification. A single amino

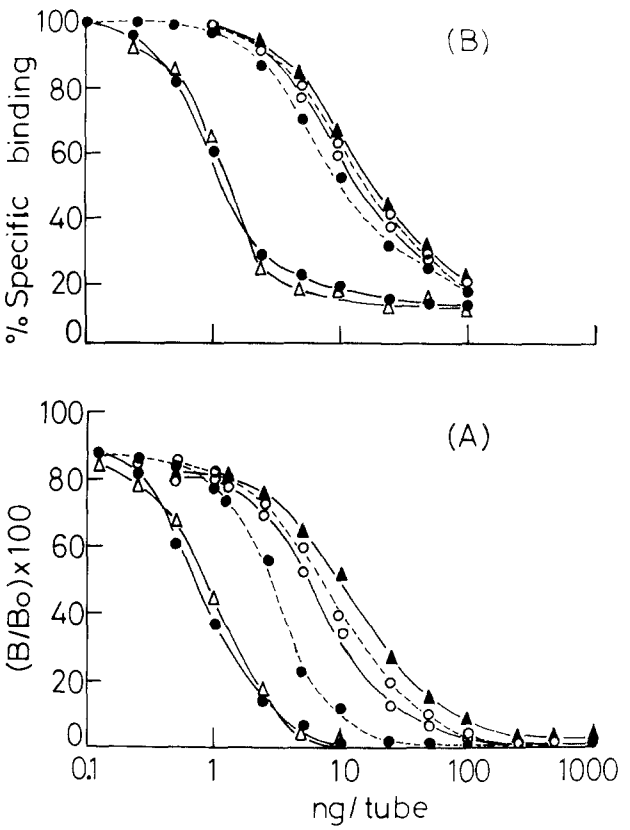

Fig. 2. (A) Immunological reactivity of oLH (△——△), recombinants of native βoLH with HPLC purified αoLH purified αoLH (●——●), αoLH-SP-1 (●---●), αoLH-SP-2 (○——○), αoLH-SP-3 (○---○) and αoLH-SP-4 (▲——▲). The oLH activity was determined by competitive displacement analysis using ^{125}I-oLH as tracer and specific antibodies as described in Method section. The recombinants used here represent the peak tube of the gel-filtration profile (Fig. 1A–E, left panel). The quantitative cross-reactivity data are recorded in Table 1. (B) Receptor binding activity of oLH (△——△), recombinants of native βoLH with HPLC purified αoLH (●——●), αoLH-SP-1 (●---●), αoLH-SP-2 (○——○), αoLH-SP-3 (○——○) and αoLH-SP-4 (▲——▲) obtained in a competitive displacement analysis as determined by RRA using rat testicular homogenate. The quantitative data are recorded in Table 1.

group modification in the α-subunit resulted in the inhibition of immunoreactivity upto 75% and receptor recognition upto 90%. The steroidogenic activity as determined by rat testicular homogenate and ^{125}I-oLH as tracer was found to be relatively unaffected in the low molar ratio modifications (ie., upto 1 : 3) (Fig. 3). Higher modification (ie., 1 : 4) affected the steroidogenic activity of the recombinant. Surprisingly, an 1 : 1 modification indeed enhanced the steroidogenic activity (Fig. 3). This may be due to the introduction of hydrophobic group in the α-subunit which might have affected the receptor recognition but might have played an

important role in the enhancement of steroidogenic activity (Table 1; Fig. 2A). Presently no appropriate explanation could be given for this observation. The retention of full biological activity may be due to the fact that only 1% receptor occupancy is required to induce and sustain full biological response [29].

The conjugation of αoLH to gelonin was carried out by SPDP as described in Method section. The thiolated αoLH-SP-1, αoLH-SP-2, αoLH-SP-3, and αoLH-SP-4, were mixed with 1.5 molar excess of gelonin-SPDP pretreated with DTT and conjugation reaction was carried out for 24 hr at room temperature (about 25° C). The reaction was monitored by recording UV-VISIBLE spectra. The liberation of pyridine-2-thione during the reaction was indicative of the conjugation of gelonin with the different thiolated oLH derivatives. The mixture was fractionated on Sephadex G-100 column and the elution profile is shown in Fig. 4 (left). The protein eluting before the ingredients was pooled and the peak (top) tube was kept separately for HPLC analysis. It may be mentioned that in conjugation process, the gelonin-SPDP was always taken about 1.5 times molar excess to ensure complete reactivity of αoLH-SPDP. The top tube of each chromatogram (Fig. 4, left) was subjected to RP-HPLC analysis and the RP-HPLC chromatograms are shown in the Fig. 4 (right). The αoLH-S-S-gelonin conjugates were devoid of contamination of either αoLH-SPDP or gelonin-SPDP as α-subunit eluted at $t_{7.4}$ and gelonin-SPDP as a sharp peak at $t_{21.0}$ were absent (Fig. 4, right). The αoLH-S-S-gelonin conjugates were dialysed against 0.1 M sodium phosphate buffer pH 7.5, containing 0.15 M NaCl and mixed with βoLH in the same buffer to initiate recombination. The recombination mixture was left for 24 hr at the room temperature and again fractionated on the same column. The protein peak emerging before the oLH elution position was pooled and further subjected to RP-HPLC analysis to ascertain the presence of βoLH in the αoLH-S-S-gelonin conjugate.

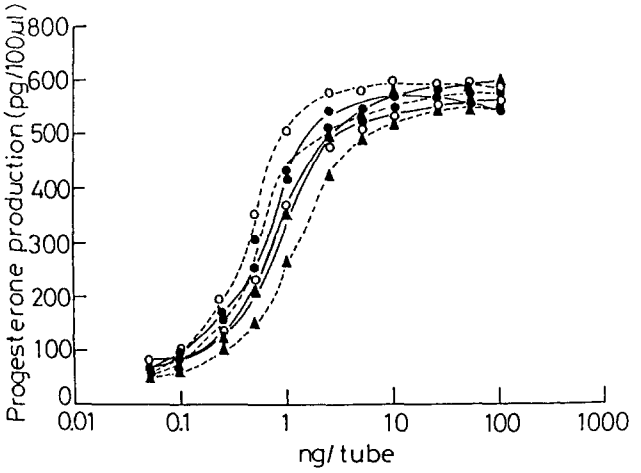

Fig. 3. Progesterone induction ability of oLH (●——●), recombinants of native βoLH with HPLC purified αoLH (●——●), αoLH-SP-1 (○——○), αoLH-SP-2 (○——○), αoLH-SP-3 (▲——▲) and αoLH-SP-4 (△---△) as determined in rat granulosa cell assay.

Table 1. Immunoreactivity and receptor binding properties of different recombination products of SPDP-modified oLH with native oLH

Code	Mol. of SPDP added/ mol of oLH	No. of -NH_2 groups modified	Immuno-reactivity		Receptor binding	
			ED_{50}	% activity	ED_{50}	% activity
oLH			0.80	100.00	1.10	100.00
αoLH-SP-1	1 : 1	0.90				
αoLH-SP-2	1 : 2	1.90				
αoLH-SP-3	1 : 3	2.80				
αoLH-SP-4	1 : 4	3.65				
αoLH.βoLH	–	–	0.70	100.00 (114.28)	0.96	100.00 (114.58)
αoLH-SP-1.βoLH	–	–	2.91	24.00 (27.50)	9.40	10.20 (11.70)
αoLH-SP-2.βoLH	–	–	5.60	12.50 (14.30)	12.60	7.60 (8.73)
αoLH-SP-3.βoLH	–	–	7.00	10.00 (11.40)	14.76	6.50 (7.45)
αoLH-SP-4.βoLH	–	–	9.33	7.50 (8.57)	16.55	5.80 (6.64)

Values in paraentheses were obtained when native oLH was taken as 100%.

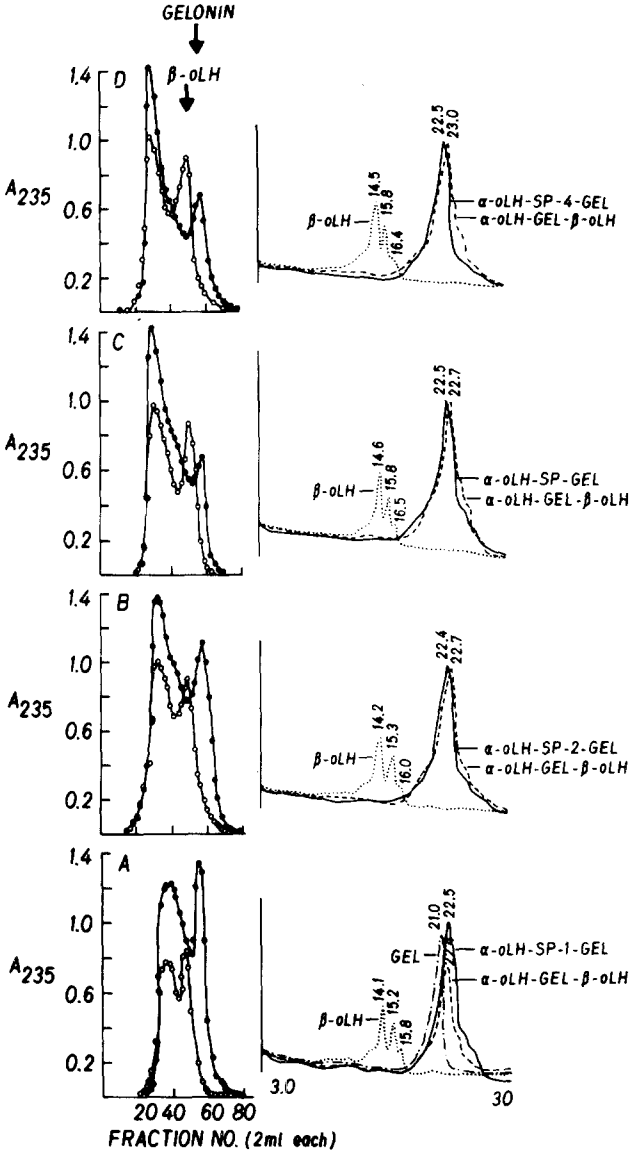

Fig. 4. (Left) Gel-filtration chromatography of conjugation mixture of SPDP-modified gelonin (●——●) with αoLH-SP-1 (A), αoLH-SP-2 (B), αoLH-SP-3 (C) and αoLH-SP-4 (D). Other details of chromatography is the same as described in Fig. 1. The protein peak eluting before the elution position of αoLH-SP1-4/gelonin-SPDP was pooled and subjected to RP-HPLC analysis (right panels) prior to recombination with the native βoLH. The elution profile of recombination mixtures (○——○) of αoLH-SP-1-gelonin (A), αoLH-SP-2-gelonin (B), αoLH-SP-3-gelonin (C) and αoLH-SP-4-gelonin (D). The protein eluting before βoLH was pooled and subjected to RP-HPLC analysis (right panels). The arrows in the upper portion of the figure show the elution position of gelonin and βoLH (right). RP-HPLC chromatograms shown by (———) in the right panels A–D indicate the profile of αoLH-SP-1-gelonin, αoLH-SP-2-gelonin, αoLH-SP-3-gelonin and αoLH-SP-4-gelonin conjugates. The conjugate analysed here represent the peak tube of gel-filtration profile (left panel ●——●). In panel A, the RP-HPLC chromatogram of gelonin-SPDP is also shown as ---. The dotted line chromatograms show the elution profile of βoLH which eluted in duplet at $t_{14.1-14.5}$ and $t_{15.2-15.8}$. The RP-HPLC chromatograms of recombinants of βoLH with αoLH-SP-1-gelonin, αoLH-SP-2-gelonin, αoLH-SP-3-gelonin and αoLH-SP-4-gelonin are shown by --- in panel A, B, C and D respectively. Note the conjugate recombinants did not possess βoLH which eluted as duplet at t_{14} and t_{15}.

The RP-HPLC chromatography of the recombinants as obtained after G-100 chromatography are shown in Fig. 4 (right). The dotted line shows the presence of βoLH under acidic conditions. Interestingly, in all the preparations, the recombinants did not show the release of βoLH which was expected due to the recombination. Since the αoLH was covalently conjugated to gelonin, the α subunit was found to be absent in the $t_{7.4-12.0}$ region of the chromatogram. The present experiment clearly demonstrate (i) αoLH-SPDP can recombine with βoLH subunit and restore full biological activity, (ii) αoLH-S-S-gelonin conjugate fail to recombine with its native βoLH as demonstrated by RP-HPLC analysis and thus excluding the possibility of generating 'universal' hormonotoxins using αoLH. However, it would of great interest to investigate other gonadotropic hormones.

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