HORMONOTOXINS: SYNTHESIS, CHARACTERIZATION AND BIOEFFICACY OF SOME αLH-GELONIN CONJUGATES

ABSTRACT

By

ANIL KUMAR MAVILA

INSTITUTE OF SELF ORGANISING SYSTEMS AND BIOPHYSICS

A THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE
OF
DOCTOR OF PHILOSOPHY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG
INDIA
1995

INTRODUCTION :

With the aim of developing a cytotoxic hybrid molecule against specific cells of gonads, the Ribosome inactivating protein (RIP), gelonin was conjugated with the Ovine luteinizing hormone (oLH) using different heterobifunctional cross-linkers. As hCG/LH receptors are highly localized on the gonadal cells and their action is mediated by internalization, the hormone carrying the toxin will be localized at the site and it would be released upon internalization. Once the hormone is internalized into the cell and the toxin is released by breaking -S-S- bond between the hormone and toxin, the toxin would terminate all key cellular activity resulting into complete cessation of cell function. Without adequate production of steroids, the reproductive events will be disrupted. If this strategy materializes and becomes effective *in-vivo*, a new class of luteolytic agents might become available. As the drug would be released only after the internalization of the conjugate, spurious toxic activity on other cells or in circulation, where gonadotropin receptors are not present (or not expressed) would be minimal.

MATERIALS :

Ovine luteinizing hormone (oLH) :

oLH was purified from the sheep pituitary powder according to the procedure described in literature. Purified hormone was subjected to physico-chemical, immunological and biological characterization especially for receptor binding and steroidogenic activity prior to use for covalent coupling to gelonin. The hormone was stored as dry powder at 4°C. This preparation was used for the subsequent experiments during the study.

Ribosome inactivating protein, Gelonin :

Dry seeds of *Gelonium multiflorum* belonging to *Euphorbeaceae family* was used for isolating gelonin using aqueous extraction, ammonium sulfate precipitation, cation-exchange and finally gel-filtration chromatography. Gelonin used in the present investigation was judged to be over 98% pure, as analysed by RP-HPLC and retained its ability to inhibit protein synthesis in an *in-vitro* cell free translation system. All other reagents were of highest purity available commercially.

RESULTS

Ovine luteinizing hormone (oLH) :

Purified oLH was subjected to extensive biological activity prior to its applicationin experiments. The apparent molecular weight was determined as 37kDa. Purified oLH was found to retain its immunoreactivity, receptor binding activity and steroidogenic potential compared to standard oLH.

Ribosome inactivating protein, Gelonin :

Gelonin, purified from the dry seeds of *Gelonium multiflorum* by three different methods. The ammonium sulfate fractionation method although yielded less amount of gelonin, but was completely devoid of non-proteinous material which absorbs at 280nm. Gelonin was further subjected to extensive physico-chemical, immunological and ribosome inactivating property studies. The molecular weight of gelonin was 30kDa and was homogeneous in nature. Purified gelonin showed high degree of reactivity against specific anti-gelonin antibodies. The ribosome inactivating property of gelonin was fully preserved after purification as shown by a cell-free translation assay.

Modification of oLH and gelonin :

Prior to conjugation of oLH with gelonin, the effect of modification of oLH and gelonin by the heterobifunctional cross-linking reagents, SPDP, LC-SPDP and 2-IT were studied. The modified αoLH derivative recombined fully with the βoLH to generate a complete hormone. Sequential modification of α- and β-subunits by cross-linking reagents led to progressive reduction in the immunoreactivity and receptor binding but the steroidogenic ability of βoLH-SPDP.αoLH was relatively comparable.

Modification of amino groups of gelonin led to progressive reduction in immunoreactivity property of gelonin. When the ribosome inactivating property was determined in a cell-free translation assay, like immunoreactivity, modification of a single $-NH_2$ group amounted to a loss of 90% RIP activity.

oLH-gelonin conjugates :

The conjugates of oLH to gelonin were prepared with the use of three types of heterobifunctional cross-linking agents, SPDP, LC-SPDP and 2-IT. Using SPDP/LC-SPDP four conjugates were synthesized having spacer arms of variable lengths ranging from 13.6A°-31.2A°. The conjugation mixture was subjected to extensive characterization to confirm its purity, homogeneity and bioefficacy. The gel-filtration chromatography shows that the oLH-gelonin conjugates were devoid of any ingredients. SDS-PAGE analysis done both under reduced and unreduced conditions using purified conjugates demonstrated that the conjugates were devoid of unreacted oLH or gelonin. Upon reduction of the conjugates, the disulfide bond between the oLH and gelonin was reduced and the oLH and gelonin were separated. RP-HPLC analysis performed on the conjugates clearly showed that the coupling occurs via the α-subunit of oLH. The conjugates retained hormone immunoreactivity and gelonin immunoreactivity and was comparable to native oLH and gelonin respectively. The receptor binding ability of the conjugate ranged from 1-4% when compared to the native oLH. The steroidogenic activity was well preserved. The protein

synthesis inhibitor activity of the conjugate was in the range of 60-80% as determined in a cell-free translation assay. The conjugates were shown to bind to the leydig tumor cells via the hormone leaving gelonin free as determined in a competitive displacement assay. In short, the hormonotoxins internalized to a sufficient degree to inhibit protein synthesis activity.

Role of positive charge :

Since the positive charge on the lysine residues of oLH plays an important role in the high affinity of oLH to its receptors and subsequently its biological activity, the oLH-gelonin conjugates were prepared with the use of 2-IT and were subjected to physico-chemical and biological characterization in order to assess its purity, homogeneity and bioefficacy upon internalization. SDS-PAGE analysis under unreduced conditions clearly showed the conjugate at a range of ~ 57kDa. and was devoid of any ingredients such as unreacted oLH, gelonin or modified oLH or modified gelonin. When subjected to immmunoreactivity studies, it is found that, single $-NH_2$ group modification of oLH by 2-IT resulted in a loss of 72% immunoreactivity, but after conjugation, this was

not much altered. Like immunoreactivity, receptor binding ability was also reduced by a single $-NH_2$ group modification prior to conjugation. But steroidogenic potential was preserved may be due to the fact that, even a 1% receptor binding is enough to elicit a response in terms of progesterone induction. Binding and and cytotoxic studies using competitive displacement analysis showed that the hormonotoxin conjugates bind to the receptor cells via the oLH part, leaving gelonin free. When oLH-gelonin conjugate synthesized by using 2-IT was compared for cytotoxicity with that of conjugates synthesized with SPDP/LC-SPDP, the earlier exhibited higher cytotoxic potential, possibly due to its ability to preserve the positive charge on the ε-NH_2 groups of ovine luteinizing hormone (oLH).

HORMONOTOXINS: SYNTHESIS, CHARACTERIZATION AND BIOEFFICACY OF SOME oLH-GELONIN CONJUGATES

By
ANIL KUMAR MAVILA

INSTITUTE OF SELF ORGANISING SYSTEMS AND BIOPHYSICS

A THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE
OF
DOCTOR OF PHILOSOPHY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG
INDIA
1995

TO

MY

ACHAN AND AMMA

पूर्वोत्तर पर्वतीय विश्वविद्यालय
पू० प० विवि० परिसर, शिलांग-७९३०२२ (मेघालय)
North-Eastern Hill University
NEHU Campus, Shillong-793022 (Meghalaya)

Phone :
Grams : NEHU

INSTITUTE OF SELF-ORGANISING SYSTEMS & BIOPHYSICS

CERTIFICATE

****

This is to certify that the thesis entitled "HORMONOTOXINS : SYNTHESIS, CHARACTERIZATION AND BIOEFFICACY OF SOME oLH-GELONIN CONJUGATES", submitted by Mr. ANIL KUMAR MAVIILA in fulfillment of the requirements for the degree of Doctor of Philosophy of North-Eastern Hill University, Shillong embodies the record of original research work carried out by him under my supervision. He has been duly registered and the thesis submitted is worthy of being considered for the award of the Ph.D. degree.

This work has not been submitted for any other degree to any other university or Institution.

Vinod Singh

Dated 14 December, 1994

Dr. Vinod Singh
Supervisor
Reader
Institute of Self-Organising Systems & Biophysics, NEHU, Shillong.

पूर्वोत्तर पर्वतीय विश्वविद्यालय
पू० प० विवि० परिसर, शिलांग-७९३०२२ (मेघालय)
North-Eastern Hill University
NEHU Campus, Shillong-793022 (Meghalaya)

Phone :
Grams : NEHU

INSTITUTE OF SELF-ORGANISING SYSTEMS & BIOPHYSICS

Pre-Ph.D. Course Grade Certificate
****

This is to certify that Mr. ANIL KUMAR MAVILA secured the grades mentioned below in the following Pre-Ph.D. courses.

	COURSES OFFERED	GRADES AWARDED
1.	Self-Organising Systems	A
2.	Methods of Science	A
3.	Living state and Biophysics of living organisms	A
4.	Condensed Matter Physics	O

RP Bajpai

Dr. R.P. Bajpai
Professor

for Director
Institute of Self Organising Systems & Biophysics, NEHU, Shillong.

CONTENTS

CHAPTER-THREE : RESULTS & DISCUSSION 67-150

PART-ONE : RESULTS

One who GIVES, shall dominate

- Professor R.P. Bajpai

ACKNOWLEDGEMENT

Words are literally insufficient for me to express my heart felt gratitude to Dr. Vinod Singh, under whose brilliant supervision I completed this research work. His most cheerful, simple and helping nature made me possible to achieve the goals of my research work. I am extremely grateful to him for the training he had offered me all these years for both academic and personality development.

I express my deep sense of gratitude to Professor R.P. Bajpai, who had taken special care to see that my research work is never hindered from the very first day of my arrival at Shillong. Apart from the excellent academic training, the encouraging and fruitful suggestions of Dr.Satish Kumar is gratefully remembered. I thank most warmly Dr. P.K. Bajpai, for his cheerful attitude and helpful suggestions all through these years. I thank Dr. (Mrs.) Debjani Roy and Dr (Miss) P. Nongkynrih for their constant encouragement and suggestions.

I am extremely grateful to Dr. Anis Alam at the Department of Biochemistry, who permitted me to work in his laboratory and devoted his valuable time for discussions and supervision, especially when Dr. Vinod Singh was out of station.

I am deeply indebted to Dr. Santosh Kar, Coordinator of Centre for Biotechnology, JNU, New Delhi, for permitting me to work in his laboratory for a long period and for the time he has spent for me for suggestions and guidance.

I am thankful to Dr. Victor Rebois of NIH, USA, for his generous gift of Mouse leydig tumor (MLTC-1) cells.

I am grateful to Dr. Harishchandra, Dr. B.B.P. Gupta, for their valuable comments about my research work.

I remember Mr. M. Kiran, my friend at JNU, New Delhi, as a rare blend of knowledge and kindness. It was alone his extreme helpful and inspiring nature that made my long stint at JNU, New Delhi very happy.

The affectionate concern of Prof. M.A. Sudhir, Prof. P. Gokulanathan, Dr. Y.S. Jain, Dr. P.N. Ram, Dr. M.K. Parida, Dr. Sameer Durani, Mr. Rajiv Kalra, Mr. Parthasarathi Adak, Mr. Nasirul Alam and experts of Waters India Ltd. are gratefully acknowledged.

I am deeply indebted to my friends, Mr. K.M. Dhananjayan, Mr. C.K. Anil Kumar, Mr. C.K. Mathew, Dr. B.G. Mathew, Mr. Shaji N. George, Mr. P. Divakaran and Mr. Wilfred for their help and encouragement during these years.

NEHU administrative Staff members Mr. R.C. Chyne, Mr. R.S. Choudhary, Mr. C.K. Singha, and Mr. Kushal Sinha, Mr. P. Unnirajan are gratefully remembered for their extreme helpful and kind nature.

I specially mention Miss. Jayati Sengupta, my colleague for her help during these years and especially at the time of preparation of thesis. Also I express my heart felt gratitude to my colleagues Mr. Vijay Kumar Sharma, Mr. Paul Sansan Dkhar, Mr. James Haorei, Mr. Biswajit Paul, Dr. (Mrs) Anjana Munshi, Mr. V.A. Sivadasan, Mr. Amerandar Sharma, Miss. Bijoy Lakshmi Borah, Miss. Priyoasha Chingakham and Miss. Bakor for their extreme helpful nature.

I am extremely grateful to Mr. Bruhaspathy, Mr. Sarat K. Dalai, Dr. Arshad Jilani and Miss. V. Radha at the Centre for Biotechnology, JNU, New Delhi for their help and suggestions.

It is my pleasure to express my heart felt gratitude to Mr. N. Bhattacharjee, Mr. N.S. Nongkynrih, Mr. Tarun K. Rakshit and Mr. T. Warjri and Mrs. Malina Wahlang for the help they offered me during the research work.

The financial assistance offered to me in the form of Junior and Senior Research Fellowships of the Council of Scientific and Industrial Research (CSIR), New Delhi is gratefully acknowledged.

I am extremely grateful to Mrs. Manju Singh for her constant encouragement and bless all through these years. In fact, many a times of distress during my stay at Shillong, Dr. Vinod Singh and his family members came as a savior to me. I sincerely express my gratitude and respect to them.

I am extremely grateful to Mr. T.H. Badwar and family who had offered me a very comfortable stay at their residence during my research work.

I remember my Parents, brothers, sister and sister in-law for their constant prayer for me, which made me optimistic in my career.

And as the moment demands, I pray and thank GOD who made me fit for this effort.

Dated 14 December, 1994 ANIL KUMAR MAVILA

ABBREVIATIONS

αoLH	:Alpha subunit of oLH
BMV	:Brome mosaic virus
βoLH	:Beta subunit of oLH
BSA	:Bovine serum albumin
CD_4	:Cluster of differentiation
CEA	:Carcinoembryonic antigen
CHO	:Chinese hamster ovary
CMC	:Carboxymethyl cellulose
CFA	:Complete freund's adjuvant
DNA	:Deoxyribonucleic acid
DTNB	:5,5'dithiobis(2-nitrobenzoic acid)
DTT	:Dithiothreitol
eCG	:equine chorionic gonadotropin
EF-2	:Elongation factor-2
EGF	:Epidermal growth factor
FPLC	:Fast protein liquid chromatography
FSH	:Follicle stimulating hormone
GnRH	:Gonadotropin releasing hormone

hCG	:Human chorionic gonadotropin
HEPES	:(N-[2-hydroxyethyl]piperazine N'-{2-ethanesulphonic acid})
HIV	:Human immunodeficiency virus
HPLC	:High protein liquid chromatography
IFA	:Incomplete freund's adjuvant
i.p	:intraperitoneal
2-IT	:2-iminothiolane
LC-SPDP	:N-succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexaonate
LH	:Luteinizing hormone (lutropin)
MLTC-1	:Mouse leydig tumor cells
mRNA	:messenger RNA
MSH	:Melanocyte stimulating hormone
PAP	:Pokeweed anti viral protein
PDP	:2-Pyridyldithiopropionate
RIA	:Radioimmunoassay
RIP	:Ribosome inactivating protein
RNA	:Ribonucleic acid
RNAse	:Ribonuclease
RP-HPLC	:Reverse phase-high performance liquid Chromatography

RPMI :Rosewell Park Memorial Institute

RRA :Radioreceptor assay

rRNA :ribosomal RNA

SDS-PAGE :Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis

SMCC :N-succinimidyl 4(N-maleimidomethyl)cyclo-hexane-1-carboxylate

SPDP :N-succinimidyl-3-(2-pyridyldithio)propionate

TCA :Trichloroacetic acid

TPCK :Tosylphenylalanine chloromethyl ketone

TRH :Thyrotropin releasing hormone

CHAPTER - ONE

INTRODUCTION

1.1: INTRODUCTION :

As a means of drug delivery, there has been much interest in attaching drugs to various carriers. Long ago P. Ehrlich advocated "*using bodies with a particular affinity for a certain organ..... as a carrier by which to bring therapeutically active groups to the organ in question*". During the past decade investigations in this direction have been intensified with a goal of developing cytostatic or cytotoxic agents as a treatment for certain cancers. The basis underlying this development rests on the fact that a number of substances can act as cytotoxins, mainly due to their ability to interfere with key elements of cellular activity namely, membrane integrity, energy metabolism, DNA/RNA or protein synthesis. A new strategy in cancer chemotherapy thus utilizes hybrid molecules possessing dual functions of recognition and cytotoxicity towards tumor cells. As antibodies (particularly monoclonal) are highly specific, many efforts are underway to attach them to drugs, such as daunomycin or plant toxins such as ricin-A subunit or single chain RIP such as, gelonin (Vitteta and Uhr, 1985). This idea is very attractive and

of potential value. Thus, an antibody carrying a covalently linked drug or toxin will convey the drug to the cell expressing the antigen against which the antibody is raised.

The pituitary and placental glycoprotein hormones of which LH, FSH, hCG and eCG are a part, constitute a family with unique structural features (Sairam 1983; Pierce and Parson, 1981). They are all similar hormones with distinctly different functions; LH and hCG are similar structurally and functionally. The hormone consists of two non-covalently linked subunits α and β, both of which are glycosylated. (Sairam, 1983; Pierce and Parson, 1981). The perfect union of the two subunits is absolutely essential for effective interaction with the receptor and expression of full biological activity. Methods for the preparation of the hormones and subunits from various species, including human are available. The complete amino acid sequence of the subunits and carbohydrate arrangement are known (Sairam, 1983; Pierce and Parson, 1981) While the α subunit within species are identical, the β subunit is designated as hormone specific because its interaction with the α-subunit determines hormonal specificity. Thus, the α-subunit in these hormones

can be interchanged, giving rise to a complex, which has hormonal activity and the specificity is dictated by the choice of the β-subunit (Sairam, 1983; Pierce and Parson, 1981).

Extensive investigations have been carried out to understand the role of different functional amino acid side chains in the α- and β-subunits in the assembly of hormone structures and biological actions, viz. receptor-binding, cyclase activation, intra cellular response etc. (Sairam, 1983). All these studies point out the critical importance of the full integrity of the α-subunit for complete biological activity. This common α-subunit is most sensitive to chemical modifications and thus the protein backbone must be preserved intact to generate a complex with full biological activity. In contrast, the hormone specific β-subunit particularly that of LH and hCG can tolerate many chemical alterations in the polypeptide moiety without seriously compromising biological functions. Thus the amino groups of LH and hCG β-subunits can be modified to produce a derivative whose ability to recombine with an intact α-subunit is not reduced and subsequently the complex

will interact with the receptor. This is mentioned in particular because this point is key to the development of toxic gonadotropins (only to gonadal cells), as done in this research thesis.

Among many compounds, that have been considered useful for immuno-conjugate therapy, daunomycin, an antibiotic and plant toxins have been studied the most. Daunomycin, a nitrogenous anthracycline glycoside, is believed to inhibit cellular activity by interaction with DNA. This drug has been attached to hormones such as MSH (Varga *et al*, 1977) and prolactin (Blossey *et al*, 1986) for possible application to melanoma cells and mammary cancers. Their cytotoxic actions are clearly receptor mediated, such as no toxic effects can be seen in cells such as 3T3 cells, which do not have MSH receptors (Varga *et al*, 1977).

The plant toxins are naturally occurring cytotoxins, which are extremely potent and exert their cytotoxic action at very low concentrations and in this regard are like the hormones which are biologically active at nM or pM concentrations. Many of these toxins from seeds are like the glycoprotein hormones, containing A and B subunits, which are

linked by a S-S bond. The B-subunit contains the structural information necessary for the interaction with virtually all cells (no specificity), but the toxic actions manifest only when the catalytically active A-subunit enters the cells. Upon entry, A-subunit interacts with the elongation factor-2 (EF-2) and shuts down the protein synthetic machinery, resulting eventually in cell death. The most interesting aspect of these toxins is that the isolated A-subunit is non-toxic by itself. When it is coupled with a carrier, such as a hormone or antibody, which can selectively interact with the respective hormone receptor or antigen on the cell membrane, entry of the A-subunit is facilitated. Once entry is gained into the cell, toxic action on protein synthesis are initiated.

The mode of action of ricin-A chain has been a topic of extensive investigations. Studies show that this toxic lectin has a single S-S- bond linking two functionally and structurally distinct N-glycosylated polypeptides each of approximately 30kDa. The A-chain catalytically inactivates the 60S subunit by the cleavage of a single N-glycosidic bond between adenine and the ribose in a

universally conserved loop structure in domain VII of 26S/28S rRNA. Using aniline cleavage assay and primer extension, it has been proved that ricin-A removes A_{3024} from yeast *(Saccharomyces cerevisiae)* 26S rRNA, and this removal shows a positive correlation with the inhibition of protein synthesis in cell free lysates (Osborn and Hartley, 1990). Studies have also shown that the high concentrations of eEF2 partially protect the ribosomes from the action of ricin A-chain, suggesting that they share a common binding site. Purified wheat germ eEF2 using aniline cleavage shows that it can protect wheat germ ribosomes from depurination.

Most of the toxins that have been considered for immunoconjugate therapy are the so called two-chain toxins, which are linked by an -S-S-bond. The other class of toxins called as single chain toxins deserve a great deal of importance. Gelonin and PAP are such plant toxins, which are non-toxic to intact cells, owing to the fact that, they lack a receptor binding domain. When gelonin was added in a cell-free translation assay using reticulocyte lysate system, the toxin inactivated the ribosomes, which is needed for

protein synthesis.

Many antibodies have been coupled to plant toxins to produce immunotoxins (Vitetta and Uhr, 1985). Several conjugates of ricin-A-subunits with hormones or growth factors or asialoglycoproteins have been prepared and tested (Simpson *et al*, 1982). To illustrate this strategy, the following examples may be mentioned: A conjugate of epidermal growth factor (EGF) and ricin-A subunit shows potent toxin effect on 3T3 cells, where as isolated ricin-A subunit was not active indicating that the toxic effect manifests only when the toxin is delivered into the cell by means of a receptor mediated membrane transport (Cawley *et al*, 1980). The EGF-ricin-A subunit conjugate was toxic to the mouse 3T3 cells analogous to as the whole toxin thereby indicating the power of this approach. In a similar study, TRH coupled to another toxin-A-subunit became toxic to GH3 rat pituitary cells in culture (Bacha *et al*, 1983).

Extensive investigations have been made to design and synthesize showing different carrier based immunoconjugates, their bioefficacy in *in-vitro* and *in-vivo* systems. Pre-clinical assessments of an immunoconjugate using ricin-A

chain and CHO anti-CEA monoclonal antibody against human colon cancer shows that the above conjugate has an improved therapeutic limit for human colon cancer and that it may have a direct relevance for i.p treatment of peritoneal carcinomatosis from colorectal cancers (Griffin *et al*, 1990).

In one study related to the glycoprotein hormones, Oeltman and Heath, (1979 a & b) linked the β-subunit of hCG to ricin-A subunit with an S-S-bond. The complex was toxic to the R2C cells from a Leydig cell tumor line, which have receptors for hCG but non toxic to mouse L-cells which lack hCG receptors. However, when compared to native ricin, the hCG-β-subunit-toxin-A hybrid was very weak even to Leydig cell carrying hCG receptors. The hybrid was less efficient than the native ricin in transporting the active A-subunit into the R2C cells to cause inhibition of protein synthesis.

The advent of heterobifunctional cross-linking resulted in the ability to design more specific coupling methods for cross-linking two proteins/peptides. Heterobifunctional cross-linking agents can be used to link two proteins in a

stepwise manner. This reduces the occurrence of unwanted side reactions of homopolymerization of the reacting proteins. The heterobifunctional reagents, SPDP [(N-Succinimidyl 3-(2-Pyridyldithio) propionate], LC-SPDP [(N-Succinimidyl 6-[3-(2-Pyridyldithio) propionamido] hexaonate] and 2 IT [2-iminothiolane] (Carlsson *et al*, 1978; O'Keefe and Draper, 1985; Jue *et al*, 1978; Marsh, 1988) have been to our interest. Others are MBS [m-maleimido benzoic acid], N-hydroxy succinimido ester, SMCC [N-succinimidyl 4(N-maleimidomethyl) cyclohexane 1-carboxylate] etc (Kitagawa and Aikawa, 1976; Youle and Neville, 1980; Yoshitake *et al*, 1979; Lambert *et al*, 1985).

The therapeutic efficacy of conjugates is likely to depend on a large number of intrinsic and physiological variables that affect both the stability of the conjugate and its ability to target tumor cells and induce selective toxicity. The question of *in-vivo* stability (immunogenecity, linker chemistry) of the conjugates has been of importance. In addition, several additional factors may be considered. In case of immunotoxins, the antigen may be shed into blood, antigen density may vary the antigen

modulation by target cells after interaction with conjugates; tumor cells may be antigenically heterogenous; the ability of immunoconjugates to penetrate tumor cell mass may vary etc. The ideal conjugate is that which can evade capture of host-immune system and can easily localize in solid tumors. To date no such construct exists.

Regarding the future prospects of improving the hormone-toxin or antibody-toxin conjugate, genetic engineering and art of development of new heterobifunctional agents stand on top. The latest development of new chemical cross linker shows that instability of -S-S- bond joining toxin to carrier is the major factor influencing the pharmacokinetics and the efficacy of these conjugates *in-vivo*. A newly designed linker (Cetus Corporation, 1400 Fifty Third Street, CA, USA) shows an improved half life of conjugate in mice, rats and monkeys. The efficacy was assessed by both intravenous administration in nude mice bearing subcutaneous MX-1 breast cancer implant, and locally in the mice bearing intraperitonial OVCAR-3 ovarian cancer cells.

Several other ideas for improving immunoconjugates using genetic engineering techniques have been proposed. Recombinant DNA technology could be used to construct a sequence that encode both antibody and toxin and to express as a single molecule thereby eliminating the conjugation step.

The manipulation of genetic material coding for proteins used as anti-cancer agents is becoming a more widely used tool in the development of novel hybrid proteins.

In short, the idea of using the specificity of hormone-receptor system to deliver drug/toxin to selected cells in the gonads is attractive and important. Although, sufficient knowledge of the biochemistry of the hormone and drug/toxin are available, very little effort has so far been made to selectively target the gonadal cells by hormonotoxins.

The experiments conducted here are logical and within the realm of possibility of extension. If the hormonotoxins are effective *in-vivo*, the HORMONOTOXINS may pave the way for the design of a new class of luteolytic agents in the near future.

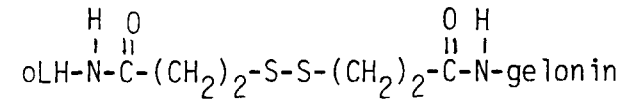
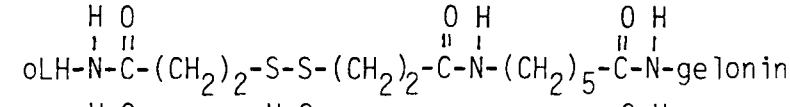
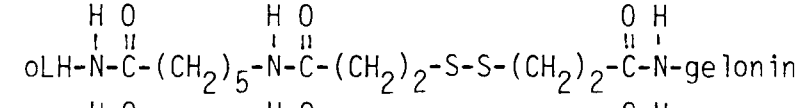
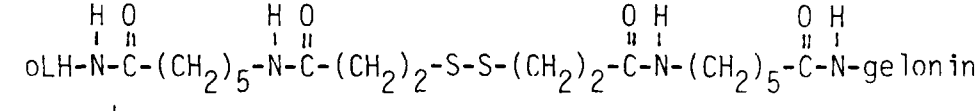
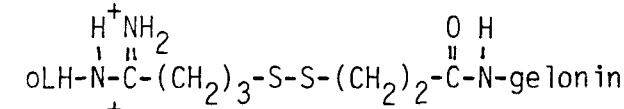
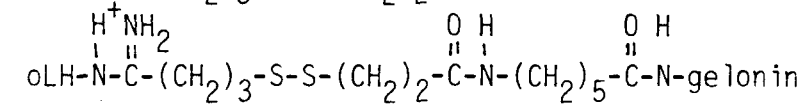
1.2: PROBLEM DEFINITION :

The success of immunotoxins, in the treatment of certain cancers (Nevile, 1987) encouraged us to use the similar strategy to link toxin to the gonadotropin and test them on the gonadal cells *in-vitro* and *in-vivo*. As hCG/LH receptors are highly localized on the gonadal cells and their action is mediated by internalization, the hormone carrying the toxin will be localized at the site and it would be released upon internalization. Once the hormone is internalized into the cell and the toxin is released by breaking -S-S- bond between the hormone and toxin, the toxin would terminate all key cellular activity resulting into complete cessation of cell function. Without adequate production of steroids, the reproductive events will be disrupted. If this strategy materializes and becomes effective *in-vivo*, a new class of luteolytic agents might become available. As the drug would be released only after the internalization of the conjugate, spurious toxic activity on other cells or in circulation, where gonadotropin receptors are not present (or not expressed) would be minimal.

The proposed structure of hormonotoxins to be synthesised with the use of different heterobifunctional cross-linking agents are shown in TABLE 1.1. The table also shows the activation of oLH/gelonin with different reagents prior to initiate the conjugation reaction. In oLH-S-S-gelonin conjugates, the cross-linking will be carried out by using SPDP following the procedures described in detail in the Methodology chapter (SECTION 2.7). Moreover, both SPDP and LC-SPDP will be used to design the conjugates of variable spacer arm length.

The role of positive charge on the ε-NH_2 groups of luteinizing hormone has been a subject of extensive investigation. It has been found that the retention of positive charge on the -NH_2 groups preserves the affinity of the hormone to its receptors and subsequently biological activity. Not all heterobifunctional cross-linking reagents preserve the positive charge on the reacting proteins during modification, but the exception being Traut's reagent (2-iminothiolane). In case of the hormonotoxins, where the positive charge on the lysine residues are retained (oLH^+-S-S-gelonin conjugates), the oLH will be activated with

TABLE 1.1: Proposed structures of the hormonotoxins to be synthesised with the use of different heterobifunctional cross-linking agents.

Conjugate/ Cross-linking reagents	Cross-linking agents used to activate		Structure of the conjugates
	oLH	Gelonin	
oLH-S-S-gelonin			
SPDP	SPDP	SPDP	
SPDP and LC-SPDP	SPDP	LC-SPDP	
SPDP and LC-SPDP	LC-SPDP	SPDP	
LC-SPDP	LC-SPDP	LC-SPDP	
oLH^{+}-S-S-gelonin			
2IT and SPDP	2IT	SPDP	
2IT and LC-SPDP	2IT	LC-SPDP	

2-IT to thiolate the $-NH_2$ groups by preserving the positive charge on the lysine residue and gelonin will be modified with SPDP-or LC-SPDP (TABLE 1.1). Mixing of oLH 2IT with SPDP/LC-SPDP activated gelonin will result into the conjugation of oLH to gelonin by liberating pyridine-2-thione. The concentration of which will be determined by measuring the absorption at λ_{343} nm (Carlsson *et al*, 1978). The role of positive charge of gelonin on the cytotoxicity of the conjugates will also be investigated in oLH-S-S-gelonin$^+$ conjugates. Such conjugation will be carried out by mixing of 2-IT activated gelonin with the SPDP/LC-SPDP activated oLH.

The conjugates synthesized above will be purified by gel-filtration chromatography on Sephadex-G-100 and the purified conjugates will be subjected to extensive physico-chemical, immunochemical and biological characterization. SDS-PAGE and RP-HPLC will be carried out to determine the purity of the conjugates and the site of conjugation. Receptor binding property of the conjugates will be determined by following the standard RRA protocol.

Polyclonal anti-gelonin and anti-oLH antbodies will be generated by immunizing Newzealand strain rabbits, with highly purified gelonin and oLH respectively.

Reactivity of anti-gelonin and anti-oLH antibodies to the hormonotoxins will be carried out by using competitive displacement analysis.

The ribosome-inactivating property of gelonin, modified gelonin and conjugates will be measured in a cell free translation assay by using rabbit reticulocyte lysate assay system according to the standard protocols.

Finally the binding property and the cytotoxicity test of the conjugates will be carried out using leydig tumor (MLTC-1) cells which express the gonadotropin receptors and in response to their binding they secrete progesterone.

It is expected that the successful completion of the above work may pave the way for the possible design of a new class of luteolytic agents.

1.3: REVIEW OF STATUS OF RESEARCH AND DEVELOPMENT :

The glycoprotein hormones from the pituitary (such as TSH, FSH and LH) and the placenta (chorionic gonadotropin) are composed of two non-covalently associated non-identical glycoprotein subunits (Sairam, 1983; Pierce and Parson, 1981). Each hormone has a common subunit, the α-subunit which within a species is identical in amino acid sequence and apparently the product of a single gene (Fiddes and Goodman, 1979, 1980; Talmadge *et al*, 1989; Boime *et al*, 1982; Jameson *et al*, 1986a&b; Carr and Chin, 1985; Boothy *et al*, 1983; Otani *et al*, 1988; Milsted *et al*, 1986; Matzuk and Boime, 1989). A unique β-subunit has been found specific for each glycoprotin hormone and has been shown to play the primary role in determining the hormone specificity, species specificity and potency of the intact heterodimer. The subunits are themselves in terms of functional roles determined for the glycoprotein hormone. However, there may be other functions under the control of individual subunits.

The complete amino acid sequence of the subunits are known. While the α-subunit within the species are identical, the β-subunit is designated as hormone specified because its

interaction with α-subunit determines hormonal specificity. Thus the α-subunit in these hormones can be interchanged, giving rise to a complex, which has hormonal activity, dictated by the choice of the β-subunit (Sairam, 1983; Pierce and Parson, 1981).

Several extensive studies have been carried out to understand the role of different amino acid side chains in the α- and β-subunits in the assembly of hormone structure and biological action, viz. receptor binding, cyclase activation and intracellular response (Gordon *et al.*, 1985; Sairam, 1983). These studies have indicated that the α-subunit plays an important role in the biological activity. It is further demonstrated that the hormone specific β-subunit, (particularly that of LH and hCG) can tolerate many alterations in the polypeptide moiety without seriously compromising biological functions (Gordon, 1985, Sairam, 1983). Therefore, it is concluded that the amino groups of oLH β-subunits can be modified to produce a derivative which can effectively recombine to the intact α-subunit and subsquently the annealed subunit complex will interact efficiently with the receptor.

One of the most interesting and exciting developments in the biochemistry of LH, hCG and FSH are the recent spate of studies, which showed the crucial role of carbohydrate moiety in biological functions (Sairam, 1983). By chemical or enzymatic means, it is possible to remove 75-80% of the sugar without altering the protein part of either subunit. The quarterary structure of hormone is maintained. The modified hormones designated as DG-hormones (de-glycosylated) bind to the receptor with very high specificity and in many instances with greater affinity to ovarian and testicular cells (Sairam, 1983; Sairam and Bhargavi, 1985). Despite this enhanced binding to the receptors, the biological activity of DG-hormone in producing cellular response is very poor (Sairam, 1983). Thus the receptor binding can be dissociated from cellular activation. Because of these differential characteristics, the DG-hormones behave as potent antagonists of the action of native hormone in *in-vitro* and *in-vivo* situations (Sairam, 1983). Studies have also shown that glycosylation of α-subunit in LH and FSH (and probably hCG) are key to signal transduction (Sairam and Bhargavi, 1985). This means that it is adequate to deglycosylate the α-subunit

alone and then combine with a fully glycosylated β-subunit to produce as antagonistic derivative. If this idea is successfully implemented in designing a hormone-toxin conjugate using a deglycosylated α-subunit of hormone, it would result in a "DOUBLE MAGIC BULLET" against gonadal cells.

A variety of biological organisms make toxic substances that act on mammalian cells and cause deleterious changes, ranging from metabolic abnormalities to immediate cell death. Of late is a family of protein toxins made by plants and bacteria that act within cell cytoplasm to inhibit protein syntesis. Among the toxins, the important being Diphtheria toxin, Pseudomonas exotoxin, ricin, gelonin etc. (TABLE 1.2).

Some of the common properties of toxins make them attractive for the use in cancer therapy. These toxins are enzymes and they have proved to be potent cell-killing agents. A single molecule delivered to cytoplasm will catalytically inactivate the ribosome and eventually kill the cell. Most of the toxins, that have been employed for the construction of immunoconjugates are composed of two functionally distinct protein chains, the A chain and B chain. A chains are single polypeptides that exhibits cell killing

TABLE 1.2: Toxins of interest for targeted toxin therapy

TOXIN	SOURCE	EFFECT ON INTACT CELL	BINDING TO CELL	CATALYTIC EFFECT
Gelonin	*Gelonium multiflorum*	Non-toxic	NO	Inactivates ribosomes
PAP[1]	*Phytolacca americana*	Non-toxic	NO	Inactivates ribosomes
Ricin	*Ricinus communis*	Toxic	Yes	Inactivates ribosomes
Abrin	*Abrus precatorius*	Toxic	Yes	Inactivates ribosomes
Modeccin	*Adenia digitata*	Toxic	Yes	Inactivates ribosomes
DT[2]	*Corynebacterium diphtheria*	Toxic	Yes	ADP-ribosylates EF-2
PE[3]	*Pseudomonas aeruginosa*	Toxic	Yes	ADP-ribosylates EF-2

1:Pokeweed antiviral protein
2:Diphtheria toxin
3:Pseudomonas exotoxin

property by shutting down protein synthesis (Stripe *et al*, 1980). For example, the A chain of plant toxin ricin can inactivate upto 1500 ribosomes per minute, by removing a single adenine residue from the 28S ribosomal RNA (Endo *et al*, 1987). B-chains, bind to the cell surface and mediate translocation of A-subunit into the cytosol (Youle and Colombatti, 1986). For instance, diphtheria toxin is endocytosed and when exposed to low pH, hydrophobic domains on the B-chain are inserted into the endosomal membrane. This insertion facilitates the transfer of A-chain into the cytosol, presumably through pore formation by B-chain (Zalman and Wisinesky, 1984).

The mechanism by which RIPs inactivate 60S subunit was investigated (Endo *et al*, 1987, Endo and Tsurugi, 1987). In the beginning the mechanism was studied using ricin-A chain but it is now shown that it is operative for all plant RIPs (Endo *et al*, 1987). Ricin-A-chain is RNA specific N-glycosidase that hydorolytically cleaves a single N-glycosidic bond from among that over four thousand nucleoside residues present in 28S rRNA. The adenosine-ribose bond at the nucleotide 4323 (in case of rat liver 28S rRNA) is

cleaved which forms a surface exposed stem-loop structure (Endo *et al*, 1987, Endo and Tsurugi, 1987). RIP-depurinated ribosomes are deficient in two protein synthesis partial reactions: the formation of 80S initiation complex during inhibition which is slowed down in the order of six folds and in the translocation step of the elongation cycle.

The site-directed mutagenesis using molecular biology has recently been used to eliminate altogether/or reduce the non-specific toxicity of ricin and other similar toxins by deleting or altering the binding unit of the protein (Vitetta *et al*, 1987, Pastan *et al*, 1986, Greenfield *et al*, 1987). Even more exciting prospects to be considered in the future of possibility of generating fusion protein incorporating essential features of the carrier and toxin component. This is demonstrated in a publication in which a genetically engineered fusion protein containing the soluble form of CD4 protein (receptor of T-cell) was fused with Pseudomonas exotoxin, a protein active region to obtain a product which was active in killing of HIV infected cells (Choudhary *et al*, 1988). The scenario in this instance was internalization of toxin via CD4 and killing by the internalized exotoxin.

1.4: PROTEIN MODIFICATION: AT A GLANCE :

With roots in ancient formulations, methods for the chemical derivatization of proteins continue to expand and develop. The tremendous growth in publications and creation of new journals in the area of protein modification itself is an indicator of the scope and promise of this area with its possible pharmacological and medical diagnostic applications, apart from its influence to understand the fundamental questions in biochemical structure and function.

Protein modifications in the past often involved the same organic chemistry reagents i.e acetylation, iodination, deamination reaction with formaldehyde etc., but the scene is changed drastically now: we have specific reagents meant for specific purpose in protein chemistry. These specialised reagents include photoaffinity labels and affinity labels and other site directed reagents (Colman, 1983; Bayley, 1983) group selective reagents that are exclusively reactive for the specific chain of amino acids and some other agents reacting non-specifically [with no specificity] (Knowles, 1972).

The reagents have been designed to preserve electrostatic charge (Hunter and Ludwig, 1962; Means and Feeney, 1968), to alter the electrostatic charge (Goldstein *et al*, 1964), and to increase electrophobicity (Nishikawa *et al*, 1986, Ampon and Means, 1988). Reagents and procedures have been developed to decrease immunogenecity (Abuchowski *et al*, 1977; Veronese *et al*, 1985), to increase and decrease the susceptibility of proteolysis (Raftery and Cole, 1966; Dixon and Perham, 1968; Rice *et al*,1977), to decrease UV or visible absorbancy (Parkinsons and Redshaw, 1984).

The chemistry of protein had its origin in the chemistry of amino acids and only later concerned the amino acid side chains of intact proteins. For practical purpose a variety of reagents have been developed and used prior to any significant understanding of protein chemistry. Interest in quantitative determinations of protein and their modified constituent amino acid s was a major impetus for many early studies of chemical modification of protein.

By the end of World War II, the interest had turned in determining the role of particular amino acid residue for its specific biological activity of protein. This was identified

by reactions with selective chemical reagents. (For reviews on protein modification in 1947, see Olcott and Frankel-Conrat, 1947, Herriot, 1947). Subsequent developments resulted in the design and use of chemical reagents for site-specific modifications (Wofsy *et al*, 1962) and sooner the compound TPCK was developed (Schoellmann and Shaw, 1963) thereby providing a direct evidence for the presence of histidine in the active centre of chymotrypsin. TPCK was shown to effect the modification of a particular histidine residue of chymotrypsin with the complete elimination of catalytic activity. The strong affinity for a particular site concentrates a reactive group like the chloromethyl ketone moiety of TPCK at a specific site, where its reaction with a nearby amino acid chain is promoted by mutual proximity.

The advent of side chain selective reagents (those react under certain specified conditions, with a single or at least a limited number of side chain groups in a fairly predictable manner) was however a simpler development. Numerous reagents recognising the amino (Lysine), carboxy (Asp, Glu), guanidino (Arg), imidazole (His), indole (Trp), phenol (Tyr), thiol (Cys-SH), thioether (Met) are leading in

the list. The disadvantage was the loss of essential activity of a given protein upon the treatment with these reagents, but this often was taken as an evidence for the need of group modified for biological activity.

The spectroscopic fluorescent labels has also been developed to characterise the structural features of protein (Hudson and Weber, 1973, Lacowics, 1983).

1.5: CROSS-LINKING OF PROTEINS :

Cross-linking of proteins have a long and important history. It is some times employed to increase the stability of proteins or of certain conformational relationships in proteins to couple two or more different proteins (eg. to join different activities into a single molecule) to identify or characterize the nature and extent of certain protein-protein interactions and in other cases, to determine the distance between two reactive groups in or between two protein sub-units (Han, *et al*, 1984, Staros, 1988; Wold, 1972; Wang and Richards, 1974; Uy and Wold, 1977; Das and Fox, 1979; Ji, 1983).

A large number of different types of cross-linking agent or as they are called, bifunctional reagents have been described. They include the so called zero length cross-linking agents that bring about direct formation of covalent bonds between existing amino acid chain groups. The use of water soluble carbodiimides bring about the formation of amide linkages between the carboxyl groups or and the ε-amino groups of lysine side chains appear to be most prominent zero-length cross-linking agents (Uy and Wold, 1977; Weare and Reichert, 1979; Waldmeyer and Bosshard, 1985; Willing *et al*, 1989). Disulfide bonds obtained from existing thiol groups would also be considered zero-length cross-links (Korodi *et al*, 1986; Huston, 1988). Such linkages appear to be formed only when the reacting groups are in close proximity.

Other cross-linking agents may be organized according to the type(s) of reactive groups, their side chain reactivity, their hydrophobicity or hydrophilicity and length or distance between the reactive groups, whether the reactive groups are same or different (homobifunctional or hetero- bifunctional reagents), whether the structure connecting the reactive groups is readily cleavable and whether the groups are

membrane permeable or impermeable and many other criteria. A list of most widely used types of cross-linkers are given in TABLE 1.3. A much more extensive list of cross-linking agents has been presented by Ji (Ji, 1983).

The reactivities of cross-linking agents, except for one or two special cases are very similar to those of corresponding monofunctional reagents. The initial reaction is presumably in most cases, a simple second order process, not affected by second reactive groups. The latter's reaction, however is completely dependent on the availability of a second appropirate side chain, which for fast efficient cross-linking must be both nearby and in proper orientation. Cross-linking agents with different lengths, different stereochemical configuration (some with little and others with great deal of conformational flexibility) and with different side chain specificities have been developed to fulfill different needs.

Of the 20 or so amino acids, normally present in protein side chains, lysine residues (specifically, ε-amino groups of lysine), are usually among the most abundant and most accessible of the potentially reactive groups. A relatively

large proportion of the most commonly used cross-linking agents are therefore amino group selective reagents (i.e., imidoesters, N-hydroxysuccinimide esters, activated arylfluorides etc.). Most of them undergo fairly rapid hydrolysis, in addition to their reaction with amino group, which seriosuly limits the yields of the modified protein.

A large number of heterobifunctional cross-linking agents have been developed which usually contain a thiol reactive and an amino reactive moiety. Among the thiol reactive groups, N-alkyl or N-arylmaleimide groups and in amino reactive groups, N-hydroxysuccinimides are the front-runners (Staros, 1982). In addition to the reactive groups, a variety of different types of connective structures or spacer arms have been employed. The nature of spacer-arm has special consequences. Longer spacer arms are assumed to be more effective for coupling to larger proteins or those where the reactive side chains are sterically protected.

Heterobifunctional cross-linking agents are particularly useful for conjugating two proteins. The different side chain reactivities of the two reactive groups, for example, usually permit to carry out the entire reaction of coupling in a

step-wise manner, which allows in some cases, for partial purification and if desired characterization of intermediates prior to the actual conjugation. Due to the hydrolytic instability of the most important groups directed at amino side chains, the first step usually involves addition of the cross-linker to the amino groups of one member of the future hybrid pair. The removal of unreacted or hydrolyzed reagent and other substances are possible at this stage. The resultant derivative is then coupled directly to the thiol containing member of the intended hybrid pair.

An artificial ricin-antibody pair has been prepared following the above way of protein conjugation (Youle and Neville, 1980). This was followed by the synthesis and characterization of different protein conjugates using various heterobifunctional cross-linking agents.

Several reagents have been employed to introduce thiol groups into proteins, which may be employed for conjugation to other proteins or various other bioactive substances. SPDP, LC-SPDP, 2-IT (Carlsson *et al*, 1978; Jue *et al*, 1978) etc., can be used under mild conditions to introduce thiol groups into proteins (TABLE 1.3). Several 2-pyridyl

TABLE 1.3: Heterobifunctional reagents for chemical cross-linking of proteins of interest

CROSS-LINKING REAGENT	DESCRIPTION	REFERENCE FOR DETAILS
SPDP[1]	A water insoluble solid; must be dissolved in a organic solvent, initial reaction with amino compound then to coupling to a thiol component at pH 7.0 or above or treatment with DTT followed by coupling; 7.6A° spacer length.	*Biochem. J.*, *173*, 723-738, 1978; *J.Biol. Chem*, *260*, 932-937, 1985
LC-SPDP[2]	Same as that of SPDP; 13.6A° spacer length.	
2-IT[3]	A water soluble solid; reacts only with amino group at pH 7.0-10.0 without eliminating their charge. May be coupled to to SPDP, LC-SPDP, SMCC or MBS treated proteins; 8.0A° spacer length.	*Biochemistry*, *17*, 5399-5406, 1978; *Biochemistry* *13*, 2334-2340, 1974
SMCC[4]	A water insoluble solid; reaction characteristics are similar to MBS; 12.0A° spacer length; more water soluble sulfosuccinimide derivative ester derivatives also available	*Eur. J. Biochem.*, *101*, 395-399, 1979; *J.Biol.Chem*, *260*, 12035-12041, 1985

1: N-succinimidyl-3(2-pyridyldithio)propionate
2: N-succinimidyl-6-[3-(2-pyridyldithio)propionamido]hexanoate
3: 2-iminothiolane
4: N-succinimidyl 4(maleimidomethyl)cyclohexane 1-carboxylate

disulfide-protein conjugates have been prepared and tested for its bioactivity. The susceptibility of disulfide linkages to cleavage by low molecular weight thiols, however appears to preclude many applications of such conjugates, including those involving exposure to physiological conditions.

1.6: HORMONOTOXINS/IMMUNOTOXINS :

The idea of constructing an antibody-toxin or hormone-toxin complex has vibrated the entire immunological research scenario with an optimistic touch. The conjugate of ricin to various hormones, growth factors or asialoglycoproteins have been prepared and tested. Asialofetuin-ricin A conjugate which binds to hepatocytes through the galactose specific receptors was toxic in cultured rat hepatocytes (Simpson *et al*, 1982). A conjugate of EGF and ricin-A showed potent cytotoxicity to the 3T3 cells, where as ricin-A alone was not active (Cawley *et al*, 1980). Human transferrin-ricin-A chain was highly active to human leukamia cells (Raso and Basala, 1984). In an analogous study, thyrotropin releasing hormone (TRH) coupled to diphtheria toxin subunit-A became toxic to GH3 rat pituitary cells in culture (Bacha *et al*, 1983). In one study related to

glycoprotein hormones, Oeltman and Heath in 1979, linked the β-subunit of hCG to the ricin A-chain via disulfide bond (Oeltman and Heath, 1979, a&b). The complex was toxic to R2C cells to cause inhibition to protein synthesis. Unfortunately, the most serious flow in the design of these studies was that the important α-subunit was excluded altogether (Oeltman & Heath, 1979 a&b). The recent studies reveal the critical role of α-subunit in the binding of hCG (and other glycoprotein hormones) to the receptors. Thus it is not surprising that the omission of the α-subunit from the recombinant was responsible for the low inhibitary activity of the conjugate. But our work has taken into consideration the importance of α-subunit before constructing the hormonotoxin.

To overcome the non-specific toxicity owing to the B-chain of the two chain toxins mentioned above, many investigators have explored the possibility of utilizing plant toxins that have only a single chain that inactivate ribosome (Barbieri & Stripe, 1986) and the status of gelonin (Stripe *et al*, 1980) gains momentum in this context. Other RIPs of this category are PAP (Ramakrishnan & Houston, 1984a), saporin (Thorpe *et al*, 1985; Cavallaro *et al*, 1993; Barthelemy *et al*,

1993). Because gelonin is isolated from an Indian plant *Gelonium multiflorum*, which could be obtained in abundance here and toxicity excercised in the test system was relatively comparable (Lambert *et al*, 1985), gelonin is used in the present thesis.

To bring this research problem into the perspective of current research arena, it should be indicated that the concept of targeting toxic drugs to certain tumors in the body has opened a new era in the development of potential therapeutic agents for cancer treatment (Ghose *et al*, 1987; Vitetta *et al*, 1987; Vitetta and Uhr, 1985). The idea of magic bullet has now been utilised in the construction of hybrid compounds called as immunotoxins (Vitetta *et al*, 1987; Vitetta and Uhr, 1985) where target specific antibodies were coupled to a variety of toxins of either bacterial or plant in origin (Pastan *et al*, 1986). The high specificity of antibodies were generally utilized to deliver toxins to the specific cells, where they could exert their extreme cytotoxic potential. Most of the work in immunotoxin field has concentrated in utilizing potent toxins like ricin and diphtheria toxin (Vitetta *et al*, 1987; Vitetta and Uhr, 1985, Pastan *et al*, 1986). As mentioned

above, all these toxins belong to the class of two chain RIPs (referred as A and B chain), where the A chain is responsible for the cytotoxic activity but, the cell binding capacity lies with the B chain. Since, the non-specific toxic effect due to the binding of B chain in almost all cells is a potential complicating factor, numerous efforts are underway to utilize only the toxic A chain using protein chemistry tools and later molecular biology has allowed the reconstruction of new immunotoxins that combine the high potency of toxin to the tumor cells, while being non-toxic to normal cells (Vitetta *et al*, 1987, Greenfield *et al*, 1987). A key element in such design is the elimination of cell receptor binding site in the B chain by site directed mutagenesis without seriously compromising its function in mediating entry into the cell (Vitetta *et al*, 1987; Greenfield *et al*, 1987).

An alternative to the two chain toxins have been to employ single chain RIPs, like saporin or gelonin as these toxins are non-toxic to the intact cells (Stripe and Barbieri, 1986; Cavallaro *et al*, 1993; Barthelemy *et al*, 1993). The hormone-toxin conjugate also referred as

HORMONOTOXINS, analogous to immunotoxins, as the hormone component of the construct provides the specificity for the toxic action of the conjugate. As receptors of hormone, such as gonadotropins are highly localized in the body, than that of other metabolic hormones, targeting of toxin to specific gonadotropin receptors stand at a better possibility.

By carefully scaning the cross-linking section of this review, one could easily identify the scope of specific reagents for specific functions. As a prelude to the research work, we have first investigated the effect of SPDP reaction on oLH and could demonstrate the following:-

(a) It is possible to introduce the sites for cross-linking in the α-subunit of the hormone without disrupting the quarternary structure of the hormone.

(b) The β-subunit remains unaltered.

(c) The hormone, with one or two modified lysine, retains appropriate receptor binding activity and virtually complete biological activity which is to be a useful carrier of the toxin. This is mentioned in particular because, one has to do careful studies

with these modifications (with other gonadotropins) while preparing the conjugates of gonadotropin to gelonin or with other toxins.

In designing a hormonotoxin conjugate, gelonin was found suitable for us as it is available in sufficient quantity and is highly effective in terminating protein synthesis, but relatively easy to isolate. We have adopted three different menthods with a slight modification and achieved highest yield (described in detail in methodology section).

CHAPTER - TWO

MATERIALS AND METHODS

2.0: MATERIALS :

2.0.1: Ovine luteinizing hormone (oLH) :

oLH was isolated from the lyophilized sheep pituitary powder as described in SECTION 2.1.

2.0.2: Ribosome inactivating protein, Gelonin :

Dry seeds of *Gelonium multiflorum* belonging to *Euphorbeaceae family* were purchased from United Chemicals and Allied Products, Calcutta. Gelonin was isolated from the dry deshelled seeds of *Gelonium multiflorum* using aqueous extraction, ammonium sulfate precipitation, cation-exchange and finally gel-filtration chromatography (Stripe *et al*, 1980; Singh and Sairam, 1989). Gelonin used in the present investigation was judged to be over 98% pure, as analysed by RP-HPLC and retained its ability to inhibit protein synthesis in an *in-vitro* cell free translation system.

2.0.3: Reagents :

Heterobifunctional cross-linking reagents (SPDP, LC-SPDP and 2-IT) and DTT were obtained from PIERCE, Rockford, USA. Sephadex G-25, G-100 and CMC-52 were purchased from Pharmacia Fine, Chemicals, Sweden. BSA (Cohn fraction IV), FCA and IFA were obtained from Sigma, Chemicals St. Louis MO. Radioactive Iodine ($Na^{125}I$, IMS-30) was obtained from Amersham,

Corporation. L-(3,4,5-^{3}H)leucine of specific activity 143 Ci/mmol was purchased from Dupont-NEN. All other chemicals were of highest purity available commercially.

2.0.4: oLH and gelonin antibodies :

Polyclonal antibodies against oLH and gelonin were developed in rabbit by immunizing them following the protocol described earlier (Singh *et al*, 1989; Stripe *et al*, 1989). Sera were collected after the appropriate booster injections and the immunoglobulin fraction was obtained after ammonium sulfate precipitation (15,24). The immunoglobulin fractions were dialysed extensively, lyophilized and stored as powder at 4°C.

2.0.5: Mouse leydig tumor cell line :

Mouse leydig tumor cell line, MLTC-1 used in the present investigation was gifted by Dr. R. Victor Rebois, NIH, Bethesda. The cells were propagated and maintained in culture according to the published procedure (Rebois, 1982). The cells grown in 24 multiwell plates were used to determine steroidogenic and cytotoxic activity of the hormonotoxins.

2.1: Methods :

Purification of oLH :

oLH was purified from the sheep pituitary powder according to the procedure described in literature (Bousfield and Ward, 1984; Ward *et al*, 1967; Glenn *et al*, 1981). The purified hormone was subjected to physico-chemical, immunological and biological characterization especially for receptor binding and steroidogenic activity prior to use for covalent coupling to gelonin. The hormone was stored as dry powder at 4°C. This preparation was used for the subsequent experiments during the study.

2.2: Modification of oLH and its subunits :

2.2.1: SPDP modification of α and β subunits of oLH :

The SPDP modification of ovine αLH and ovine βLH was carried out in 0.1M sodium phosphate buffer, pH 7.5, containing 0.15M NaCl (reaction buffer). Typically, the subunit dissolved in reaction buffer was mixed with different molar concentrations of SPDP (dissolved in minimal volume of absolute ethanol). The reaction mixture was kept for 30min at room temperature and passed through Sephadex G-25 column, equilibrated and eluted with PBS buffer. The SPDP modified derivatives were eluted with 0.1M sodium phosphate buffer (pH

6.0, containing 0.15M NaCl). The number of ε-NH_2 groups modified by SPDP was determined by the liberation of pyridine-2-thione after the treatment with 50 times excess of DTT. Pyridine-2-thione thus released was quantitated by using extinction coefficient $\varepsilon_{343}=8.08\times10^{3}M^{-1}$ (Carlsson *et al*, 1978).

2.3: Recombination of modified oLH subunits :

The recombination experiments were performed in 0.05M sodium phosphate buffer (pH 7.2, containing 0.01M NaCl). Equal amounts by weight of αoLH, αoLH-SPDP were mixed with the native βoLH and the mixture was left at room temperature for 24 hr. The recombination mixture was fractionated on Sephadex G-100 column using 0.05M ammonium bicarbonate. Similarly βoLH-SPDP derivatives were mixed with native αoLH and allowed to recombine. The mixture was fractionated as described above. αoLH, αoLH-SPDP and their recombinants were also subjected to RP-HPLC on a Water's μBondapak phenyl column using a continuous gradient as described earlier (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991 a&b; Singh and Das, 1991).

2.4: Purification of gelonin :

Ribosome inactivating protein, gelonin isolated from an Indian plant *Gelonium multiflorum*, of *Euphorbiaceae* family has

been used for the synthesis of oLH based hormonotoxins. The toxin was purified by using three different methods as described below.

2.4.1: Method I :

Gelonin was isolated by the method originally described by Stripe *et al* (Stripe *et al*, 1980) with minor modifications as described by Singh and Sairam (Singh and Sairam, 1989). The deshelled seeds were soaked overnight in the extraction buffer (0.05M sodium phosphate buffer, pH 7.2 containing 0.1M NaCl) and blended with ten volumes of the extraction buffer. The smooth slurry stirred overnight at 4°C, was centrifuged in the cold and precipitate was re-extracted with the same buffer. The supernatant of both extractions were dialysed extensively against 0.05M sodium phosphate buffer (pH 6.5, without NaCl) and passed through CMC-52 column (3.2x40cm). The column was extensively washed with the equilibration buffer until eluate showed absorbance less than 0.05 at 280nm and the bound material was eluted by applying a linear gradient of 0-0.30M NaCl in the same buffer at a flow rate of 30ml/hr at 4°C. The protein eluting between 0.15-0.20M NaCl gradient was pooled, concentrated and subjected to gel-filtration chromatography on a pre-calibrated Sephadex G-100 (3.4x110cm) column. The column

was washed with 0.05M ammonium bicarbonate. The protein eluting at ~30 kDa was collected and lyophilised directly. The lyophilized protein was rechromatographed on the same G-100 column.

2.4.2: Method II :

As described above, the deshelled seeds were soaked with four times of extraction buffer (0.05M sodium phosphate buffer containing 0.01M NaCl and 0.001M EDTA) and blended in the presence of seven times more of extraction buffer. The recovery of supernatant and re-extraction were the same as described in the Method I. The supernatant was precipitated with ammonium sulphate and the precipitate of 45-95% fraction was dissolved in 0.01M sodium phosphate buffer (pH 6.5) containing 0.02M NaCl and dialysed extensively against the same buffer. The dialysed protein was centrifuged to remove the precipitated protein prior to its passing through CMC-52 column. Bound material was eluted by applying 0.02-0.30M NaCl gradient in the same buffer. Further purification was carried out on the gel-filtration column as described above for the Method I.

2.4.3: Method III :

In this method, gelonin was isolated by a single step procedure using Cibacron blue F_3GA-Sepharose column (Sreenivasan *et al*, 1985). Cibacron blue was coupled to Sepharose by the methods described earlier (Hughes *et al*, 1982). The deshelled seeds were homogenized with ten volumes of 0.01M sodium phosphate buffer (pH 7.2) containing 0.10M NaCl. The slurry was centrifuged and the supernatant was dialysed extensively against 0.01M sodium phosphate buffer (pH 7.2, without NaCl). The supernatant was passed over F_3GA-Sepharose column and washed with 0.01M sodium phosphate buffer (pH 7.0). The bound protein was eluted with a gradient of NaCl (0.0-1.0M) in the same buffer. The appropriate fractions were pooled, concentrated, fractionated and re-fractionated on a pre-calibrated Sephadex G-100 column as described in the above methods.

2.5: Modification of $-NH_2$ groups of gelonin :

The modification of gelonin by SPDP was carried out by the standard procedure as described (Singh and Sairam, 1989; Carlsson *et al*, 1978). The gelonin was reacted with SPDP in molar ratio varying from 1:1 to 1:30 (mole/mole) in the initial reaction. In a typical reaction, gelonin dissolved in

sodium phosphate buffer (0.10M, pH 7.5, containing 0.15M NaCl) was activated with SPDP at varying concentrations and the modified gelonin was passed through Sephadex G-25 (1.5x30cm), using 0.05M ammonium bicarbonate. The protein peak was pooled and lyophilized directly. The extent of amino group modification was determined on the basis of liberation of pyridine-2-thione upon DTT treatment which has λ_{max} at 343 nm. The UV-VISIBLE spectra were recorded and the concentration of pyridine-2-thione was calculated by using extinction coefficient of $\varepsilon_{343}=8.08x10^{3}M^{-1}$. The absorption at 280nm was also corrected for pyridine-2-thione contribution (Carlsson *et al*, 1978).

2.6: Liposome entrapment of gelonin :

2.6.1: Preparation of liposomes :

Three different lipid compositions were used in the preparation of liposmes. Egg PC : CHOL : DCP; DPPC : CHOL : DCP; DMPC : CHOL : DCP. The lipids were taken at a molar ratio of 1.0 : 0.9 : 0.25 and dissolved in 2ml of chloroform and kept in a 10ml round bottom flask. The flask was hand shaken for a few minutes to ensure the complete solubility. The aqueous solution of gelonin (2mg/ml) in 0.01M tris-buffered saline pH 7.4 was added dropwise to the liquid

dispersion. During addition, the flask was hand shaken to avoid frothing or formation of chloroform : H_2O emulsion. The round bottom flask was then fitted with a Buchii type rotary evaporator. The chloroform was removed by applying vacuum and raising the temperature of water bath to 30-35°C. At this temperature, the gelonin neither looses its immunological or ribosome-inactivating property. As a control experiment, 2ml of $CHCl_3$ was mixed with equal concentration of gelonin and all other treatments were carried out as mentioned in the liposome preparation except the addition of lipids. The remaining traces of $CHCl_3$ was removed during the dialysis and gel-filtration chromatography.

2.6.2: Separation of liposomes from free gelonin :

Liposomes were separated from the unentrapped gelonin using following methods:

a. Centrifugation :

Liposomal suspension prepared as mentioned above was centrifuged at 14,000rpm at 4°C for 30 min. in a refrigerated centrifuge (SORVAL RC-5C). The supernatant was carefully aspirated and the pellet was washed for 2-3 times with the same buffer. The supernatant was pooled together and concentrated by ultra filtration on YM-10 filter (Amicon) and

subjected to FPLC and SDS-PAGE analysis to determine molecular weight and homogeneity.

b. Gel-filtration on Sepharose CL-4B column :

After complete removal of solvent, the liposomal suspension was passed through a Sepharose CL-4B column (30x15cm) equilibrated with tris-buffered saline (10mM, pH 7.4 containing 0.02% sodium azide). The column was developed with the same buffer. Fractions were collected and read at 280nm.

2.7: Conjugation of oLH with gelonin :

2.7.1: Conjugation by using SPDP/LC-SPDP :

To initiate conjugation, the SPDP/or LC-SPDP modified gelonin in 0.1M sodium phosphate buffer, pH 6.0 containing 0.15M NaCl was treated with DTT to generate -SH group and then the treated mixture was passed through a Sephadex G-25 column to remove small molecular weight reduced products. Prior to G-25 chromatography, the UV-VISIBLE spectra was recorded in order to determine the number of -SH groups generated as described above. Gelonin-SH was then mixed with oLH-SPDP/or oLH-LC-SPDP in 0.10M sodium phosphate buffer, pH 7.5 containing 0.15M NaCl. The mixture was left at 25°C for 24 hr, later concentrated by Centriprep-10 and fractionated on G-100 column at 4°C using 0.05M ammonium bicarbonate buffer. Prior

to G-100 chromatography, the UV-VISIBLE spectra was recorded to determine the liberation of pyridine-2-thione during the conjugation reaction. The contribution of pyridine-2-thione absorption at 280nm was corrected in calculating its molar concentration and thus the hormone:gelonin molar ratio was determined. Appropriate fractions of the conjugates were pooled, dialysed and lyophilized.

2.7.2: Conjugation by using 2-IT :

The oLH-gelonin hormonotoxin was prepared with 2IT by the following method essentially described for immunotoxin synthesis (Thorpe *et al*, 1987; Wawrzynczak *et al*, 1987). The oLH was dissolved in 0.05M triethanolamine-HCl containing 0.10M NaCl, 0.001M EDTA and the pH was adjusted to 8.0 with 0.2M NaH_2PO_4. This solution was added to 0.5ml of freshly prepared solution of 2IT. 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 1hr at 25° C and the reaction was stopped by adding 100μl of borate buffer saline containing 2.2M glycine. An aliquot of the derivatized oLH was then treated for 1hr at room temperature with 0.002M DTNB Ellman's reagent (Ellman, 1959). Both DTNB treated and untreated fractions were then applied to a Sephadex G-25 column

equilibrated with nitrogen 0.1M sodium phosphate buffer (pH 7.4), containing 0.1M NaCl and 0.001M EDTA. The number of activated disulfide groups introduced into the oLH was determined by reducing the DTNB treated sample with DTT and measuring the absorption of the released 3-carboxylate 4-nitrothiophenolate ion which has molar absorptivity of $1.36x10^{4}M^{-1}cm^{-1}$ at 412nm (Ellman, 1959). To initiate conjugation with gelonin, the SPDP-modified gelonin (as described in Section 2.7.1) in 0.1M phosphate buffer pH 7.4 containing 0.1M NaCl was added in 2IT derivatized oLH (oLH-2-IT) solution. The conjugation mixture was left for 24 hr at 25°C. The mixture was concentrated with Centriprep-10 before fractionation on a Sephadex G-100 column at 4°C in 0.05M NH_4HCO_3. The protein eluting before the ingredients was 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.

2.8: Charactrization of oLH and modified oLH :

Extensive physico-chemical, immunological and biological characterization was carried out on the purified oLH and the modified oLH. These are described below :

2.8.1: RP-HPLC analysis :

Reverse phase high performance liquid chromatography (RP-HPLC) of native and modified oLH were carried out as described recently (Singh, 1991; Singh and Das, 1991; Singh and Sairam, 1989 a,b&c; 1990; Singh *et al*, 1989). The analysis was performed by using Water's μBondapak phenyl column (4x250mm) and stepwise gradient of HPLC grade H_2O and CH_3CN having 0.1% TFA.

2.8.2: Immunoreactivity :

The immunoreactivity of oLH, SPDP or LC-SPDP modified oLH, gelonin or its modified derivatives was assessed by competitive displacement method (Singh, 1985). For this the radioiodinated oLH and gelonin were required. The radioiodination was carried out by IODOGEN method essentially following the procedure described for GnRH (Singh, 1985). Free $Na^{125}I$ and ^{125}I-oLH or ^{125}I-gelonin was separated by gel-filtration chromatography on a sephadex G-75 (1.0x50cm). The specific activity of ^{125}I-oLH was in the range of 50-80μCi/μg as determined by auto displacement method (Singh, 1986, 1987; Talwar *et al*, 1985). The labelled oLH/gelonin was stored in the aliquotes at -20°C and was used within a month of the preparation.

The anti-oLH or anti-gelonin antibodies were initially screened using a series of dilutions in 0.05M sodium phosphate buffer, pH 7.5 containing 0.1% BSA and a single concentration of radioiodinated oLH/gelonin. The assay system consisted of 0.02ml normal horse serum, 0.05ml ^{125}I-oLH/gelonin and 0.05ml of different dilutions of anti-oLH/anti-gelonin antibodies. The tubes were incubated at 20°C for 2 hr followed by 20 hr at 4°C. Bound and unbound ^{125}I-oLH/^{125}I-gelonin were separated by addition of 0.2ml of 1:5 diluted Pansorbin™ (Calbiochem, LaJolla, USA). The tubes were further incubated at room temperature for 20-30 min and centrifuged at 3000xg 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.

The competitive displacement assay was performed essentially following the procedure described earlier with minor modifications. All the samples and reagents were diluted in RIA buffer (0.05M sodium phosphate buffer, pH 7.5 containing 0.15M NaCl and 0.1% each BSA and sodium azide). The appropriately diluted anti-oLH antibody (giving 30-50% of binding in the absence of unlabelled hormone) was mixed with the ^{125}I-oLH were separated by the addition of 0.2ml of 1:5

diluted Pansorbin™ to separate bound and unbound radioactivity. The tubes were further incubated for 30 min and centrifuged at 3,000xg for 15 min. The supernatant containing unbound ^{125}I-oLH was removed by aspiration and the pellet was counted in a LKB-Rack mini gamma counter (~ 70% efficiency). The inhibition lines were obtained by plotting $(B/B_o)x100$ against the logarithm of the dose of competitor (where B_o represents the binding of radiolabelled oLH/gelonin in the absence of cold oLH/gelonin and B in the presence of the competitor). The extent of cross-reactivity (as percentage) was expressed as : $C=(L_{50}/U_{50})x100$, where U_{50} is the dose of competitor which shows 50% inhibition and L_{50} is the dose of oLH which shows 50% inhibition.

2.8.3: Receptor binding activity :

The radioreceptor assay was performed by using rat testicular homogenate as a source of receptor. Briefly, the assay was performed in 12x75mm polystyrene tubes. The radioiodinated oLH (0.5-1ng; ≈ 60,000-80,000 CPM/tube), the samples and RRA buffer (25mM Tris-HCl, pH 7.5,containing 10mM magnesium chloride and 1mg/ml BSA) were incubated at 20^{o}C for 16-20hr. After incubation, the reaction was terminated by addition of 2ml RRA buffer and centrifuged in cold at 3,000xg

for 20min. The supernatant was removed by aspiration and pellet was counted. The receptor binding is expressed as a percentage with respect to native oLH and modified oLH which were taken as 100%. ED_{50} is the dose causing 50% displacement.

2.8.4: Steroidogenic activity :

The progesterone induction ability was determined with leydig tumor cells, MLTC-1 which possess receptors for gonadotropins and by following the procedure described earlier (Singh and Sairam, 1989, 1990). Upon binding of gonadotropins to MTLC-1 cell, the progesterone is secreted into the medium. The tumor cells were cultured in a 24-well plates in RPMI-1640 media supplemented with 10% FCS and antibiotics as described. After culturing ~ 50,000 cells/well for 48 hr, the wells were washed three times with serum free medium and incubated with different concentrations of test samples. The incubation was carried out in a humidified chamber with 95% oxygen and 5% carbon dioxide. An aliquot was removed for progesterone estmiation by RIA and using specific anti-progesterone antibodies as described earlier (Singh and Sairam, 1989a, b&c; 1990; Singh *et al*, 1989; Singh and Das, 1991; Singh, 1991).

2.9: Characterization of gelonin :

2.9.1: Gel-permeation chromatography :

Gel-permeation chromatography on Sephadex G-100, LKB 2135 Ultro Pac TSK-G4000SW and Superose-12™ were used to determine the molecular weight and homogeneity of purified gelonin. Gel-permeation [LKB 2135 Ultro Pac TSK-G4000SW (7.5x600mm)] column was connected with LKB 2135 Ultro Pac TSKWP (7.5x75mm) column and then calibrated by the known molecular weight proteins calibration kit. Chromatography was run by Beckman HPLC having 450 controller and monitored at 280nm. Superose-12™ was connected to Pharmacia FPLC system. All the columns were calibrated with known molecular weight proteins prior to running purified gelonin. A plot of V_e/V_o (where V_e=elution volume; V_o=the void volume) *versus* log Mw was plotted and V_e of gelonin was used to calculate its molecular weight.

2.9.2: RP-HPLC analysis :

Reverse phase high performance liquid chromatography (RP-HPLC) was used for further analysis of homogeneity and purity of gelonin obtained from different methods. RP-HPLC was performed on a Water's μBondapak phenyl column (4x250mm) using a continuous gradient as described in literature (Singh and

Sairam, 1989).

2.9.3: SDS-PAGE analysis :

Homogeneity and molecular weight were also determined by SDS-PAGE analysis. The gel slab (20x12cm) was cast with acrylamide gradient (5-15%, w/v) according to Laemmli (Laemmli, 1969) and run under reducing and non-reducing conditions. The gel stained with Commassie blue was scanned on a densitometer equipped with computer for analysis. Low and high molecular weight reference standards from BioRad served as markers.

2.9.4: Gelonin antibody production :

The anti-gelonin antibodies were raised in rabbits (about 3 kg weight; New Zealand strain) by immunizing them with 1mg of RP-HPLC purified gelonin dissolved in saline and emulsified with CFA. The subsequent injections were given at fortnightly intervals with IFA. The animals were bled after one week, and the sera was separated. The immunoglobulin fraction was prepared by precipitation with ammonium sulphate at 50% final concentration. The precipitate was dissolved in 0.01M sodium phosphate buffer (pH 7.2), dialysed extensively and lyophilized.

2.9.5: Liposome entrapped gelonin :

a. Protein concentration determination :

The concentration of protein entrapped at any step was determined by using Bradford reagent (PIERCE, Rockford, USA) as described by the manufacturers.

b. Lysis of liposomes :

In order to determine the quantity of gelonin entrapped in the liposome, the liposomes were lysed with 10% Triton X-100 and centrifuged at 14,000 rpm at $4^{o}C$ for 20 minutes. The supernatant was passed through the Extracti Gel-D (PIERCE, Rockford, USA) in order to remove Triton X-100. The protein collected was concentrated by ultra filtration on YM-10 filter (Amicon) and passed through FPLC on a gel-filtration column Superose 12^{TM}. The protein eluting at 30kDa was pooled and the protein concentration was determined by the method described above. The gelonin thus obtained was subjected to SDS-PAGE analysis and further for its immunoreactivity and bioactivity.

2.10: Characterization of Hormonotoxins :

The conjugates of oLH with gelonin as prepared above were subjected to extensive physico-chemical, immunochemical and biological characterization. SDS-PAGE and RP-HPLC analysis were carried out to establish purity and molecular

weight determination of the conjugate.

2.10.1: RP-HPLC analysis :

RP-HPLC analysis was used to analyse the purity of the ingredients and hormonotoxins as described above. RP-HPLC was performed on a Water's μBondapak phenyl column (4mmx250mm) using a continuous gradient as described (Singh *et al*, 1989; Singh and Sairam, 1989 a&b; Singh, 1991; Singh and Das, 1991; Singh and Kar, 1992). The samples dissolved in water 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 2ml/min on a DuPont's HPLC and data analysed by a computer.

2.10.2: SDS-PAGE analysis :

The purity and the molar compositions of conjugates were compared with those of ingredients, oLH and gelonin by SDS-PAGE analysis. The gel slab (20x12cm) was cast with acrylamide gradient (5-15%, w/v) according to Laemmli (Laemmli, 1969) and run under reducing and non-reducing conditions. The gel stained with Commassie blue was scanned on a densitometer equipped with computer for analysis. Low and high molecular weight reference standards from BioRad served as markers.

2.10.3: Immunoreactivity :

The immunoreactivity of oLH, gelonin, their SPDP and LC-SPDP modified derivatives and hormonotoxins was determined by a competitive displacement analysis as described earlier (Singh *et al*, 1989; Singh and Sairam, 1989 a&b; Singh, 1991; Singh and Das, 1991; Singh and Kar, 1992). ^{125}I-oLH required for RIA was radioiodinated by IODOGEN method (Fraker and Speck, 1978) essentially following the procedure described for GnRH (Singh, 1986, 1987; Talwar *et al*, 1985). Free Na^{125}I and ^{125}I-oLH/or ^{125}I-gelonin was separated by gel-filtration chromatography on a Sephadex G-75 (1.0x50cm) column. The specific activity as determined by autodisplacement method was in the range of 50-80μCi/μg. The labelled hormone/gelonin was stored in aliquots at -70°C and used within a month of preparation.

The competitive displacement assay of the hormone was performed essentially following the procedure described earlier (Singh *et al*, 1989; Singh and Sairam, 1989 a&b; Singh, 1991; Singh and Das, 1991). Briefly, the test samples, labelled and unlabelled oLH, and anti-oLH antibody were diluted in assay buffer (0.05M sodium phosphate pH 7.5, containing 0.15M NaCl and 0.1% each of BSA and sodium azide).

One hundred microlitre of diluted anti-oLH antibody giving 30-50% binding (as determined in **SECTION 2.8.2**) in the same buffer was incubated with 100μl of different concentrations of test samples (competitors). After 12 hr of incubation at $4^{o}C$, 100μl of ^{125}I-oLH was added and further incubated for 12 hr at $4^{o}C$ was carried out. Bound and unbound ^{125}I-oLH were separated by addition of 0.2ml of 1:5 diluted Pansorbin^{TM} (Calbiochem, USA). The tubes were incubated further at room temperature for 20-30min and centrifuged at 3000xg for 15 min. The supernatant containing unbound ^{125}I-oLH was removed by aspiration and the pellet was counted in a LKB-Rack mini Gamma counter. The inhibition lines were obtained by plotting $(B/B_{o})x100$ against the log dose of the competitor (where B_{o} represent the binding of ^{125}I-oLH in the absence of cold oLH and B in the presence of competitor). The extent of percentage cross-reactivity was expressed as: $C= (L_{50}/U_{50})x100$, where the L_{50} is the dose of the competitor which shows 50% inhibition and U_{50} is the dose of native oLH which shows the 50% inhibition .

Immunoreactivity of gelonin was also determined by a competitive displacement assay as described earlier (Singh and Sairam, 1989; Singh and Kar, 1992) with minor modifications. The radioiodination of gelonin was carried out by IODOGEN

method as described for oLH. Five micrograms of gelonin was radioiodinated with 1mCi of $Na^{125}I$ and iodinated gelonin was purified on a Bio-Gel P6DG disposable column. The specific activity of the ^{125}I-gelonin, calculated by the incorporation efficiency, was ~ 80μCi/μg. The immunoreactivity of the labelled gelonin was estimated by measuring the binding ability (>80%) in the presence of excess of anti-gelonin antibody (Singh and Sairam, 1989; Singh and Kar, 1992).

Antibody binding and competitive binding assays were carried out following the methods described earlier (Singh and Sairam, 1989; Singh and Kar, 1992) with the difference that double antibody precipitation was used instead of precipitation by Pansorbin™. The method of calculation of the extent of cross-reactivity was the same as described earlier (Singh and Sairam, 1989; Singh and Kar, 1992)

2.10.4: Receptor binding property :

The receptor binding of oLH, SPDP/LC-SPDP modified oLH and their conjugates with gelonin was determined by RRA using rat testicular homogenate as a source of gonadotropin receptors as described earlier (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991). In brief, 100μl of each of labelled oLH (~ 70,000 CPM/tube), test

samples, unlabelled oLH and assay buffer (0.025M, Tris-HCl, pH 7.5, containing 0.001M $MgCl_2$ and 1mg/ml BSA) were incubated at 22-25°C for 16-20 hr. The reaction was terminated by addition of 2ml of assay buffer and centrifugation in cold at 3000xg for 30 min. Supernatant was aspirated and the pellet was counted in a Gamma counter as described above.

2.10.5: Steroidogenic activity :

The progesterone induction ability of oLH, SPDP or LC-SPDP modified oLH and their conjugate to gelonin was evaluated by incubation with Leydig tumor cells, MLTC-1 as described earlier (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991). The leydig tumor cell line was cultured in 24-well plates with some modifications of earlier procedures (Rebois, 1982) using 10% horse serum and OPTI-MEM medium (Gibco, NY, USA) for 72 hr. The cultures cells were washed with serum free medium and incubated with samples for at 37°C for 4hr. Progesterone secreted in the medium was estimated by a specific RIA as reported earlier (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991).

2.10.6: *In-Vitro* cell free translation assay

The inhibitory activity of gelonin and their SPDP modified derivatives on cell-free protein synthesis was determined using a system consisting of nuclease treated rabbit reticulocyte lysate (Singh and Curtiss, 1991; Singh, 1991). The extent of protein synthesis was determined by measuring radioactive leucine incorporated into the TCA insoluble protein fraction. Briefly, in a total of 15μl reaction mixture, 2μl of test sample was incubated with 1μl of BMV mRNA (0.5μg/μl), 2μl each amino acid mixture (minus leucine) and tritiated leucine, 0.5μl each 0.50M potassium acetate, 0.20M magnesium acetate and 7μl nuclease treated rabbit reticulocyte lysate. The positive control was incubated with mRNA (no test sample) while negative control received equal volume of RNAse free water. After incubation at 30^{o}C for 60 min, 5μl of reaction mixture was spotted at least 10-15 places on the 3mm whatman filter paper and dipped in cold 5% TCA containing 0.2% leucine. Three more washings of two minutes each was carried out with cold TCA. The paper was heated at 90^{o}C in 5% TCA for 2min, then quickly washed with cold ethanol and dried in the air. The protein precipitated was counted in a Scintillation counter (Beckman of ~70%

efficiency) after adding 8ml of scintillation cocktail. Percentage of protein synthesis was calculated on the basis of radioactivity incorporated in the precipitate. The protein synthesis inhibition = $(B/B_o)x100$ where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and B_o is in the absence of any inhibitor. IC-50 is the dose of inhibitor required for 50% inhibition of protein synthesis.

2.10.7: Binding and cytotoxicity to Leydig cells :

The binding and cytotoxicity experiments were carried out as described earlier (Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991). Briefly, for binding experiments, MLTC-1 cells (~ 1.5×10^5 cells/well) were plated in 24 well plates (flat bottom, Flow laboratory) and grown for 24-48 hr. When the cells occupied most of the space, the wells were washed two times with serum free medium. The cells received different concentrations of hormonotoxins and appropriate concentration of gelonin as a control. The plates were incubated for 2-4 hr at 37°C in a humidified chamber containing 95% O_2 and 5% CO_2. After incubation, the wells were washed three times with serum free medium and each well received 50μg of gelonin antibody purified by 50% ammonium

sulphate fractionation as described above. After 2hr 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 iodination of protein-A was carried out by IODOGEN method (Fraker and Speck, 1978) essentially described above for oLH. The wells were washed extensively and the bound radioactivity was counted after solubilizing cells with 0.1N NaOH. For competition experiments, 10μg/well concentration of the conjugate was used. This was determined after preliminary binding experiments. Fifty micrograms/well of native oLH was added together with the 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 test for cytotoxicity of the conjugate was carried out on MLTC-1 cells as described before (Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991) using ^{3}H-Leucine incorporation assay. Briefly, the cells were plated in RPMI-1640 medium in a 24 multiwell plates and after 24 hr culture, the medium was replaced with the fresh medium. Different concentrations of test samples were added and plate was further left for 18-24 hr at 37°C in a humidified chamber

containing 95% O_2 and 5% CO_2. The cells were washed after 24 hr with serum free medium and pulsed for 2 hr with ^{3}H-leucine (0.5μCi/well) in a leucine free RPMI-1640 medium. After 4 hr incubation, the cells were extensively washed with 5% TCA to remove non-specifically bound radioactivity. The cells were solubilized with warm 0.5ml of 0.1N NaOH. The solubilized protein was mixed with 5ml of scintillation fluid and counted in the scintillation counter.

CHAPTER - THREE

RESULTS AND DISCUSSION

PART - ONE

RESULTS

3.1: Purification of oLH :

Ovine LH was purified from the sheep pituitary by following the standard procedure as mentioned in SECTION 2.1. The purified hormone was subjected to physico-chemical, immunological and biological characterizations. The α and β-subunits of oLH were prepared with the use of salt precipitation technique. The physico-chemical characterization was carried out by SDS-PAGE, amino acid and RP-HPLC analysis. The native oLH reacted with the anti-oLH antibodies. The biological characterization of the hormone involved the receptor binding and steroidogenic activity determination. The native oLH used for subsequent cross-linking studies had comparable receptor binding and steroidogenic activity as compared to standard oLH. The α and β-subunits were completely devoid of the contamination of respective subunits as judged by RP-HPLC. The subunits recombined and regained full biological activity as determined by receptor binding and steroidogenic activity.

3.2: Modification of oLH and its subunits :

3.2.1: SPDP modification of α and β subunits of oLH :

Salt precipitation technique was used to isolate α and β subunits and RP-HPLC analysis was carried out to further establish the purity of the subunits. The HPLC purified

subunits were used in subsequent SPDP modification and recombination studies. Ten different molar ratios of SPDP were used to modify αoLH. The initial molar ratio of SPDP used in the modification and the ε-NH_2 groups of αoLH is shown in TABLE 3.1. A 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 molar ratio of αoLH:SPDP yielded modification of 1:0.69, 1:1.60, 1:2.10, 1:2.90, 1:3.20, 1:4.50, 1:5.70, 1:6.00 and 1:6.10 respectively (TABLE 3.1). The degree of modification was determined by DTT treatment of SPDP modified derivatives. For βoLH modification with SPDP, a 1:1, 1:2, 1:3, 1:4 and 1:5 molar ratio of βoLH:SPDP was used which resulted into the ε-NH_2 modification of 1:0.5, 1:0.90, 1:1.40, 1:2.40 and 1:3.60 respectively (TABLE 3.1). The number of -NH_2 groups modified in the protein was determined by using extinction coefficient of pyridine-2-thione as described above. The extent of modification is directly proportional to the increase in the absorbance at 343nm. The spectrophotometric method is reproducible as several experiments gave comparable values (TABLE 3.1). This indicates that the degree of modification of -NH_2 groups was relatively more than the native oLH therefore indicating that the ε-NH_2 in the isolated subunits are more reactive than the native oLH. A relationship between the the number of -NH_2 groups modified *versus* the mole of SPDP used

TABLE 3.1: Relationship between the moles of SPDP added and mumber of amino groups modified in the α and β subunits of oLH

Code	Subunit (mM)	SPDP (mM)	Initial ratio	Calculated number of amino groups modified*	Ratio obtained
αoLH-SP1	0.265	0.265	1:1	0.69	1:0.69
αoLH-SP2	0.265	0.530	1:2	1.60	1:1.60
αoLH-SP3	0.265	0.795	1:3	2.10	1:2.10
αoLH-SP4	0.265	1.06	1:4	2.90	1:2.90
αoLH-SP5	0.265	1.32	1:5	3.20	1:3.20
αoLH-SP6	0.265	1.59	1:6	4.50	1:4.50
αoLH-SP7	0.265	1.85	1:7	5.00	1:5.00
αoLH-SP8	0.265	2.12	1:8	5.70	1:5.70
αoLH-SP9	0.265	2.38	1:9	6.00	1:6.00
αoLH-SP10	0.265	2.65	1:10	6.10	1:6.10
βoLH-SP1	0.120	0.12	1:1	0.50	1:0.50
βoLH-SP2	0.120	0.24	1:2	0.90	1:0.90
βoLH-SP3	0.120	0.36	1:3	1.40	1:1.40
βoLH-SP4	0.120	0.48	1:4	2.40	1:2.40
βoLH-SP5	0.120	0.60	1:5	3.60	1:3.60

*Values are calculated spectrophotometrically as described in Materials and Methods SECTION 2.2

in the reaction is shown in the inset of Figure 3.1A and 3.1B. A progressive release of pyridine-2-thione at 343nm of αoLH-SPDP and βoLH-SPDP was observed (Figure 3.1A and 3.1B). Based on the modification, ε-NH_2 groups in αoLH may be divided into different groups: A 1:1-4 molar ratio reacted to highly accessible (1-3 NH_2 groups); 1:8-10 to less (~6 NH_2 groups) accessible groups. Introduction of 5 or more SPDP groups in the hydrophilic α-subunit renders the modified derivatives hydrophobic and precipitation occurred during the modification procedure. Therefore estimation of -NH_2 modification by using spectrophotometric technique may be treated as tentative. Five different molar ratios of βoLH.SPDP which resulted into all lysine ε-NH_2 modifications. Higher molar ratio was not used as the introduction of more SPDP groups induced hydrophobicity in the molecule resulting into precipitation. (Gordon and Ward, 1985). Different molar ratio of oLH:SPDP were used to modifiy ε-NH_2 groups of oLH. The extent of modification was determined with the use of extinction coefficient as described above for the subunits. TABLE 3.2 shows the relationship between the moles of SPDP added per mole of oLH and the members of amino groups modified. The twelve lysine available could be classified into three categories, as highly, moderating and less accessible.

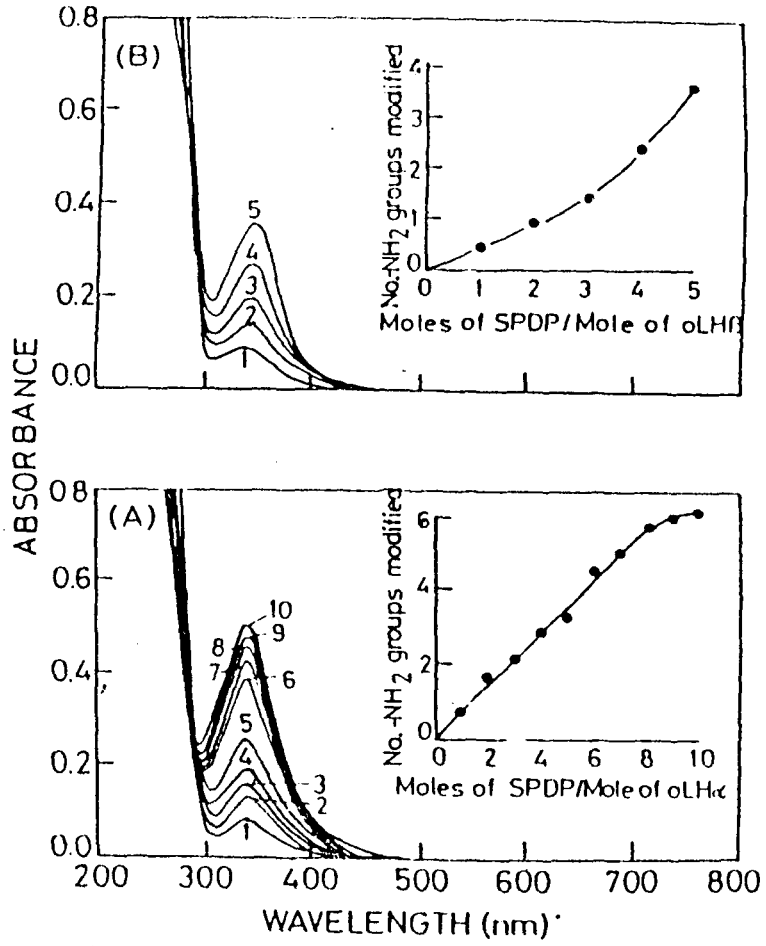


Figure 3.1: UV-VISIBLE absorption spectra of DTT treated SPDP modified αoLH derivatives. Inset shows a relationship between the number of amino groups modified by SPDP added per mole of αoLH/βoLH *versus* the number of $-NH_2$ groups modified. (A) UV-VISIBLE spectra of (1) αoLH-SP1, (2) αoLH-SP2, (3) αoLH-SP3, (4) αoLH-SP4, (5) αoLH-SP5, (6) αoLH-SP6, (7) αoLH-SP7, (8) αoLH-SP8, (9) αoLH-SP9, (10) αoLH-SP10. (B) (1) βoLH-SP1, (2) βoLH-SP2, (3) βoLH-SP3, (4) βoLH-SP4, (5) βoLH-SP5. The spectra show progressive release of pyridine-2-thione. [λ_{max} at 343nm]. The number of $-NH_2$ groups modified by SPDP was calculated on the basis of extinction coefficient ε =8.08 x $10^3 M^{-1}$.

TABLE 3.2: Relationship between the moles of SPDP added and number of amino groups modified in oLH

Code	oLH (mM)	SPDP (mM)	Initial ratio	Calculated number of amino groups modified*	Ratio obtained
oLH-SPDP-0.5	0.192	0.096	1:0.5	0.50	1:0.50
oLH-SPDP-1.0	0.192	0.196	1:1	0.80	1:0.80
oLH-SPDP-2.0	0.192	0.384	1:2	1.40	1:1.40
oLH-SPDP-4.0	0.192	1.768	1:4	2.10	1:2.10
oLH-SPDP-6.0	0.192	1.152	1:6	3.60	1:3.60
oLH-SPDP-10.0	0.192	1.920	1:10	6.10	1:6.80
oLH-SPDP-30.0	0.192	5.76	1:30	7.90	1:7.90

*Values are calculated spectrophotometrically as described in Materials and Methods SECTION 2.2

3.2.2: LC-SPDP modification of oLH :

Similar to SPDP modification studies, the LC-SPDP modified oLH showed the progressive release of pyridine-2-thione at 343nm immediately after reduction by DTT. A 1:1 concentration of oLH:LC-SPDP resulted into a 0.70 modification. A molar ratio of 1:2 and 1:3 yield 1:1.1 and 1:1.8 molar ratio which are some what less than the SPDP modification. Higher modifcations of 1:5, 1:6, 1:8 and 1:10 resulted into 1:2.20, 1:3.00, 1:5.20 and 1:5.60 respectively (TABLE 3.3). Although further molar ratios were attempted but immediately after addition of LC-SPDP solution, the precipitation of protein occurs which may be due to the introduction of higher hydrophobic LC-SPDP groups. Less number of $-NH_2$ group modifications with LC-SPDP comparative to SPDP may also attribute to the increase in the carbon chain length in LC-SPDP.

3.3: Recombination of modified oLH subunits :

In order to determine whether αoLH-SPDP or βoLH-SPDP recombines with native βoLH or αoLH respectively, the recombination studies were carried out. For a typical recombination experiment, equal amount of βoLH and αoLH-SPDP or native αoLH and βoLH-SPDP were dissolved in 0.05M sodium phosphate buffer pH 7.2 containing 0.15M NaCl and mixed

TABLE 3.3: Relationship between the moles of LC-SPDP added the number of amino groups modified in oLH

Code	oLH (mM)	SPDP (mM)	Initial ratio	Calculated number of amino groups modified*	Ratio obtained
oLH-LC-SPDP-0.5	0.192	0.096	1:0.5	0.50	1:0.50
oLH-LC-SPDP-1.0	0.192	0.192	1:1	0.70	1:0.70
oLH-LC-SPDP-2.0	0.192	0.384	1:2	1.10	1:1.10
oLH-LC-SPDP-3.0	0.192	0.576	1:3	1.80	1:1.80
oLH-LC-SPDP-5.0	0.192	0.962	1:5	2.20	1:2.20
oLH-LC-SPDP-6.0	0.192	1.152	1:6	3.00	1:3.00
oLH-LC-SPDP-8.0	0.192	1.536	1:8	5.20	1:5.20
oLH-LC-SPDP-10.0	0.192	1.920	1:10	5.60	1:5.60

*Values are calculated spectrophotometrically as described in Materials and Methods SECTION 2.2

together. Due to hydrophobic nature of βoLH and its SPDP modified derivatives, the solution was not clear. However, when left in solution along with αoLH, the annealing process begins and upon completion most of the subunits recombine and the solution becomes more or less clear. Similar observations were obtained in different SPDP modified derivatives. The recombination mixture was fractionated on a pre-calibrated G-100 column. Native oLH, αoLH and βoLH were run separately on the same column as a control experiment at a flow rate of 18ml/hr. Figure 3.2 shows the gel-filtration chromatography of the recombination mixtures. All αoLH-SPDP derivatives recombined to βoLH and all βoLH-SPDP recombined to αoLH subunits.

3.4: Purification of gelonin :

Since the Method I and Method II involve several common steps, their results are presented together and the Method III separately. Purification of gelonin by Method I involves aqueous extraction, cation-exchange and gel-filtration chromatography. Method II has an additional step of ammonium sulphate fractionation prior to cation-exchange and gel-filtration chromatography. As described in the Methods, the supernatant was applied to CMC-52 column. The unadsorbed fraction was removed by washing and the bound fraction was

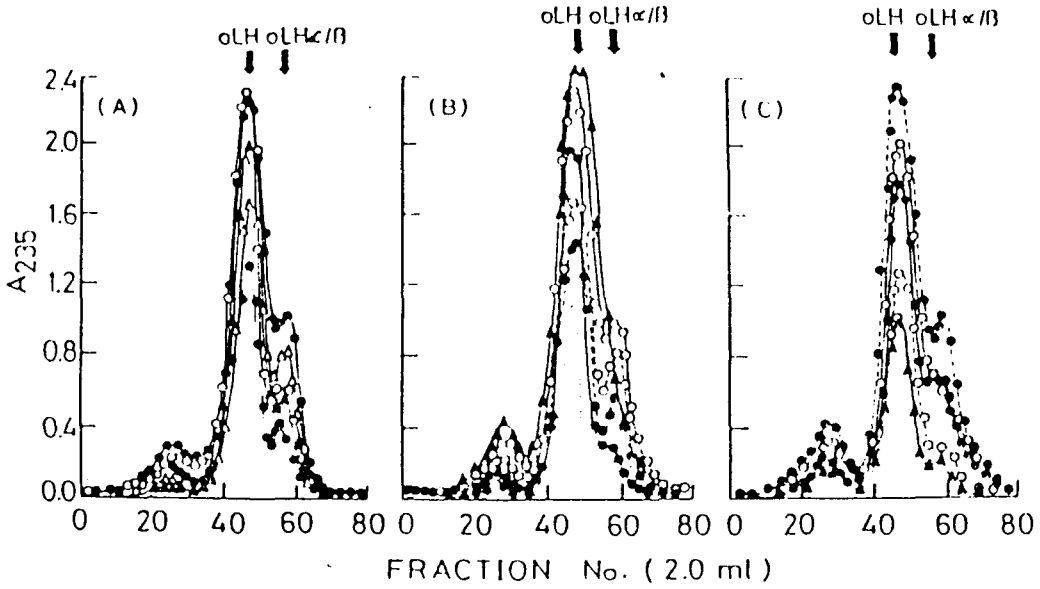


Figure 3.2 : Gel-filtration chromatography of the recombination mixture of native αoLH and βoLH; αoLH-SPDP derivatives and βoLH; βoLH-SPDP derivatives and αoLH on Sephadex G-100 column in 0.05M ammonium bicarbonate at a flow rate of 2ml/hr. The arrows on the upper portions of the chromatograms show the elution positions of native oLH and its α and β subunits. (A) Chromatograms of recombination mixtures of native βoLH with native αoLH-SP1 (●---●), αoLH-SP2 (●—●), αoLH-SP3 (▼—▼) and αoLH-SP4 (Δ—Δ). (B) Chromatograms of recombination mixtures of βolH with αoLH-SP6 (O—O), αoLH-SP7 (●---●), αoLH-SP9 (▼—▼) and αoLH-SP10 (---). (C) Chromatograms of recombination mixtures of αoLH with βoLH-SP1 (●---●), βoLH-SP2 (●—●), βoLH-SP3 (O—O) and βoLH-SP4 (●—●).

eluted by applying a linear gradient of 0-0.3M NaCl in Method I and 0.2-0.3M NaCl in Method II. It may be noted that in Method I, the extract was dialysed against 0.05M sodium phosphate buffer (pH 6.5, without NaCl), while in Method II the dialysis was against 0.05M sodium phosphate buffer (pH 6.5, with 0.02M NaCl). A representative Figure 3.3 shows the purification steps of Method I which involves aqueous extraction, cation-exchange and gel-filtration chromatography. The protein eluting between 0.15-0.20M NaCl gradient showed ribosome inactivating activity as determined in a cell-free translation assay (See Section 3.10.6). The CMC-52 elution profile of Method I was relatively different from that reported by Stripe *et al* (Stripe *et al*, 1980), where several peaks were observed. Possibly the seed batch may also attribute to such variation. The peak eluting at 0.15-0.20M NaCl gradient was pooled, concentrated and run on a pre-calibrated Sephadex G-100 column. The column was calibrated using standard gel-filtration molecular weight markers. A standard curve was obtained by plotting V_e/V_o *versus* the log Mw as described in the Methods. Using this standard curve, the molecular weight of gelonin was determined (Figure 3.3D). Figure 3.3B shows the elution profile of a representative batch of gelonin on G-100 column. The marked

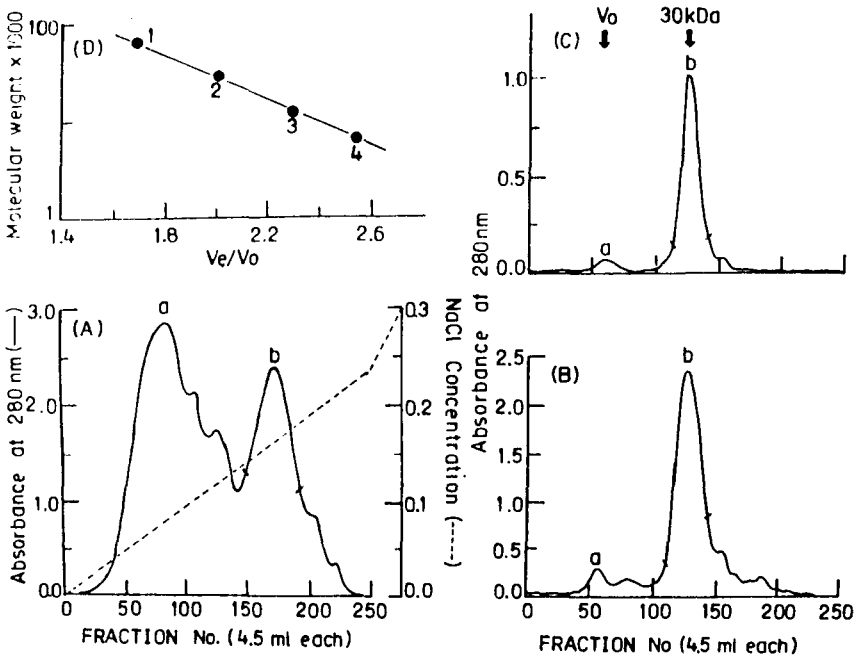


Figure 3.3: Purification of gelonin from the dry seeds of *Gelonium multiflorum*. A. The dialysed extract was passed through a column of CMC-52 (3.2x40cm) as described in the Materials and Methods section. The bound fraction was eluted with a 0.0M-0.3M NaCl gradient (---) at a flow rate of 20ml/hr at 4°C and the fraction was read at 280nm. Two major peaks `a' and `b' were obtained. B. The marked portion of peak `b' was concentrated and chromatographed on a Sephadex G-100 (3.4x100cm). Two peaks `a' and `b' along with few minor peaks were obtained. Peak `b' eluted at about 30kDa was concentrated and rechromatographed on the same G-100 column. C. Rechromatography of gelonin (as obtained from B) on G-100 column. The aggregated protein as indicated (peak `b') eluted in the void volume and the marked portion of the symmetrical peak `b' was directly lyophilized. D. Calibration of G-100 column with standard molecular weight proteins: 1 = Bovine Serum Albumin; 2 = Carbonic Anhydrase; 3 = Cytochrome-C; 4 = Aprotinin. A plot of log Mw *versus* V_e/V_o is drawn where V_e is the elution volume of the standard protein and V_o is the void volume of the column.

area of major peak `b' was pooled, concentrated and re-run on the same column by controlling the flow rate (Figure 3.3C). It may be noted that the protein shows a slight tendency to aggregate during concentration and the aggregated protein eluted in the void volume of the column (peak `a' of Figures 3.3A and 3.3B). This protein was found to be immunologically reactive towards gelonin antibodies and also showed a band of Mw 30 kDa when run under the reduced SDS-PAGE. The marked area of Figure 3.3C was pooled and directly lyophilized and stored at $4^{\circ}C$.

3.5: Modification of $-NH_2$ groups of gelonin :

The amino groups of gelonin were modified by SPDP/LC-SPDP as described in the SECTION 2.5. The UV-VISIBLE spectra of DTT treated gelonin-SPDP derivatives obtained from different molar ratio of SPDP are shown in Figures 3.4. A clear concentration dependent increase of λ_{280} and λ_{343} was observed in modified gelonin-SPDP derivatives . The relationship between the number of $-NH_2$ groups modified *versus* the molar concentration of SPDP added is shown in the inset of Figure 3.4 and the quantitative data are recorded in TABLE 3.4. Higher modification ratios (over 6) usually caused the precipitation of the protein during the reaction. Therefore, in these cases, the reaction was terminated as soon as the

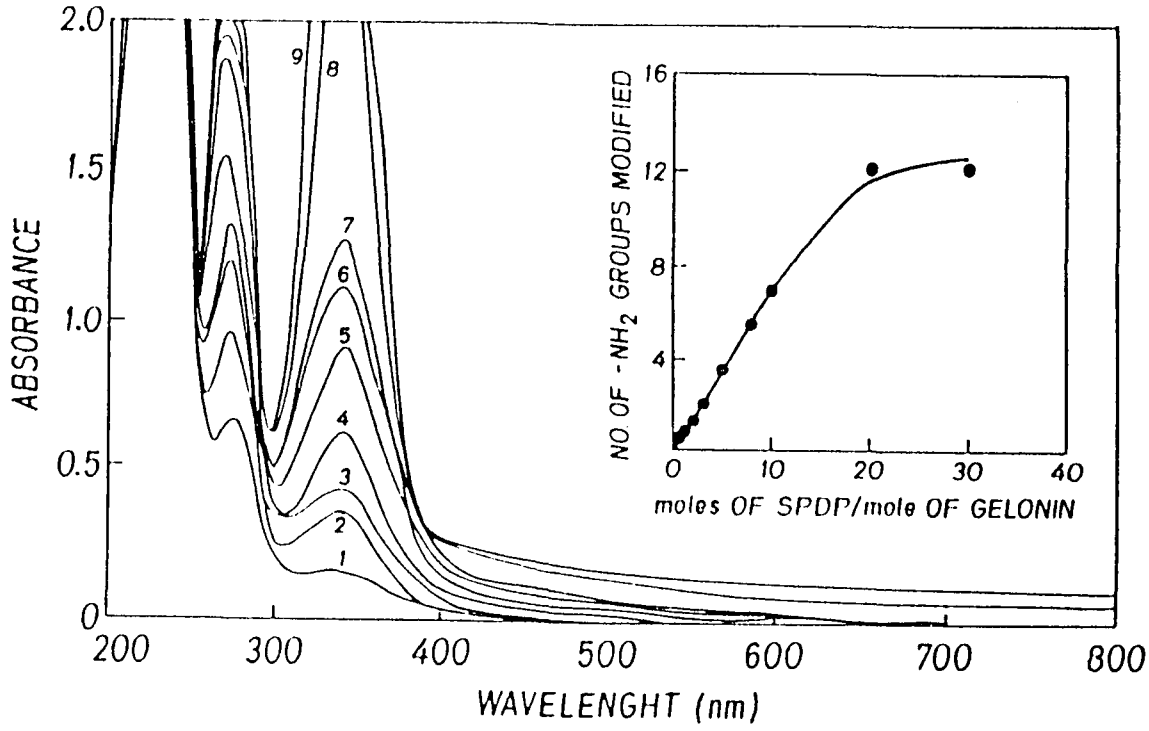


Figure 3.4: UV-VISIBLE absorption spectra of DTT treated gelonin-SPDP derivatives. 1. Gelonin; 2. G-SP-0.5; 3. G-SP-1.0; 4. G-SP-2.0; 5. G-SP-4.0; 6. G-SP-5.0; 7. G-SP-8.0; 8. G-SP-10.0; 9. G-SP-30.0. The gelonin-SPDP samples were treated with DTT for 30 min and spectra was recorded. The spectra shows the progressive release of pyridine-2-thione [λ_{max} at 343 nm]. On the basis of the extinction coefficient, [ε=8080], the number of amino groups modified by SPDP were calculated. Inset shows a relationship between the moles of SPDP added per mole of gelonin *versus* the number of amino groups modified in the protein. The quantitative data based on this observation is recorded in TABLE 3.4.

TABLE 3.4: Relationship between the moles of SPDP added and number of amino groups modified in gelonin

Code	Gelonin (mM)	SPDP (mM)	Initial ratio	Calculated number of amino groups modified*	Ratio obtained
Gelonin	0.166	--	--	--	--
G-SP-0.5	0.166	0.083	1:0.5	0.4	1:0.5
G-SP-1.0	0.166	0.166	1:1.0	0.8	1:0.8
G-SP-2.0	0.166	0.332	1:2.0	1.7	1:1.7
G-SP-4.0	0.166	0.664	1:4.0	3.2	1:3.2
G-SP-5.0	0.166	0.830	1:5.0	3.8	1:3.8
G-SP-8.0	0.166	1.328	1:8.0	6.6	1:6.6
G-SP-10.0	0.166	1.660	1:10.0	7.6	1:7.6
G-SP-20.0	0.166	3.320	1:20.0	12.0	1:12.0
G-SP-30.0	0.166	4.980	1:30.0	11.6	1:11.6

Gelonin was thiolated with SPDP and the number of amino groups modified was determined by estimating the release of pyridine-2-thione after reducing with DTT.

solution appeared to be cloudy. After the desalting on a Bio-Gel P6DG column, the highly modified derivatives did not completely dissolve in 0.1M sodium phosphate buffer, pH 6.0, even after adding excess DTT. Thus, it is presumed that the protein must have been denatured after the modification of many of the accessible ε-lysine residues. In view of this the values shown for ε-NH_2 group modification for higher SPDP-gelonin derivatives should be considered tentative.

3.6: Liposome entrapment of gelonin :

Liposomes made using three different lipids had different compositions in same molar ratio as shown in **TABLE** 3.5. A total of 8.0-8.6mg of lipids were taken to entrap 1.0mg of gelonin. Although, the preparations were heterogeneous and multilamellar in nature, a significant population of liposomes was found to be larger in size when observed in phase-contrast microscopy. A consistent and reproducible entrapment of gelonin in liposomes was obtained as observed after separating free from entrapped protein by centrifugation or on sepharose 4B column (**TABLE** 3.6). Each experiment was repeated three times and the values of liposome encapsulated gelonin are represented as mean ± SD. A 75-80% entrapment efficiency was obtained from three different lipid compositions indicating that the entrapment of gelonin appeared to be independent of

TABLE 3.5: Liposome composition and LIPID/GELONIN ratio used in liposome preparations

Lipid composition of liposomes	Molar ratio	Lipid/Gelonin ratio
Egg PC : CHOL : DCP	1:0.9:0.25	8.6
DPPC : CHOL : DCP	1:0.9:0.25	8.5
DMPC : CHOL : DCP	1:0.9:0.25	8.0

Egg PC : Egg phospahtidyl choline; CHOL : Cholesterol; DCP : Dicetyl phosphate; DPPC : Dipalmitoyl phosphatidyl choline; DMPC : Dimyristoyl phosphatidyl choline.

TABLE 3.6: **Percentage of entrapment of gelonin in liposomes of different lipid composition after separating from unentrapped gelonin by centrifugation and on Sepharose CL-4B column**

Liposomal Phospholipids (mg)	Gelonin	Centrifugation (mean ± SD)			Sepharose CL-4B column (mean ± SD)		
		Free (µg)	entrapped (µg)	% entrapment	Free (µg)	entrapped (µg)	% entrapment
Egg PC : CHOL : DCP (5.1 : 2.5 : 1.0)	1000	213±27	770±31	77±3	188±25	789±18	79±2
DPPC : CHOL : DCP (5.0 : 2.5 : 1.0)	1000	208±31	766±28	76±3	213±23	765±17	76±2
DMPC : CHOL : DCP (4.5 : 2.5 : 1.0)	1000	21±29	752±32	75±3	232±26	745±37	74±4

the nature of the phospholipids used. A good correlation was obtained between free and liposome encapsulated gelonin as can be seen TABLE 3.6.

3.7: Conjugation of oLH with gelonin :

3.7.1: Conjugation by using SPDP/LC-SPDP :

A careful titration of molar ratio of oLH/gelonin *versus* SPDP or LC-SPDP was carried out. Experiments with SPDP revealed that a molar ratio of 1:2 for oLH/gelonin:SPDP thiolated 1.2±0.2 amino groups. Similarly, the LC-SPDP modification of ε-NH_2 groups of oLH clearly revealed that a 1:2 molar ratio of oLH and LC-SPDP thiolated 1:1.1 ± 0.2 amino groups per molecule (TABLE 3.3). The conjugation of oLH to gelonin was initiated following the protocol as described in Section 2.7. The conjugation mixtures (as prepared with the use of SPDP and LC-SPDP) were fractionated on a Sephadex G-100 column and elution profiles of the conjugates are shown in Figure 3.5. The upper portion of Figure 3.5A shows the elution positions of oLH, gelonin and the void volume (V_o) of the column. The chemical structures of four different conjugates, C200A, C210A, C220A and C230A are shown in TABLE 3.7. This table also shows the activation of oLH and gelonin with SPDP and LC-SPDP. In all the cases, SPDP/LC-SPDP modified gelonin was only treated with DTT to generate -SH group which

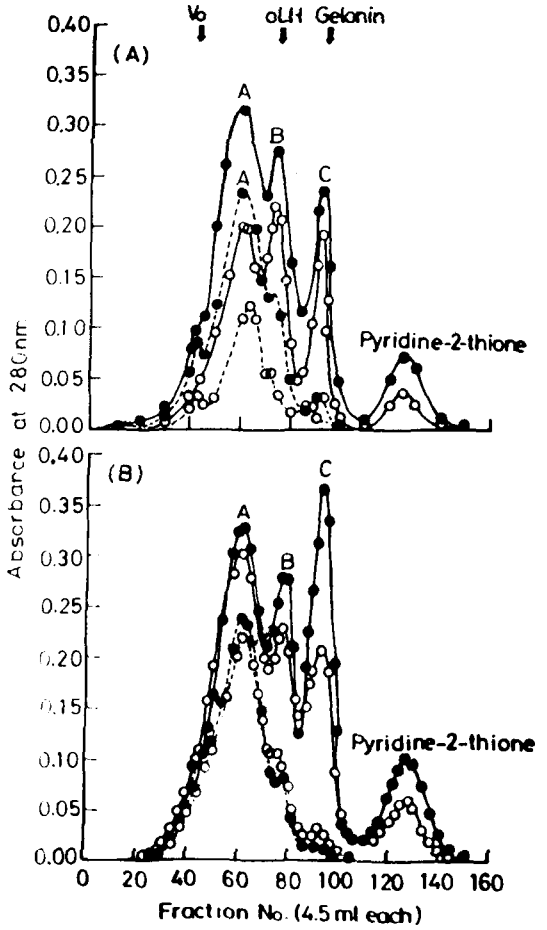


Figure 3.5: Gel-filtration chromatography of oLH-gelonin conjugation mixtures on the Sephadex-G-100 column. The protein was eluted with 50mM ammonium bicarbonate at a flow rate of 20mL/hr. Arrows in the upper portion of A indicate the void volume and elution positions of oLH and gelonin. (A) This shows gel-filtration chromatograms of the conjugation mixtures of C200 (●) and C210 (○). The major peak eluting before oLH and gelonin was their conjugate (designated as C200A and C210A). This peak was concentrated and rechromatographed on the same column. The dashed line (---) chromatograms show the rechromatographed C200A and C210A. The peak A (designated as C200ARA and C210ARA) was pooled and lyophilized directly. The unconjugated peaks of oLH and gelonin were lyophilized separately. (B) Gel-filtration chromatograms of the conjugation mixtures of C220 (● ●) and C230 (○ ○). The chromatograms shown by ●---● and ○---○ indicate rechromatography of peak A of Peak C220 (as C220A) and C230 (as C230A). The major peak of the rechromatography of C220A and C230A were designated as C220ARA and C230ARA respectively. As described above, these portions of the peaks of were directly lyophilized.

TABLE 3.7: Chemical structure of the conjugates prepared with the use of SPDP and LC-SPDP.

oLH-gelonin conjugate code	spacer (Å)	Cross-linking agent used: activation of oLH with	Cross-linking agent used: activation of gelonin with	
C200A	13.6	SPDP	SPDP	oLH-NH-CO-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-NH-gelonin
C210A	22.4	SPDP	LC-SPDP	oLH-NH-CO-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-NH-$(CH_2)_5$-CO-NH-gelonin
C220A	22.4	LC-SPDP	SPDP	oLH-NH-CO-$(CH_2)_5$-NH-CO-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-NH-gelonin
C230A	31.4	LC-SPDP	LC-SPDP	oLH-NH-CO-$(CH_2)_5$-NH-CO-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-NH-$(CH_2)_5$-CO-NH-gelonin

was subsequently used for conjugation with oLH/SPDP or LC-SPDP. In C200A conjugate both the ingredients were activated with SPDP and later the conjugation was initiated after mixing with the DTT treated gelonin-SPDP as per the methodology described in SECTION 2.7.1. C210A and C220A conjugates were prepared with the use of both SPDP and LC-SPDP while C230A was synthesised with the use of only LC-SPDP. Therefore, the spacer arm in C200A conjugate was calculated to be 13.3A^{o} long, while in C230A the spacer arm was of 31.2A^{o} long. The C210A and C220A conjugates had spacer arms of 22.4A^{o} length (TABLE 3.7). The protein peak appearing before oLH and gelonin was considered to be their conjugate and was pooled (designated as A peak). The unconjugated oLH or gelonin peaks were designated as B and C respectively. In each conjugate, the pool A peak was concentrated and rerun on the same column (Figure 3.5). In the rechromatographic run, the major peak eluting before oLH was pooled (designated as ARA) and lyophilized directly. The unconjugated peaks of oLH and gelonin were lyophilized separately. Figure 3.5A shows the elution profile of C200 and C210 conjugate whereas Figure 3.5B recorded the profile of C220 and C230 conjugation mixture. A small peak appearing between 110-140th fractions was due to the release of pyridine-2-thione which apart from its

absorption at 343nm, also absorbs at 280nm. Therefore, in order to determine the molar concentration using molar extinction coefficient of pyridine-2-thione a correction for its contribution at 280nm is applied (Carlsson *et al*, 1978).

3.7.2: Conjugation by using 2-IT :

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. Similarly, present and earlier experience (Singh and Sairam, 1989; Singh and Kar, 1992) 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 SECTION 2.7.1. The conjugation mixture was fractionated on a Sephadex G-100 column and the elution profiles are shown in Figure 3.6. The upper portion of 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 also absorbs at 280nm. Therefore, in order to estimate molar concentration using molar extinction coefficient of pyridine-2-thione a correction for its contribution at 280nm was applied as mentioned in SECTION 3.7.1. (Carlsson *et al*, 1978). Both ingredients, oLH and

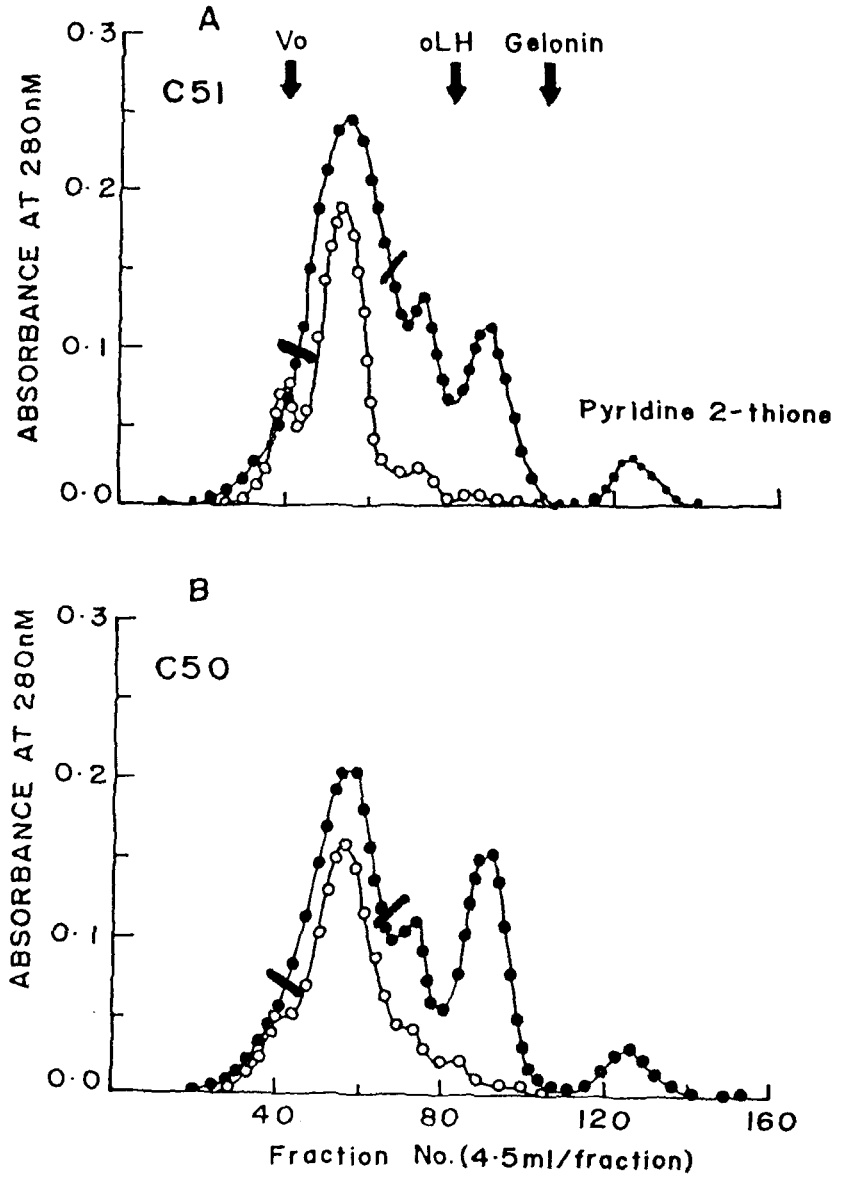


Figure 3.6: Gel-filtration chromatography of oLH-gelonin conjugates prepared with the use of 2-IT on a pre-calibrated G-100 column. A. Gel-filtration chromatography of C-51 conjugate (●——●). B. Gel-filtration chromatography of C-50 conjugate (●——●). The arrows in the upper portion of the figures indicate the elution position of oLH, gelonin and the void volume of the column. The pyridine-2-thione liberated during the conjugation is also shown. The marked portions were rerun on the same column and the elution positions are shown (○——○).

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 Figure 3.6). Pool A (coded as C50A, C51A) was the conjugate which was rerun on the same column. Typical rechromatographic elution profiles of C50A and C51A pools on the same column are also shown in Figure 3.6. This yielded free fractions C50AA, C51AA, C50AB, C51AB, C50AC and C51AC. The pooled fractions were subjected to further purification and charcterization to ensure that the fractions were devoid of ingredient contamination.

3.8: Characterization of modified oLH :

3.8.1: RP-HPLC analysis :

The SPDP modified subunits and their recombinant products were subjected to RP-HPLC analysis in order to determine the extent of $-NH_2$ modification. Left panel of Figure 3.7 shows the RP-HPLC chromatograms of αoLH and αoLH-SPDP and right panel βoLH and βoLH-SPDP derivatives. A progressive modification occurring in αoLH is evident from the emergence of new peaks (Figure 3.7, left panel). The αoLH eluted as a major peak at $t_{4.7}$. Minor modification (1:1) shows the emergence of a new peak at $t_{9.2}$ and a few shoulders. A gradual shift of $t_{7.4}$ to $t_{9.1}$, t_{10}, $t_{11.8}$ was observed in 1:2 and 1:3 molar modifications. In 1:4 modification, the peak was

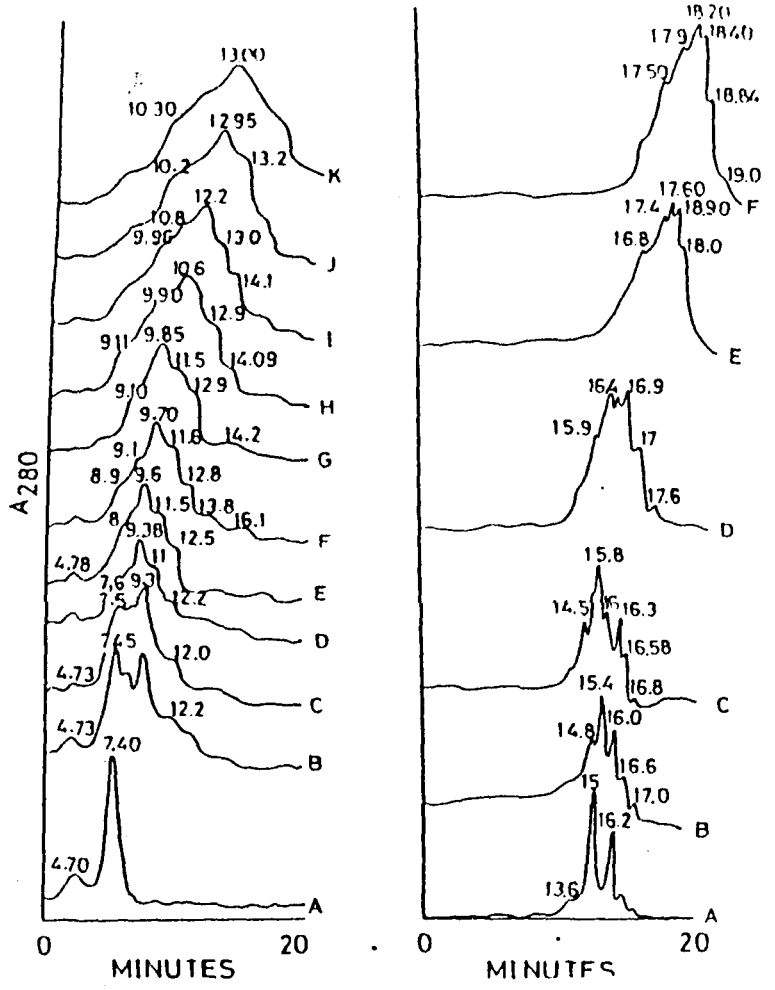


Figure 3.7: RP-HPLC analysis of αoLH (left), βoLH (right) and their SPDP modified derivatives [αoLH-SPDP (left) and βoLH-SPDP (right)]. The samples were dissolved in water containing 0.1% TFA (solvent A) and analysis was performed on a Water's μBondapak phenyl column (4x250mm) equilibrated in solvent A+25% CH_3CN and 0.1% TFA. A gradient of 25-50% was run over 30 min at a flow rate of 2ml/min and monitored at 280nm. Chromatogram was analysed between 0 and 20 min (left). RP-HPLC chromatograms shown by (——) indicate profile of αoLH (A), αoLH-SP1 (B), αoLH-SP2 (C), αoLH-SP3 (D), αoLH-SP4 (E), αoLH-SP5 (F), αoLH-SP6 (G), αoLH-SP7 (H), αoLH-SP8 (I), αoLH-SP9 (J) αoLH-SP10 (K). (Right) RP-HPLC chromatograms of βoLH (A), βoLH-SP1 (B), βoLH-SP2 (C), βoLH-SP3 (D), βoLH-SP4 (E), βoLH-SP5 (F).

broadened and eluted at the $t_{9.6}$ and the shoulders at $t_{11.5}$ and $t_{12.5}$ (Figure 3.7, left panel). Further modification resulted into more broadening of the peak and the retention time was also enhanced (Figure 3.7, left panel). The shift of $t_{7.4}$ towards the higher retention time may be due to the introduction of hydrophobic groups which abolishes the positive charges on the subunits. Under the similar conditions, the thiolation of the native oLH exhibited different RP-HPLC chromatograms.

To further confirm that the SPDP modified subunits indeed recombine with their respective subunits, RP-HPLC was carried out on the peak tube of the recombination peaks (of Figure 3.2). Figure 3.8 shows the RP-HPLC chromatograms of recombinants of native αoLH and βoLH (Figure 3.8A), native βoLH and αoLH-SPDP (Figure 3.8B), native βoLH and αoLH-SPDP (Figure 3.8C). This clearly demonstrates the presence of native βoLH doublet at $t_{14.20}$ and $t_{15.28}$ in the αoLH/αoLH-SPDP and βoLH recombinants indicating the recombination ability of the SPDP modified αoLH. Similarly, βoLH-SPDP derivatives hybridized to native αoLH at $t_{4.7}$ and $t_{7.4}$.

Similar to oLH-SPDP derivatives, the oLH-LC-SPDP derivatives were also subjected to RP-HPLC analysis to determine the site(s) of modifications. The native oLH shows

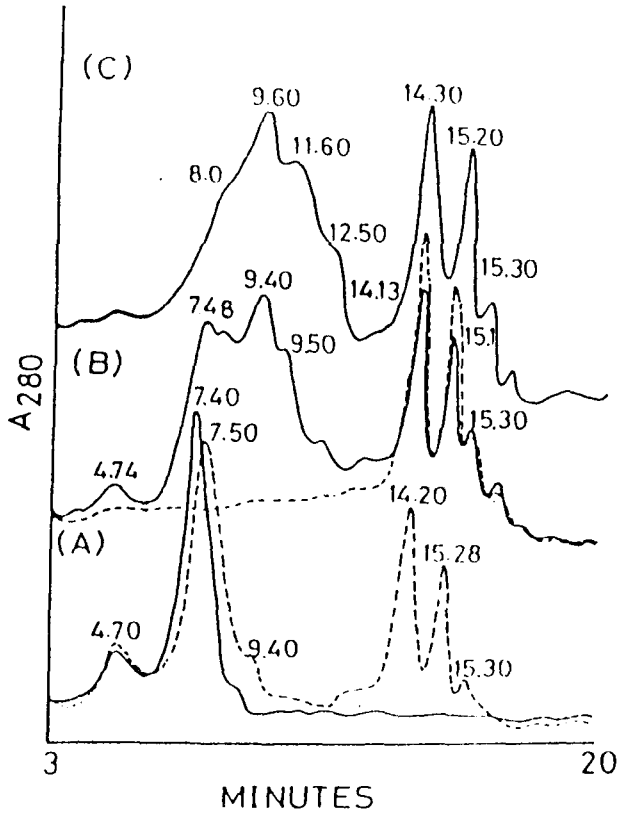


Figure 3.8: RP-HPLC chromatograms of αoLH, βoLH, their recombinants and recombinats of SPDP modified αoLH with native βoLH. HPLC conditions are same as mentioned in Figure 3.7. Chromatograms were analysed between 3 and 20 min. (A) RP-HPLC chromatograms of αoLH (——) and its recombinants with βoLH (---). (B) RP-HPLC chromatograms of βoLH (---) and its recombinants with αoLH-SP2 (——). (C) RP-HPLC chromatograms of the recombinant of αoLH-SP3 with βoLH.

three major peaks, the peak at $t_{7.4}$ was due to the α-subunit whereas $t_{14.20}$ and $t_{15.25}$ were due to β-subunit. A minor modification of 1:0.5 amino groups show the decrease of $t_{7.4}$ peak and a shoulder at $t_{8.30}$ began to appear. A modification of 1:0.70 group further indicated an appearence of an additional shoulder at $t_{8.40}$. Subsequent modifications of two $-NH_2$ groups drastically affected $t_{7.40}$ peak. In fact, this peak disappeared and some other peaks emerged in between $t_{7.4}$-$t_{12.0}$. All the peaks of α-subunit regions disappeared when two to three amino groups were modified by LC-SPDP. At this stage, the α-subunit peaks started merging with that of β-subunit dublets $t_{14.20}$ and $t_{15.20}$. It seems that the β-subunit dublets are not affected as two sharp peaks of $t_{14.20}$ and $t_{15.80}$ remain intact. When more than three amino groups are modified, the β-subunit components are drastically altered suggesting the possible involvement of β-subunit $-NH_2$ groups in the modification reaction. The reaction of ε-NH_2 groups by LC-SPDP renders the protein more hydrophobic with the result that the hydrophobic α-subunit binds more tightly to the column and eluted at higher CH_3CN concentrations. Therefore, in the higher modification chromatograms, the peaks are shifted towards the higher retention times. The RP-HPLC chromatograms were more or less similar to that of SPDP

modified oLH derivatives as described earlier.

3.8.2: Immunoreactivity :

To study the immunological and biological properties of native oLH and the recombinants, anti-oLH antibody reactivity, receptor binding and steroidogenic activity were determined. The immunoreactivity was assessed by using conformation directed anti-oLH antibodies in a competitive displacement assay and receptor recognition in a radio-receptor assay using rat testicular membrane (Talwar *et al*, 1987; Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991). The competitive displacement curve of αoLH-SPDP, βoLH and βoLH-SPDP.αoLH recombinants as shown in Figures 3.9A and 3.9B respectively. αoLH-SP9-10βoLH did not compete with the oLH antibodies. (Figure 3.9A). However, other displacement curves were parallel to native oLH. Similarly, the SPDP/LC-SPDP modified oLH were also studied for their competition ability. The quantitative data are recorded in TABLE 3.8. Upon one $-NH_2$ group modification, the immunoreactivity was reduced upto 80%. However, additional modification of 2.0 ε-NH_2 groups further reduces immunoreactivity to more than 90%. A 99% loss in immunoreactivity was observed when five $-NH_2$ groups were modified by LC-SPDP (TABLE 3.8). The immunoreactivity of different recombinant products of oLH after its modification

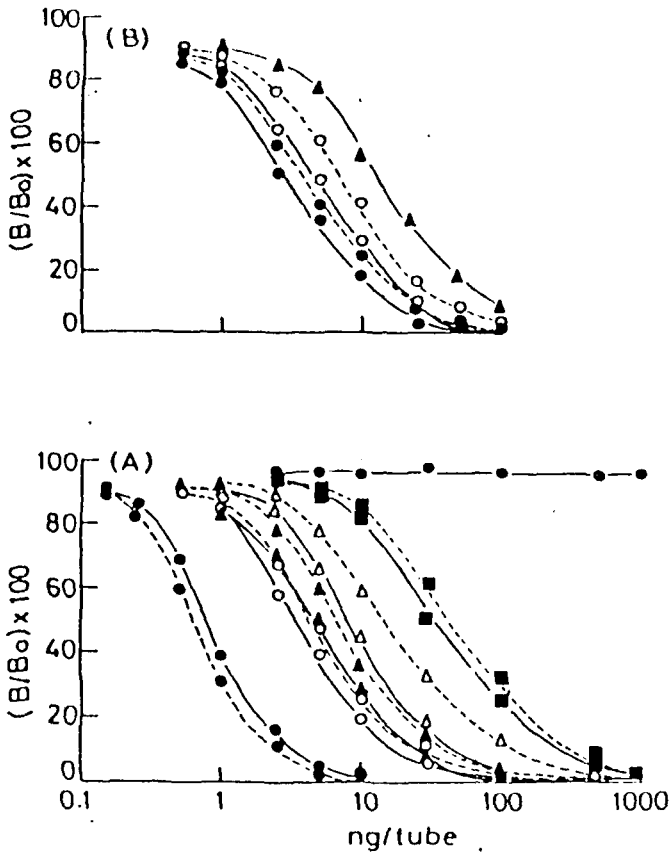


Figure 3.9: Immunological activity of oLH (●——●), αoLH.βoLH (●---●), αoLH-SP1.βoLH (O——O), αoLH-SP2.βoLH (O---O), αoLH-SP3.βoLH (▲——▲), αoLH-SP4.βoLH (▲---▲), αoLH-SP5.βoLH (Δ——Δ), αoLH-SP6.βoLH (Δ---Δ), αoLH-SP7.βoLH (■——■), αoLH-SP8.βoLH (■---■) and αoLH-SP9-10.βoLH (---). (B) Immunological activity of βoLH-SP1.αoLH (●——●), βoLH-SP2.αoLH (●---●), βoLH-SP3.αoLH (O——O), βoLH-SP4.αoLH (O---O) and βoLH-SP5.αoLH (Δ——Δ).

TABLE 3.8: Immunoreactivity of oLH, oLH-SPDP and oLH modified by different molar ratio of LC-SPDP. The activity is expressed as percentage with respect to native oLH which was taken as 100%. The method of calculation is described in SECTION 2.8. ED_{50} is the dose causing 50% displacement.

oLH/oLH-SPDP/ oLH-LC-SPDP	Molar ratio of oLH and number of $-NH_2$ groups modified	Immunoreactivity	
		ED_{50} (ng)	% cross reactivity
oLH	--	0.60	100
oLH-SPDP-0.5	1:0.5	3.00	20
oLH-SPDP-1.0	1:0.8	3.75	16
oLH-SPDP-2.0	1:1.4	5.00	12
oLH-SPDP-4.0	1:2.1	5.00	12
oLH-SPDP-6.0	1:3.6	8.00	7.5
oLH-SPDP-10.0	1:6.8	15.00	4.0
oLH-SPDP-30.0	1:7.9	--	--
oLH-LC-SPDP-0.5	1:0.5	1.66	36
oLH-LC-SPDP-1.0	1:0.7	1.87	32
oLH-LC-SPDP-2.0	1:1.1	3.30	18
oLH-LC-SPDP-3.0	1:1.8	5.45	11
oLH-LC-SPDP-5.0	1:2.2	7.05	8.5
oLH-LC-SPDP-6.0	1:3.0	1.50	4.0
oLH-LC-SPDP-8.0	1:5.2	--	--
oLH-LC-SPDP-10.0	1:5.6	--	--

The numbers 0.5-30 means the use of various molar ratios of oLH-SPDP/oLH-LC-SPDP in the reaction. The data were calculated on the basis of the release of pyridine-2-thione after the DTT treatment. Three separate experiments indicated 5-15% variation in the extent of modification and 3-7% deviation in immunoreactivity.

are also recorded in TABLE 3.9.

3.8.3: Receptor binding activity :

Receptor recognition was determined in a radio-receptor assay using rat testicular membrane (Talwar *et al*, 1987; Singh, 1991; Singh and Sairam, 1989). The receptor binding activity of different αoLH-SPDP.βoLH and βoLH-SPDP.αoLH recombinants are shown in Figures 3.10A and 3.10B respectively. The quantitative data based on Figures 3.9 and 3.10 are recorded in TABLES 3.9 and 3.10 respectively. Both immunoreactivity and receptor binding activities were drastically inhibited even after a minor modification.

Like immunoreactivity, the receptor binding ability of SPDP or LC-SPDP modified oLH derivatives was also reduced to a greater degree (TABLE 3.11). More than 80% receptor binding activity was lost with a single $-NH_2$ group was modified with LC-SPDP. One more $-NH_2$ group modification could only retain 2% receptor binding activity (TABLE 3.11). Further modifications virtually eliminated the receptor binding activity (TABLE 3.11).

3.8.4: Steroidogenic activity :

The progesterone induction ability of SPDP modified oLH derivatives is shown in Figure 3.11. The steroidogenic activity of SPDP modified oLH was (as described by SECTION

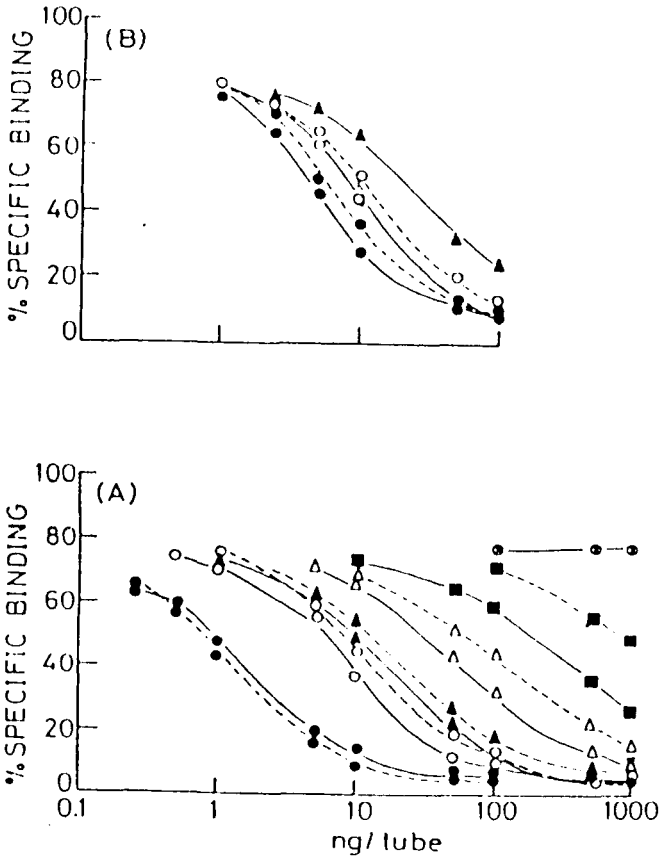


Figure 3.10: (A) Receptor binding activity of oLH (●——●), αoLH.βoLH (●---●), αoLH-SP1.βoLH (O——O), αoLH-SP2.βoLH (O---O), αoLH-SP3.βoLH (▲——▲), αoLH-SP4.βoLH (▲---▲), αoLH-SP5.βoLH (Δ——Δ), αoLH-SP6.βoLH (Δ---Δ), αoLH-SP7.βoLH (■——■), αoLH-SP8.βoLH (■---■), αoLH-SP9-10.βoLH (---). (B). Receptor binding activity of βoLH-SP1.αoLH (●——●), βoLH-SP2.αoLH (●---●), βoLH-SP3.αoLH (O——O), βoLH-SP4.αoLH (O---O) and βoLH-SP5.αoLH (▲——▲). Receptor binding activity was obtained in a competitive displacement analysis as determined by RRA using rat testicular homogenate. The quantitative data are recorded in TABLE 3.10.

TABLE 3.9: Immunoreactivity of native oLH, different recombination products of SPDP modified subunits with respective native subunits

Code	Immunoreactivity	
	ED_{50}	% activity
αoLH	0.75	100
αoLH.βoLH	0.75	100
αoLH.βoLH	0.69	100
αoLH-SP1.βoLH	3.33	22.5 (20.75)
αoLH-SP2.βoLH	4.05	18.5 (17.03)
αoLH-SP3.βoLH	4.16	18.0 (16.58)
αoLH-SP4.βoLH	6.82	11.0 (10.11)
αoLH-SP5.βoLH	7.89	9.5 (8.74)
αoLH-SP6.βoLH	13.63	5.5 (5.06)
αoLH-SP7.βoLH	34.10	2.2 (2.06)
αoLH-SP8.βoLH	37.50	2.0 (1.84)
αoLH-SP9.βoLH	--	--
αoLH-SP10.βoLH	--	--
βoLH-SP1.αoLH	3.00	25.0 (23.00)
βoLH-SP2.αoLH	3.75	20.0 (18.40)
βoLH-SP3.αoLH	4.54	16.5 (15.20)
βoLH-SP4.αoLH	6.25	12.0 (11.04)
βoLH-SP5.αoLH	12.50	6.0 (5.52)

[Number in parantheses were obtained when αoLH.βoLH was taken as 100%]

TABLE 3.10: Receptor binding property of native oLH, different recombination products of SPDP modified subunits with respective native subunits

Code	Receptor binding	
	ED_{50}	% activity
αoLH	0.90	100
αoLH.βoLH	0.90	100
αoLH.βoLH	0.85	100
αoLH-SP1.βoLH	6.20	14.50 (13.71)
αoLH-SP2.βoLH	8.03	11.20 (10.58)
αoLH-SP3.βoLH	9.57	9.40 (8.88)
αoLH-SP4.βoLH	12.50	7.20 (6.80)
αoLH-SP5.βoLH	30.00	3.00 (2.83)
αoLH-SP6.βoLH	60.00	1.50 (1.41)
αoLH-SP7.βoLH	180.00	0.50 (0.47)
αoLH-SP8.βoLH	900.00	0.10 (0.094)
αoLH-SP9.βoLH	--	--
αoLH-SP10.βoLH	--	--
βoLH-SP1.αoLH	4.39	20.50 (19.36)
βoLH-SP2.αoLH	5.45	16.50 (15.60)
βoLH-SP3.αoLH	8.18	11.00 (10.40)
βoLH-SP4.αoLH	10.00	9.00 (8.50)
βoLH-SP5.αoLH	20.00	4.50 (4.25)

[Number in parantheses were obtained when αoLH.βoLH was taken as 100%]

TABLE 3.11: Receptor binding and steroidogenic properties of native oLH, different SPDP and LC-SPDP modified oLH derivatives

Code	Receptor binding activity		Steroidogenic activity (%)
	ED_{50} (ng)	% Activity	
oLH	0.95	100	100
oLH-SPDP-0.5	3.16	30	120-130
oLH-SPDP-1.0	5.27	18	110-100
oLH-SPDP-2.0	15.80	6	--
oLH-SPDP-4.0	19.00	5	20-30
oLH-SPDP-6.0	47.50	2	5-10
oLH-SPDP-10.0	--	--	--
oLH-SPDP-30.0	--	--	--
oLH-LC-SPDP-0.5	2.50	38	120-135
oLH-LC-SPDP-1.0	4.50	21	80-100
oLH-LC-SPDP-2.0	10.50	9	12-30
oLH-LC-SPDP-3.0	23.75	4	7-21
oLH-LC-SPDP-5.0	47.50	2	--
oLH-LC-SPDP-6.0	95.00	1	--
oLH-LC-SPDP-8.0	--	--	--
oLH-LC-SPDP-10.0	--	--	--

The numbers 0.5-30 means the use of various molar ratios of oLH-SPDP/oLH-LC-SPDP in the reaction.

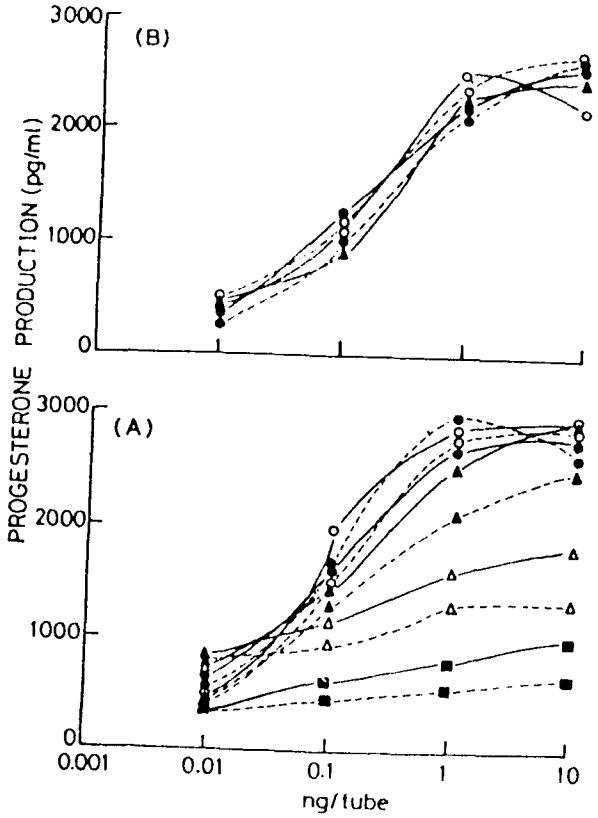


Figure 3.11: (A) Progesterone induction ability of native oLH (●——●), αoLH.βoLH (●---●), αoLH-SP1.βoLH (O——O), αoLH-SP2.βoLH (O---O), αoLH-SP3.βoLH (▲——▲), αoLH-SP4.βoLH (▲---▲), αoLH-SP5.βoLH (Δ——Δ), αoLH-SP6.βoLH (Δ---Δ), αoLH-SP7.βoLH (■——■), αoLH-SP8.βoLH (■---■). (B) βoLH-SP1.αoLH (●——●), βoLH-SP2.αoLH (●---●), βoLH-SP3.αoLH (O——O), βoLH-SP4.αoLH (O---O) and βoLH-SP5.αoLH (▲——▲). Progesterone secreted by cells was measured by specific RRA.

3.8.4) found to be unaffected in the low molar ratio modified derivatives (upto 1:3) in αoLH-SPDP-βoLH recombinants (Figure 3.11). Similar results were also observed for LC-SPDP modified oLH derivatives (TABLE 3.11). The quantitative data are recorded in TABLE 3.11.

3.9: Characterization of gelonin :

3.9.1: Gel-permeation chromatography :

The molecular weight and homogeneity were further determined by HPLC using gel-permeation column. The protein was dissolved in 0.01M sodium phosphate buffer (pH 7.5) containing 0.01M NaCl and 0.1% sodium azide and run on a pre-calibrated LKB-2135-Ultro Pac-TSK-G4000SW (7.5x600mm) column connected with LKB-2135 Ultro Pac TSKSWP (7.5x75mm) as pre-column using Beckman HPLC system connected with 450 controller. The samples were also run on a Pharmacia gel-permeation Superose-12™ using Pharmacia FPLC pump. Both columns were calibrated by running standard molecular weight proteins. A plot of V_e/V_o *versus* log Mw gave a straight line. All batches of gelonin were run on the same column and their elution profiles showed multiple minor peaks with a major peak. The peak appearing in the void volume was due to the aggregation of gelonin which was later confirmed by immunoreactivity studies. Minor peaks were the contaminated

proteins. Gelonin purified by the Method II when subjected to HPLC/FPLC analysis, showed two peaks. As usual, the peak in the void volume was an aggregated protein while the major peak showed purified gelonin. A careful comparison of these profiles reveals that gelonin purified by the Method I contains more non-proteinous material absorbing at 280nm than that of other methods. The method II yielded relatively more purified material and was devoid of non-proteinous substances than the Method III.

3.9.2: RP-HPLC analysis :

Gelonin obtained from three different methods were further subjected to RP-HPLC analysis in order to determine the purity and homogeneity. A careful analysis of elution pattern clearly reveals a hump at the ascending protein of the peak as marked by the arrow (Figure 3.12) which could not be resolved even after applying a shallow gradient. This may be due to variation in glycosylation of gelonin.

3.9.3: SDS-PAGE analysis :

SDS-PAGE analysis was further performed on the two batches of preparations of gelonin under non-reducing and reducing conditions is shown in Figure 3.13. The estimated Mw was 30 kDa which is in agreement with the literature value (Stripe *et al*, 1980). Two minor bands of 32 kDa and 28kDa were

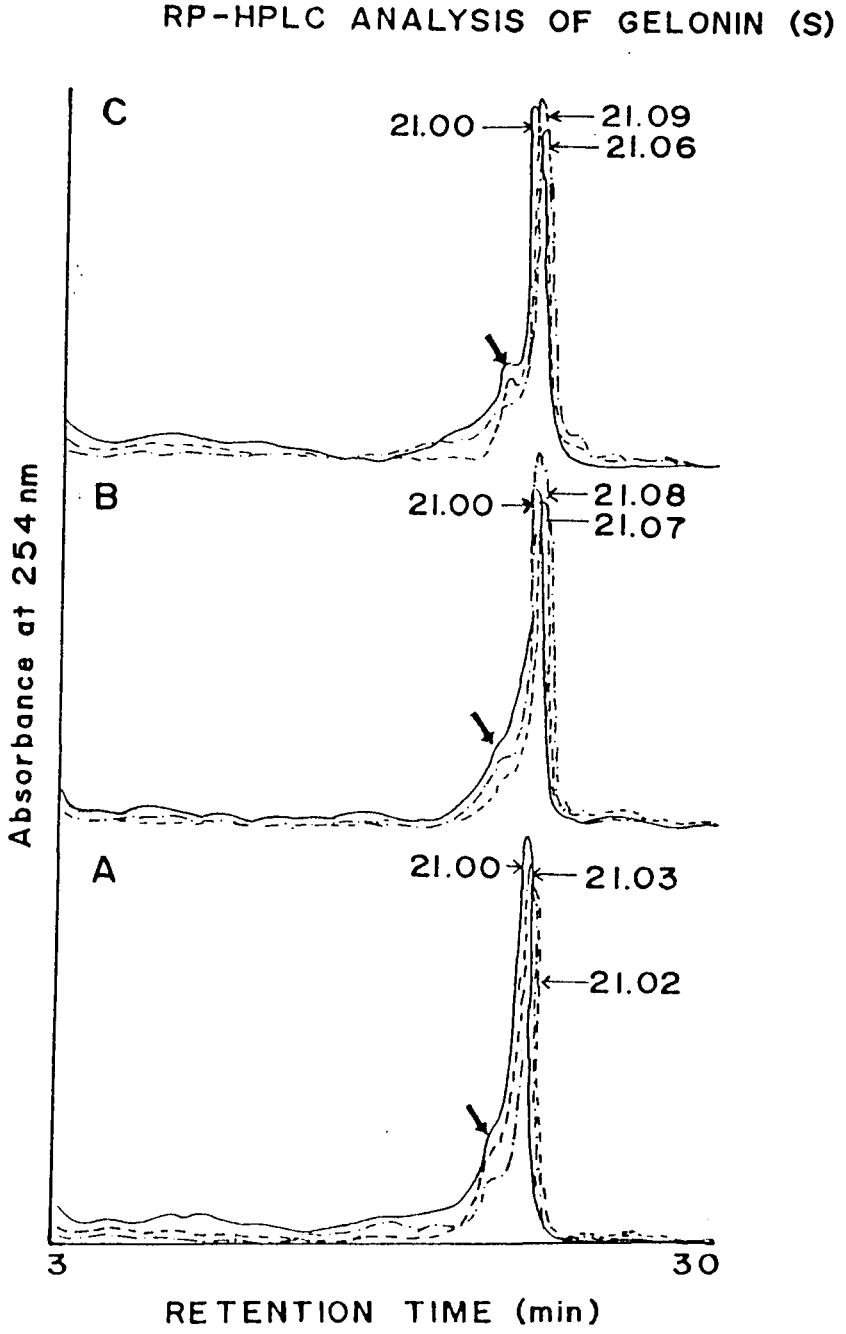


Figure 3.12: RP-HPLC analysis of different gelonin batches prepared by METHODS I (A), II (B) and III (C). Arrows show the presence of presumed isoforms of gelonin of different molecular weights. Retention times of peaks are shown. The experimental details are given in Materials and Methods section.

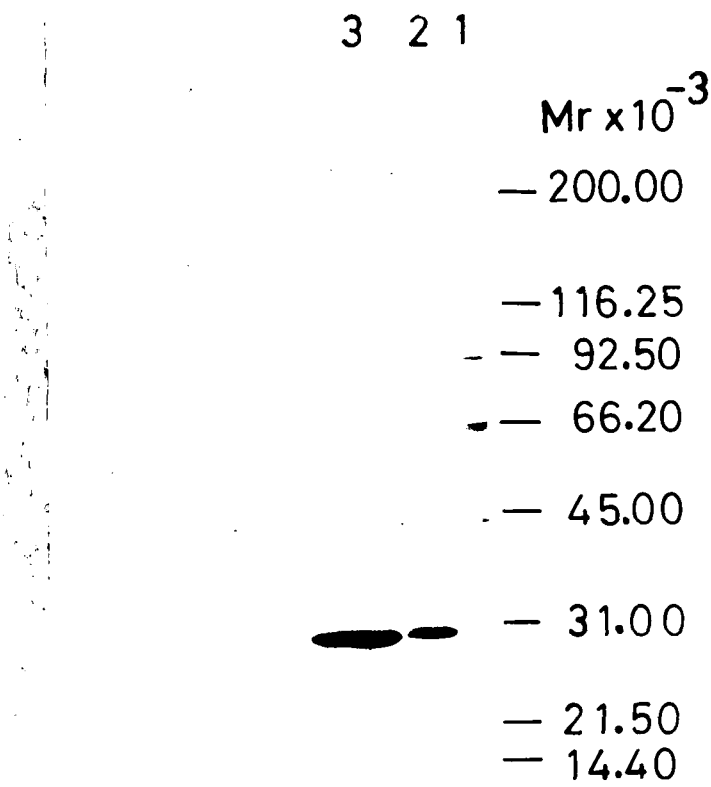


Figure 3.13: SDS-PAGE analysis of two batches (lane 2 and 3) of gelonin purified by Method II. A gel consisting of 5-15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained Commassie blue and destained with the methanol/acetic acid for 24 hr. Lane 1. (Molecular weight markers); myosin (200 kDa); β-galactosidase (116.25 kDa); phosphoamylase-B (92.5 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); soyabean trypsin inhibitor (21.5 kDa); lysozyme (14.4 kDa).

also observed which are possibly the variation of gelonin and could not be resolved. Possibly, the hump in RP-HPLC chromatogram may correspond to one or both of these components. Neither the position nor the intensity of the band was altered when run under reducing condition (Figure 3.13), thereby indicating that gelonin is a single chain polypeptide.

3.9.4: Gelonin antibody production :

The antibodies against gelonin were raised in rabbits (about 3 kg weight; New Zealand strain) by immunizing them with 1mg of gelonin dissolved in saline and emulsified with Freund's complete adjuvant. The subsequent injections were given at fortnightly intervals with Freund's incomplete adjuvant. The animals were bled after one week, and the immunoglobulin fraction was prepared by precipitation with ammonium sulphate at 50% final concentration. The precipitate was dissolved in 0.01M sodium phosphate buffer (pH 7.2), dialysed extensively and lyophilized.

3.9.5: Liposome entrapped gelonin :

The gelonin entrapped in the liposome was separated from the free gelonin on a Sepharose CL-4B column (Figure 3.14). The unentrapped gelonin obtained by FPLC (Figure 3.15) were analysed by SDS-PAGE analysis (Figure 3.15). When tested for the reactivity of entrapped and unentrapped gelonin with

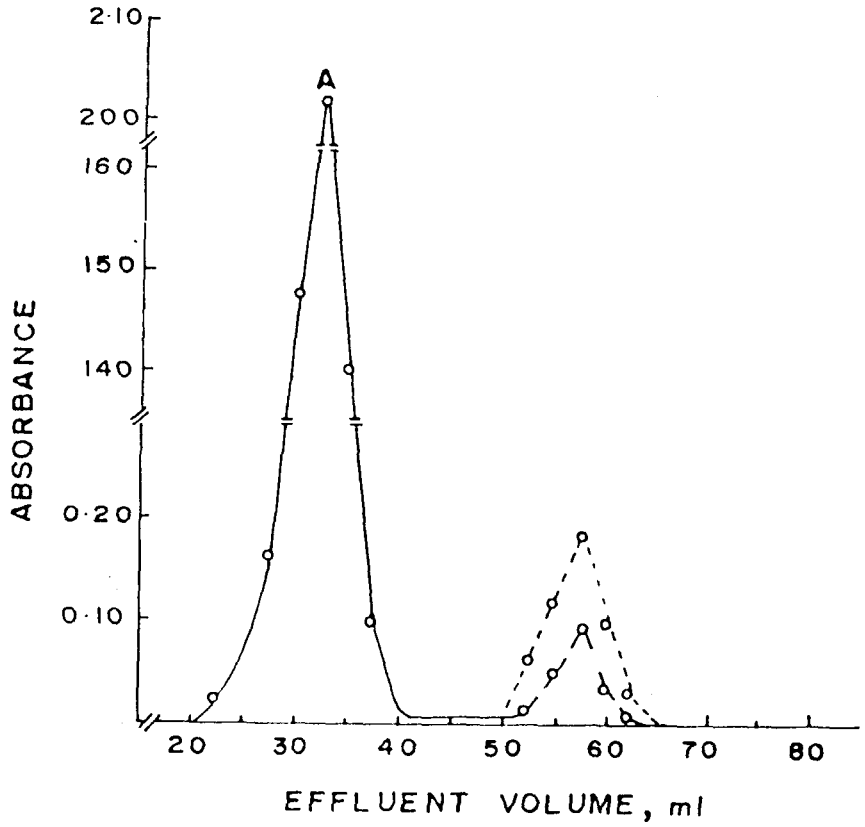


Figure 3.14: Separation of free and encapsulated gelonin on Sepharose CL-4B column. Liposome and free gelonin were eluted with tris-buffered saline (10mM, pH 7.4). A 5ml fractions were collected. Similar elution profiles were obtained for all the three liposomal compositions. Liposome eluted in the void volume (V_o) as shown in the peak 'A'. Free gelonin appeared much later (peak 'B'). Peak 'C' corresponds to elution profile of native gelonin. The elution profile of liposome was followed by measurement of absorbance at 300nm. Free gelonin was measured at 280nm.

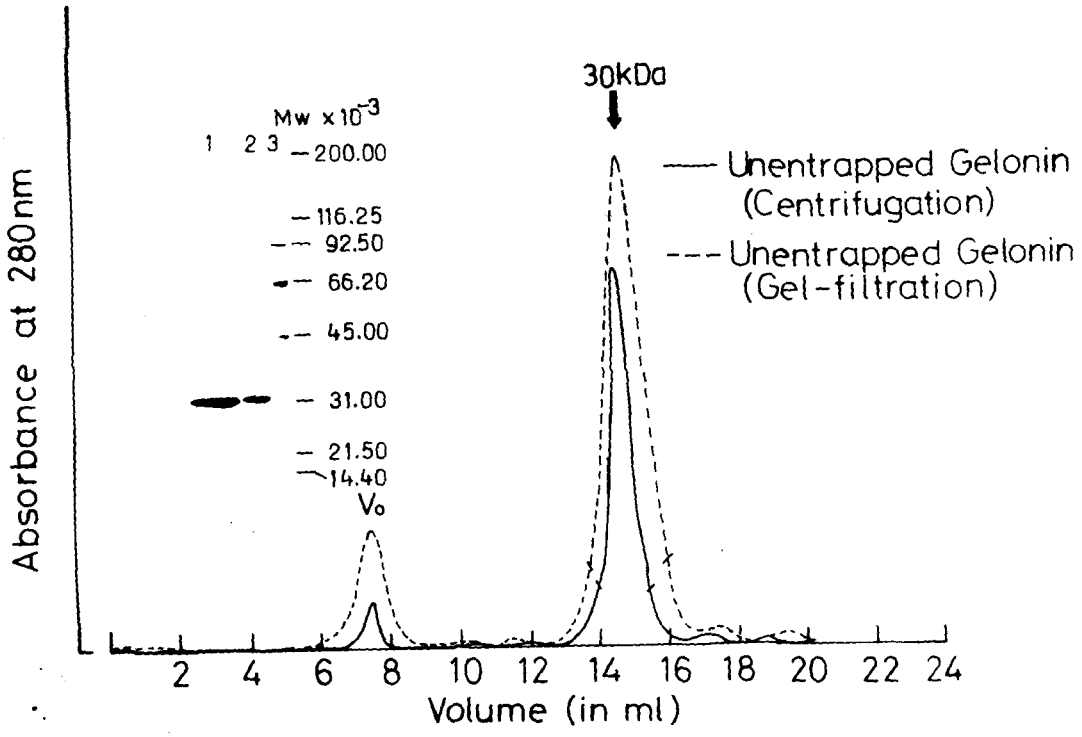


Figure 3.15: FPLC analysis of unentrapped gelonin obtained from centrifugation (——) and gel-filtration chromatography (---). Peak 'A' eluting in the void volume is the aggregated gelonin and peak'B', the gelonin eluting at 30kDa was subjected to SDS-PAGE analysis. Lane 1. 25μg unentrapped gelonin (centrifugation), Lane 2. 10μg of unentrapped gelonin (gel-filtration) and Lane 3, molecular weight markers.

gelonin antibody, it is found to retain the immunoreactivity (TABLE 3.12). The ribosome inactivating property of entrapped gelonin was found to be relatively higher than that of unentrapped gelonin. (TABLE 3.13, Figure 3.16). Possibly the unentrapped gelonin remained longer in contact with the chloroform which might have effected the protein synthesis inhibition property (TABLE 3.13, Figure 3.16).

3.10: Characterization of Hormonotoxins :

3.10.1: RP-HPLC analysis :

conjugates prepared with the use of SPDP/LC-SPDP :

The conjugates synthesised and purified as described above were further analysed by RP-HPLC. Figure 3.17 shows the RP-HPLC chromatograms of native oLH, gelonin and their SPDP and LC-SPDP modified derivatives. The analysis was carried out on the Water's μBondapak phenyl column, using a continuous gradient (as described in the legend of the Figure 3.17). A comparative study of oLH, SPDP or LC-SPDP modified oLH chromatograms clearly reveal that ε-NH_2 group modification primarily occours in the α-sub-unit of the hormone as seen by the emergence of new prominent fractions at $t_{8.2}$, $t_{9.4}$ in SPDP-oLH and $t_{8.98}$, $t_{9.4}$ and $t_{9.98}$ in LC-SPDP-oLH (Figure 3.17). In both the cases, the pattern of βoLH peaks in the elution zone at 14-16 min remain essentially unaltered (Figure

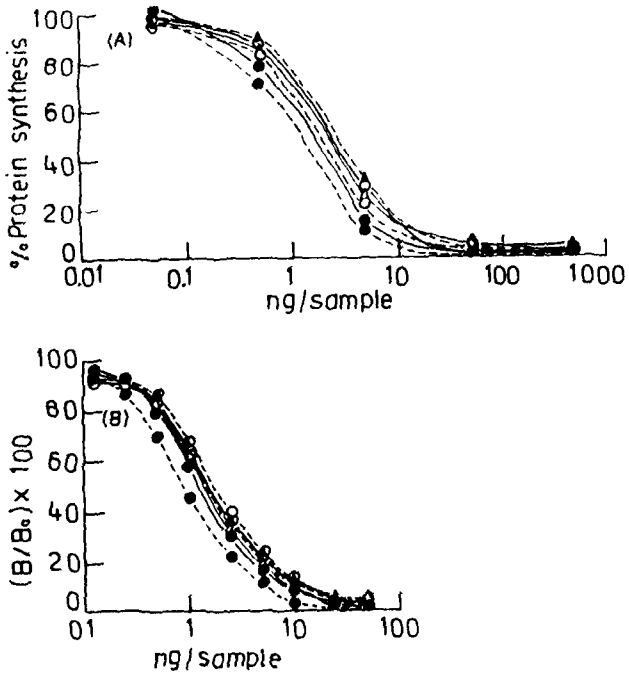


Figure 3.16: (A) Ribosome inactivating property of gelonin (●——●), HPLC-gelonin (●---●), unentrapped gelonin-C (▲——▲), gelonin-G (▲---▲), entrapped gelonin (O---O) and gelonin-G (O——O) as determined in a cell-free translation assay using rabbit reticulocyte lysate assay. (B) Immunological reactivity of gelonin antibody to gelonin (●——●), gelonin-HPLC (O---O), unentrapped gelonin-C (●---●), gelonin-G (▲——▲), entrapped gelonin-C (O---O) and gelonin-G (Δ——Δ) as obtained by a competitive displacement method described in the Methodology Section. The quantitative data of immunoreactivity and % protein synthesis inhibition are recorded in TABLES 3.12 & 3.13.

TABLE 3.12: Activity of entrapped and unentrapped gelonin with gelonin antibody. The immunoreactivity is expressed as percentage with respect to that of native gelonin, which was taken as 100%. ED_{50} is the dose causing 50% displacement.

Gelonin	Immunological activity (%)*	
	ED_{50}	% cross-reactivity
Gelonin - 200	1.20	100
Gelonin - HPLC	0.90	100
Unentrapped gelonin - C	1.62	74
Unentrapped gelonin - G	1.50	84
Entrapped gelonin - C	1.36	88
Entrapped gelonin - G	1.30	92

C indicates gelonin from centrifugation, G indicates gelonin from gel-filtration.

*Three separate experiments showed 3-7% deviation in immunoreactivity values.

TABLE 3.13: *In-vitro* ribosome inactivating property of gelonin as determined by Cell free translation using rabbit reticulocyte lysate assay. ID_{50} is the dose causing 50% inhibition

Gelonin	Biological activity (%)* (% protein synthesis inhibition)	
	ID_{50}	% activity
Gelonin - 200	1.65	100
Gelonin - HPLC	1.25	100
Unentrapped gelonin - C	2.54	65.0
Unentrapped gelonin - G	2.84	58.0
Entrapped gelonin - C	2.00	82.5
Entrapped gelonin - G	2.20	75.0

*Three separate experiments indicated 5-10% variation in the activity

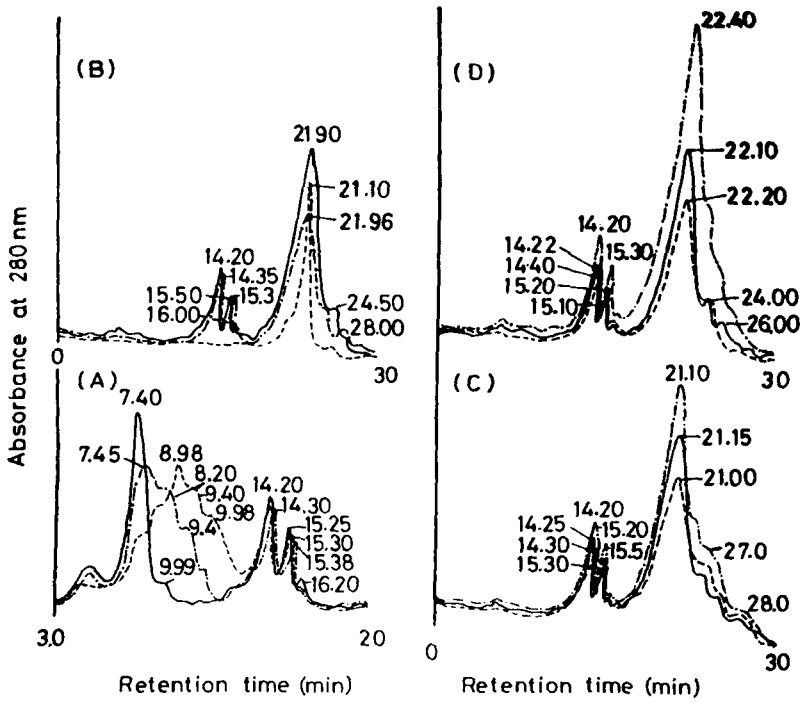


Figure 3.17: RP-HPLC analysis of oLH, SPDP and LC-SPDP modified oLH, gelonin and the conjugates. The samples were dissolved in H_2O containing 0.1% TFA (solvent A) and the analysis was performed on a Water's μBondapak phenyl column and (4x250mm) equilibrated in solvent A+25% CH_3CN and 0.1% TFA. A gradient of 25-50% was run over 30min at a flow rate of 2ml/min and monitored at 280nm. In case of oLH, oLH-SPDP and oLH-LC-SPDP, the chromatograms were analysed between 3-20 min, however in case of gelonin, oLH-gelonin conjugates the chromatograms were analysed between 0 and 30 min (panel A). The RP-HPLC chromatograms of oLH (——), oLH-SPDP (—.—) and oLH-LC-SPDP (---). Please note that the modification occurs in α-sub-unit (t_R = 7.4 min) while β-sub-unit (t_R = 14-16 min) remains unaltered (Panel B). The RP-HPLC chromatograms of gelonin-SPDP (---), oLH-gelonin conjugates C200A (—.—) and C200ARA (-.-.-) (Panel C). The RP-HPLC chromatograms of C210A (—), C210ARA(---) and C220A (-.-.-). (Panel D). The RP-HPLC chromatograms of C220ARA (——) , C230A (---) C230ARA (-.-.-). The structure of C200, C210, C220 and C230 conjugates is shown in TABLE 3.7. Note all the conjugates did not show the presence of α-sub-unit as it was co-valently conjugated to gelonin. Under the acidic condition, the β-sub-unit which is associated non-covalently was dissociated, from the complex and eluted around t_R = 14-16 min.

3.17A). The appearance of more peaks at the higher retention time may be due to the increase in hydrophobicity of LC-SPDP compared to SPDP. The elution pattern of SPDP or LC-SPDP gelonin essentially remains the same at ~ $t_{21.10}$ min, except that its extinction coefficient is higher. RP-HPLC of different conjugates are also shown in Figure 3.17B, C and D. A comparison of RP-HPLC chromatograms clearly reveal the presence of βoLH component in the expected zone, but there is a complete absence of the modified α-sub-unit, as this now remains covalently linked to gelonin in a disulfide bond. This further confirms that the conjugation selectively occurs through one of the amino groups of the α-oLH. The acidic conditions used in the RP-HPLC causes complete dissociation of the oLH sub-units which is distinctly separated by the gradient elution. The β-subunit eluted as a doublet in t_{14-16} min zone was due to the αLH-S-S-gelonin complex. Due to the higher extinction coefficient of gelonin, the conjugate peak eluting t_{21-23} min as a broad peak indicated relative higher concentration than the β-subunit (Figure 3.17B, C and D). Furthermore, the conjugates do not have free hormone contamination (LC-SPDP modified oLH) because of the lack of α-sub-unit peaks, which if present would have been easily detectable under the conditions employed here. This

observation further confirms the SDS-PAGE data in which the conjugates did not show the presence of either of the ingredients (SECTION 3.10.3).

conjugates prepared with the use of 2-IT :

The purified conjugates prepared with the use of 2-IT were subjected to RP-HPLC analysis to further assess purity and the site of linkage. Figure 3.18 shows a comparative RP-HPLC chromatograms of native oLH and oLH modified by 2-iminothiolane (Figure 3.18A). It is clear that the modification primarily occurs in the $-NH_2$ groups of α-subunit which is in agreement with those observations made for SPDP. Since, the elution zone at 12-16 min remains essentially unaltered, it may be concluded that the $\varepsilon-NH_2$ groups of beta subunit are resistant to such modification at the concentration used in the present investigation. Figure 3.18B shows RP-HPLC chromatograms of native gelonin, gelonin-25, αoLH and the oLH-gelonin conjugates (C50AA32 and C51AA35). Gelonin-25 eluted at $t_{22.40}$ as a sharp peak , which after conjugation to oLH broadened (Figure 3.18B). The βoLH peak eluted in t_{14-16} zone. The βoLH dissociated from the conjugate under the acidic conditions and can be seen in Figure 3.18B (t_{14-16}). Therefore, it is concluded that the α-subunit was cross-linked to gelonin in the conjugates which further

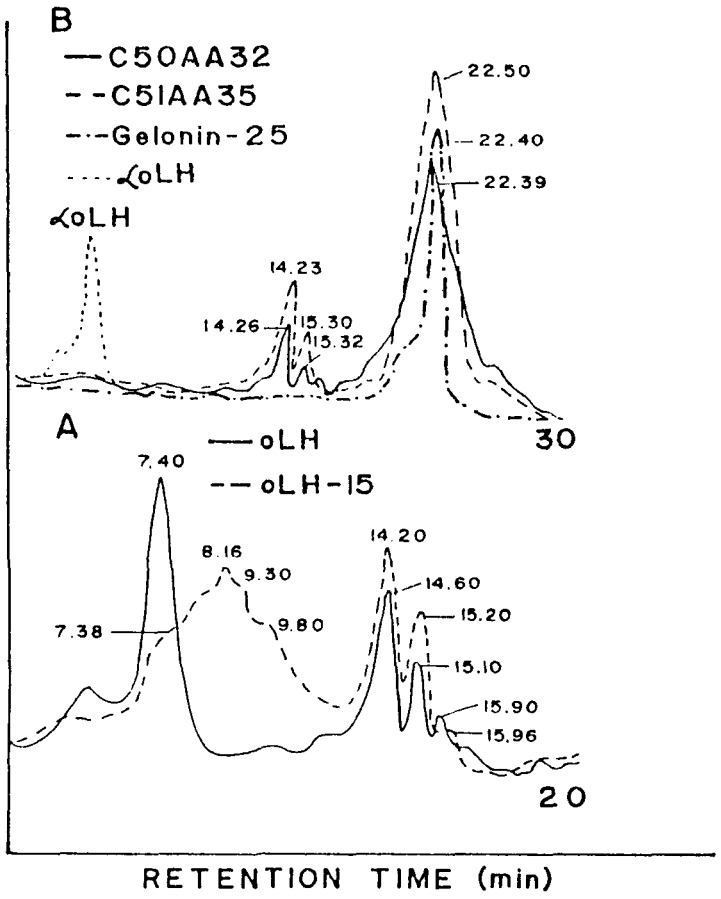


Figure 3.18: RP-HPLC analysis of oLH, oLH-2-IT and oLH-gelonin conjugates prepared with the use of 2-IT. The samples were dissolved in H_2O containing 0.1% TFA (solvent A) and the analysis was performed on a Water's μBondapak phenyl column and (4x250mm) equilibrated in solvent A+25% CH_3CN and 0.1% TFA. A gradient of 25-50% was run over 30min at a flow rate of 2ml/min and monitored at 280nm. In case of native oLH and oLH-15, the chromatograms were analysed between 0-20 min (Panel A). In case of αoLH, gelonin-25, oLH-gelonin conjugates C51AA35 and C50AA32, the chromatograms were analysed between 0-30 min (Panel B). Panel A: The RP-HPLC chromatograms of native oLH (——), oLH-15 (---). Panel B: The RP-HPLC chromatograms of αoLH (...), gelonin-25 (—.—) and oLH-gelonin conjugates; C51AA35 (---) and C50AA32 (——). Note both the conjugates did not show the presence of αoLH as it was covalently conjugated to gelonin. Under the acidic conditions, the β-subunit which is associated non-covalently was dissociated from the complex.

confirms the earlier mentioned observation where SPDP was used as a cross-linking agent. 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.

3.10.2: SDS-PAGE analysis :

conjugates prepared with the use of SPDP/LC-SPDP :

The SDS-PAGE analysis of two batches of oLH and the gelonin used in the conjugation reaction is shown in Figure 3.19. In SDS-PAGE analysis, the pattern of the conjugates were compared with the ingredients, oLH and gelonin. The samples analysed on SDS-PAGE under reduced and unreduced conditions are shown in Figure 3.20. Under unreduced conditions (Figure 3.20, left panel), the conjugates showed the absence of either free oLH or gelonin (lanes 3,4,7,8 and 11). These conjugates showed the distinct presence of both oLH and gelonin once they are reduced (Figure 3.20, right panel). The native oLH appears as a diffused band in between 31-45kDa (Figure 3.20, left panel,lane 13) and gelonin appears as a sharp band at ~ 31kDa (lane 12, left and right panel). The conjugates showed multiple bands of slower migrating component with apparent molecular weight of 55-116 kDa (lanes 3,4,7,8 and 11). The

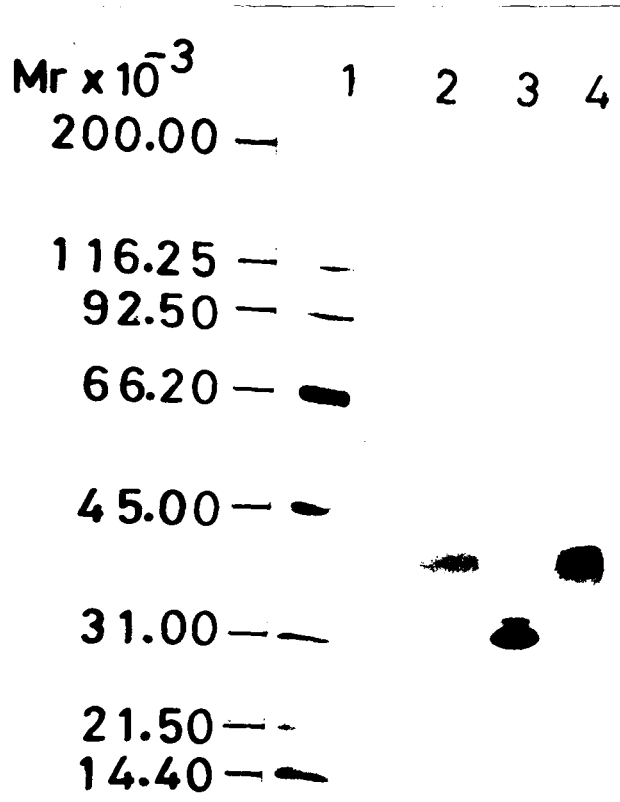


Figure 3.19: SDS-PAGE analysis of two batches of oLH (lanes 2 and 4) and one batch of gelonin (lane 3). A gel consisting of 5-15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained with Commassie blue and destained with the methanol/acetic acid for 24 hr. Lane 1. (Molecular weight markers); myosin (200 kDa); β-galactosidase (116.25 kDa); phosphoamylase-B (92.5 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); soyabean trypsin inhibitor (21.5 kDa); lysozyme (14.4 kDa).

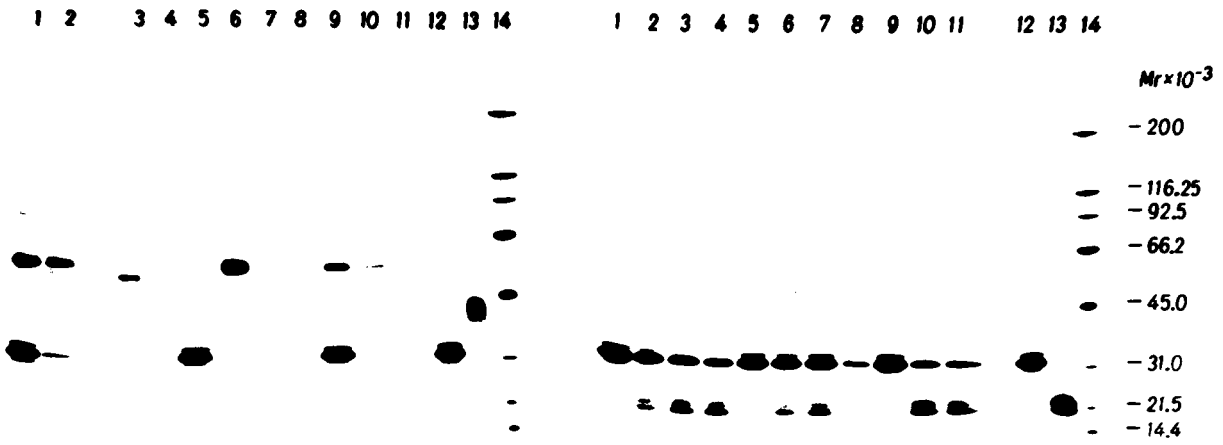


Figure 3.20: SDS-PAGE analysis of oLH-gelonin conjugate prepared by using SPDP and LC-SPDP. SDS-PAGE [reduced (right panel) and unreduced (left panel)] analysis of the conjugate and other peaks. A gel consisting of 5-15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained with Commassie blue and destained with methanol/acetic acid for 24 hr. Lane 1, C210C-P85-100; lane 2, C210B-P72-80; lane 3, C210A-P50-65; lane 4, C210ARA-P50-67; lane 5, C220C-P87-100; lane 6, C220B-P72-80; lane 7, C220ARA-P50-66; lane 8, C200A-P35-46; lane 9, C230C-P87-100; lane 10, C230B-P75-80; lane 11, C230ARA-P45-65; lane 12, Gelonin; lane 13, oLH; lane 14, molecular weight markers, myosin (200kDa); β-galacosidase (116.25kDa), phosphoamylase B (92.5kDa), bovine serum albumin (66.2kDa), ovalbumin (45kDa), carbonic anhydrase (31kDa), Soyabean trypsin inhibitor, (21.5 kDa), lysozyme, (14.4 kDa). A sample of 40ug each was run under reduced (right) and unreduced (left) panel conditions on the gel. Densitometric scan and quantitation was done on the reduced gel in order to determine molar ratio of the conjugate.

conjugates showed the distinct presence of both oLH and gelonin once they are reduced. (Figure 3.20, right panel).

conjugates prepared with the use of 2-IT :

The oLH-gelonin conjugates prepared with the use of 2-IT were subjected to SDS-PAGE analysis to ascertain the apparent molecular weight and purity. A representative analysis of conjugates under unreduced and reduced conditions are shown in Figure 3.21. The pattern of ingredients is also shown in Figure 3.21. The oLH band (Figure 3.21, lane 15) appears in between 31-41kDa and gelonin (Figure 3.21, lane 14) appears as a sharp band at ~ 30 kDa under the unreduced conditions used in the present analysis. Under the unreduced conditions, the conjugate did not show the contamination of ingredients, (lanes 10 and 13). Since the conjugate was linked by a S-S bridge, upon reduction both the ingredients were shown (Figure 3.21, lanes 3 and 6).

3.10.3: Immunoreactivity :

conjugates prepared with the use of SPDP/LC-SPDP :

Reactivity of oLH and gelonin with their specific antibodies in the different fractions of the conjugates was determined by competitive RIA (described in SECTION 2.10.3). The competitive displacement curves of oLH and gelonin are shown in Figures 3.22 and 3.23 respectively. The quantitative

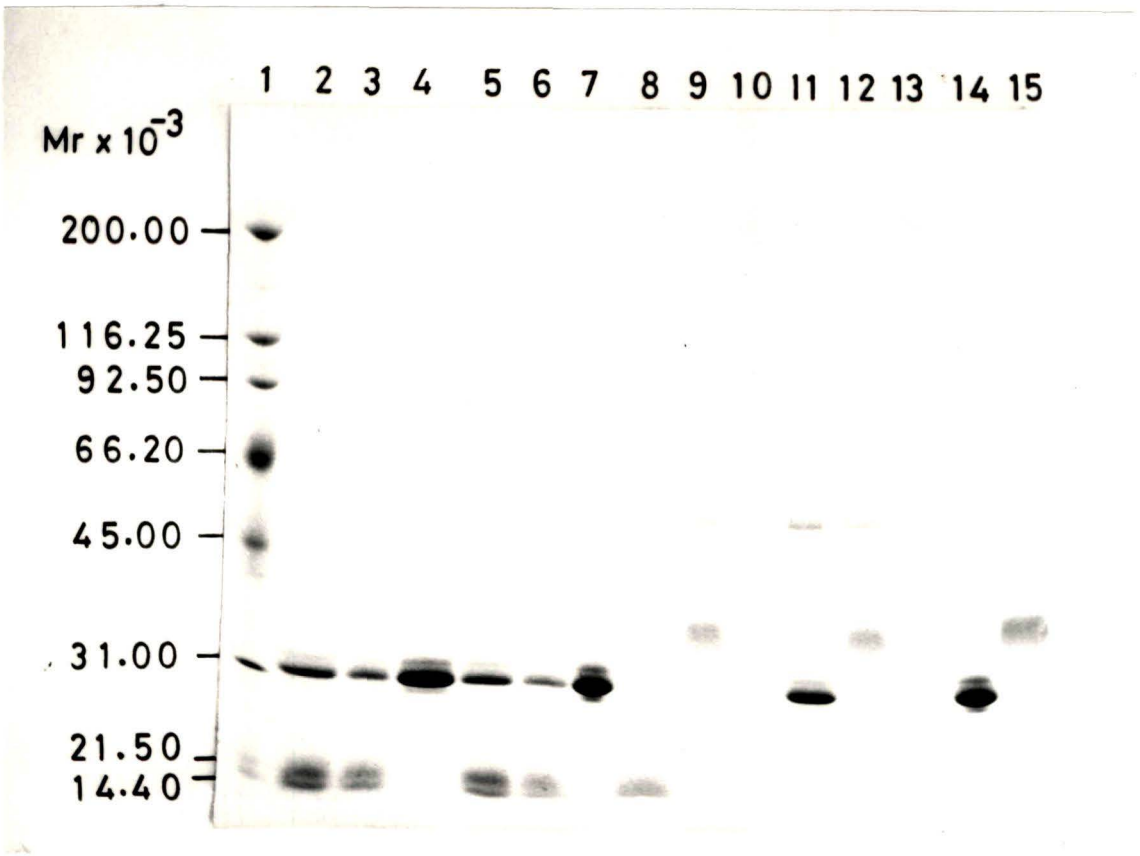


Figure 3.21: SDS-PAGE analysis of C50A and C51A conjugates prepared with the use of 2-IT. The electrophoretic behaviour of different fractions of C50 and C51 is shown under both reduced (lanes 2-8) and unreduced (lanes 9-15) conditions. A 30μg each ofthe sample ofthe different fractions were run under the unreduced (lanes 9-15) and reduced (lanes 2-8) conditions. A gel consisting of 5-15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained with Commassie blue. The lane 1 shows, the molecular weight markers, myosin (200kDa), β-galactosidase (116.25kDa), phosphoamylase (92.5kDa), bovine serum albumin (66.2kDa), ovalbumin (45.0kDa) carbonic anhydrase (31kDa), soyabean trypsin inhibitor (21.5kDa) 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. SDS-PAGE (unreduced) analysis of Lanes (9-15) : Native oLH (lane 15); gelonin (lane 14), C50AA32 (lane 13); C50B (lane 12); C50C (lane 11); C51AA35 (lane 10) and C51B (lane 9). Lanes (2-8) : SDS-PAGE (reduced) analysis of C50AA32 (lane 2); C50B (lane 3); C50C (lane 4); C51AA35 (lane 5); C51B (lane 6); C51C (lane 7) and native oLH (lane 8).

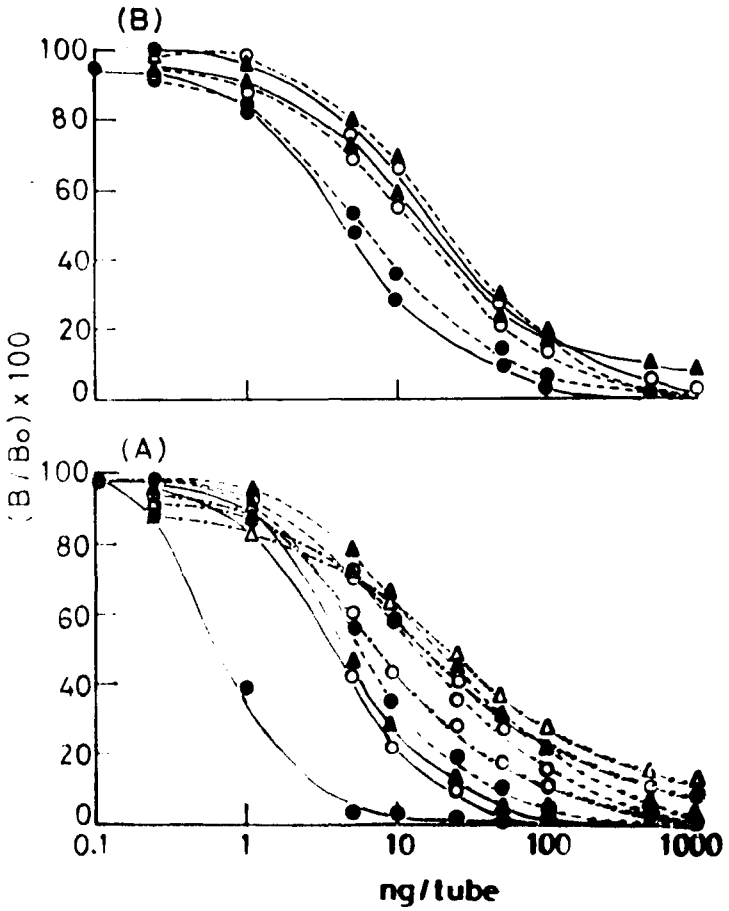


Figure 3.22: Immunological cross-reactivity of oLH antibody to oLH, its SPDP and LC-SPDP modified derivatives and their conjugates to gelonin. The percent immunoreactivity was determined by competitive displacement analysis using specific RIA as described in Method section. (Lower panel A). The displacement curves are oLH (●——●), oLH-SPDP (O——O), oLH-LC-SPDP (Δ——Δ), C200A (▲---▲), C200B(●--●), C200ARA (O---O),C230A (●-.-.-●), C230B (O-.-.O), C230ARA (Δ-.-.-Δ). Upper panel B shows the displacement curves of C210A (O—O), C210B (●——●), C210ARA (▲——▲), C220A (O--O), C220B(●--●), and C220ARA (▲---▲). The quantitative data based on these displacement curves are recorded in TABLE 3.14.

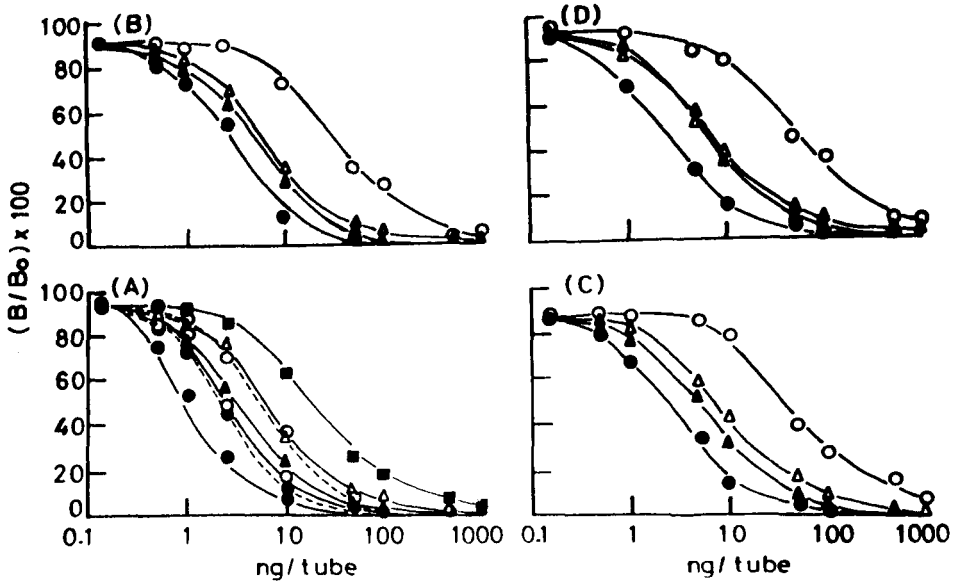


Figure 3.23: Immunological reactivity of gelonin, gelonin-SPDP, gelonin-LC-SPDP and their conjugates with oLH. The immunoreactivity was determined using specific gelonin antibodies as described in Materials and Methods SECTION 2.8. Panel A, competitive displacement curves of gelonin (●——●), gelonin-SPDP (O——O), gelonin-LC-SPDP (Δ——Δ), C200A (▲——▲), C200B (■——■), C200C (●---●), and C200ARA (O---O). Panel B shows the displacement curves of C210A (Δ——Δ), C210B (O——O), 210C (●——●) and C210ARA (▲——▲). Panel C shows the displacement curves of C220A (Δ——Δ), C220B (O——O), C220C (●——●) and C220ARA (▲——▲). Panel D indicates the displacement curves of C230A (Δ——Δ), C230B (O——O), C230C (●——●) and C230ARA (▲——▲).

immunoreactivity data of oLH and gelonin are recorded in TABLES 3.14 and 3.15 respectively. The immunoreactivity of oLH was drastically reduced (>86% activity) after a single $-NH_2$ group modification by SPDP. In case of LC-SPDP modified oLH, the activity was further reduced (>89%) (TABLE 3.14) which may be due to the increase in hydrophobicity of oLH-LC-SPDP. However, after conjugation, with gelonin, the oLH immunoreactivity was further affected which was in the range of 3-5% (TABLE 3.14). When oLH-SPDP or oLH-LC-SPDP immunoreactivity was taken as 100%, the activity of the gelonin conjugates varied from 22-41% (TABLE 3.14). In a 1:1 molar ratio conjugate, the immunoreactivity was doubled. Similarly, the gelonin immunoreactivity was reduced upto 30-36% after a single ε-NH_2 group modification with SPDP or LC-SPDP. However, the activity was not further affected after conjugation with oLH (TABLE 3.15).

conjugates prepared with the use of 2-IT :

Immunoreactivity of oLH and gelonin in their conjugates prepared with the use of 2-IT was determined by competitive RIA as described in the SECTION 2.10. The competitive displacement curves of oLH and gelonin in the conjugates are shown in the Figure 3.24A and 3.24B respectively and the quantitative immunoreactivity data are recorded in TABLE 3.16.

TABLE 3.14: Immunoreactivity of oLH in the oLH-gelonin conjugates prepared with the use of SPDP and LC-SPDP

Hormone-cross-link-gelonin	hormone assay		
		% cross-reactivity	
	ED_{50} (ng)	A	B
oLH	0.60	100	--
oLH-SPDP	4.15	14.5	100
oLH-LC-SPDP	5.45	11.0	100
oLH-S-S-gelonin			
C200A	18.70	3.2 (6.4)	22.10 (44.20)
C200B	3.75	16.0	110.60
C200C	N.D.	N.D.	N.D.
C200ARA	12.50	4.8	33.20 (66.40)
C210A-P50-65	51.80	3.8 (7.6)	26.26 (53.53)
C210B-P72-80	4.10	3.2 (6.4)	22.10 (44.20)
C200C-P85-100	N.D.	N.D.	N.D.
C210ARA-P50-67	14.30	4.20	29.00 (58.00)
C220A-P50-64	13.30	4.50 (9.00)	40.90 (81.80)
C220B-P72-80	6.00	10.00	90.83
C220C-P87-100	N.D.	N.D.	N.D.
C220ARA-P50-66	17.10	3.50 (7.0)	31.87 (63.74)
C230A-P52-65	15.80	3.80 (7.60)	34.50 (69.00)
C230B-P75-80	7.50	8.00	72.66
C230C-P87-100	N.D.	N.D.	N.D.
C230ARA-P45-65	18.75	3.20 (6.40)	29.06 (58.13)

The immunoreactivity is expressed as the percentage with respect to native oLH (column A) and oLH-SPDP/LC-SPDP (column B), which were taken as 100%. Numbers in parantheses show values when corrected for molecular weight of the conjugate assuming a 1:1 complex formation.

TABLE 3.15: Immunoreactivity of gelonin in the oLH-gelonin conjugates prepared with the use of SPDP and LC-SPDP

Hormone-cross-link-gelonin	gelonin assay			
		% cross-reactivity		
	ED_{50} (ng)	A		B	
gelonin	0.90	100		--	
gelonin-SPDP	2.50	36.0		100	
gelonin-LC-SPDP	3.00	30.0		100	
oLH-S-S-gelonin					
C200A	5.45	16.50	(33.0)	45.80	(91.60)
C200B	18.00	5.00		13.90	
C200C	2.30	39.00		108.00	
C200ARA	5.15	17.50		48.50	(97.00)
C210A-P50-65	5.00	18.00	(36.0)	60.00	(120.00)
C210B-P72-80	30.00	3.00		10.00	
C200C-P85-100	2.80	32.00		107.00	
C210ARA-P50-67	5.50	16.50	(33.0)	54.54	(109.10)
C220A-P50-64	4.73	19.00	(38.0)	52.85	(105.70)
C220B-P72-80	30.00	3.00		8.33	
C220C-P87-100	2.25	40.00		111.11	
C220ARA-P50-66	5.62	16.00	(32.0)	48.48	(98.96)
C230A-P52-65	5.62	16.00	(32.0)	53.40	(106.80)
C230B-P75-80	45.00	2.00		6.60	
C230C-P87-100	2.50	36.00		120.00	
C230ARA-P45-65	5.60	16.00	(32.0)	53.60	(107.20)

The immunoreactivity is expressed as the percentage with respect to native gelonin (column A) and gelonin-SPDP/LC-SPDP (column B) which were taken as 100%. Numbers in parantheses show values when corrected for molecular weight of the conjugate assuming a 1:1 complex formation.

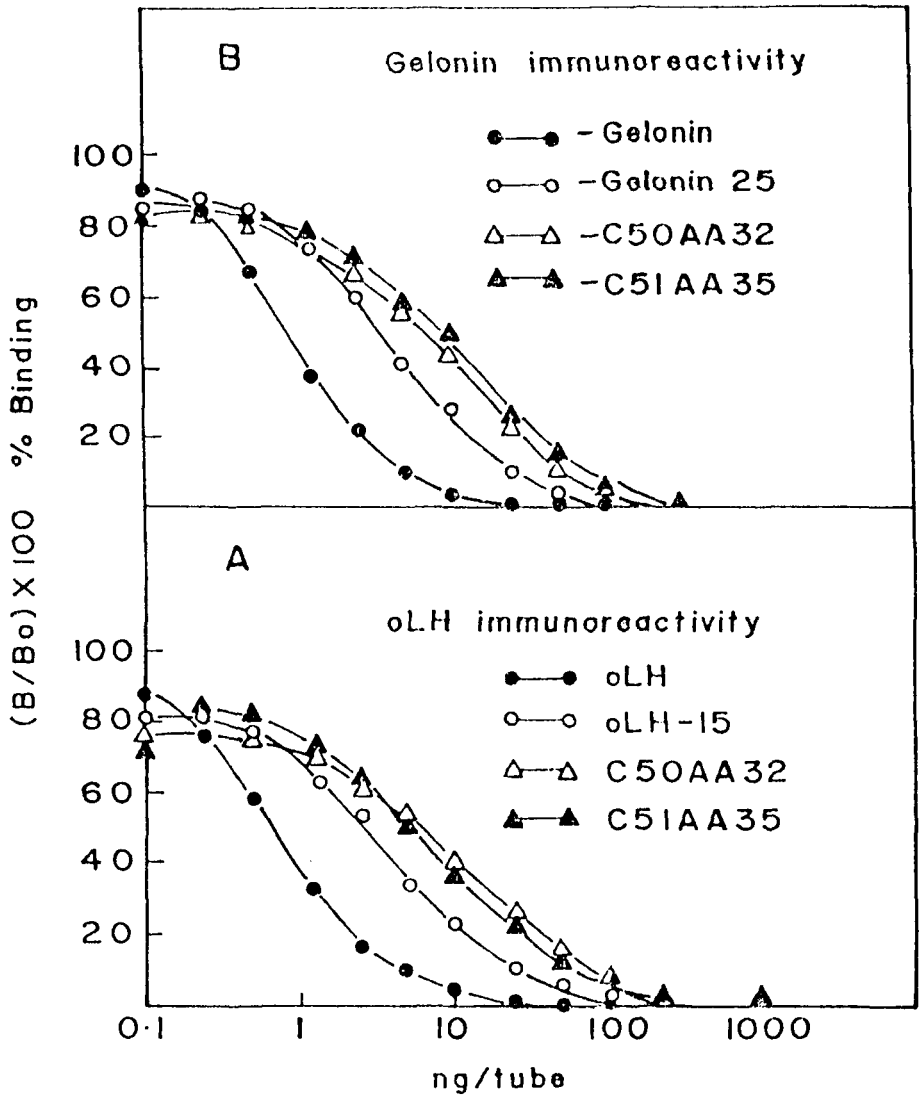


Figure 3.24: Immunological cross-reactivity of oLH and gelonin antibodies to oLH, gelonin, its modified derivatives and their conjugates prepared with the use of 2-IT (C50AA32 and C51AA35). The % immunoreactivity was determined by competitive displacement analysis using specific RIA as described in Materials and Method section. Lower panel A shows the displacement curves of oLH (●—●), oLH-15 (O—O), C50AA32 (△—△) and C51AA35 (▲—▲); upper panel B shows the displacement curves of gelonin (● - ●), gelonin-25 (O—O), C50AA32 (△—△), C51AA35 (▲—▲). oLH-15 is the code of oLH modified by 2-IT and Gelonin-25 is the code of gelonin modified by SPDP. The experimental details are given in SECTION 2.8.

TABLE 3.16: Immunoreactivity of oLH, oLH-15, gelonin, gelonin-25 and conjugates of oLH and gelonin prepared with the use of 2-IT. oLH and gelonin reactivity was determined by using specific 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 SECTION 2.8. ED_{50} is the dose causing 50% displacement.

Code	Immunoreactivity			
	oLH		Gelonin	
	ED_{50}	% Activity	ED_{50}	% Activity
oLH	0.65	100	--	--
oLH-15	2.60	25.0	--	--
Gelonin	--	--	0.90	100
Gelonin-25	--	--	3.40	26.47
oLH^{+}-S-S-Gelonin conjugates				
C50AA32	5.60	11.60 23.20[a] (46.42) (92.85)[a]	8.60	10.46 20.29[b] (39.50) (79.07)[b]
C51AA35	5.20	12.50 25.00[a] (50.00) (100.00)[a]	7.50	12.00 24.00[b] (45.30) (90.60)[b]

Values in parentheses indicate the immunoreactivity when oLH-15/ gelonin-25 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 was found to be close to 1:1 which was in agreement with the molar ratio determined by spectrophotometric and SDS-PAGE analysis. oLH-15 is the code of oLH modified by 2-iminothiolane and gelonin-25 is the code of gelonin modified by SPDP.

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 was found to be close to 1:1 which was in agreement with the molar ratio determined by spectrophotometric and SDS-PAGE analysis. oLH-15 is the code of oLH modified by 2-iminothiolane and gelonin-25 is the code of gelonin modified by SPDP.

The immunoreactivity of oLH was reduced upto 75% after a single ε-NH_2 group modification with 2-IT (TABLE 3.16, Figure 3.24). However, after conjugation, the oLH immunoreactivity was not further reduced drastically (TABLE 3.16). When oLH-15, (2-IT-modified oLH) activity was taken as 100%, the activity varied from 46-50%. 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.

3.10.4: Receptor binding activity :

conjugates prepared with the use of SPDP/LC-SPDP :

One of the primary requirment of biological activity of hormonotoxin was its binding to the hormone receptor. The receptor binding was determined by using rat testicular homogenate as a source of gonadotropin receptor. The receptor binding activity is shown in Figure 3.25. After a single -NH_2 group modification with SPDP or LC-SPDP, the activity was reduced to 8.5% and 7.0% respectively (TABLE 3.17). This was further reduced upon conjugation to gelonin (TABLE 3.17). As compared to oLH-SPDP or oLH-LC-SPDP with a single amino group modification and correction for increase in the

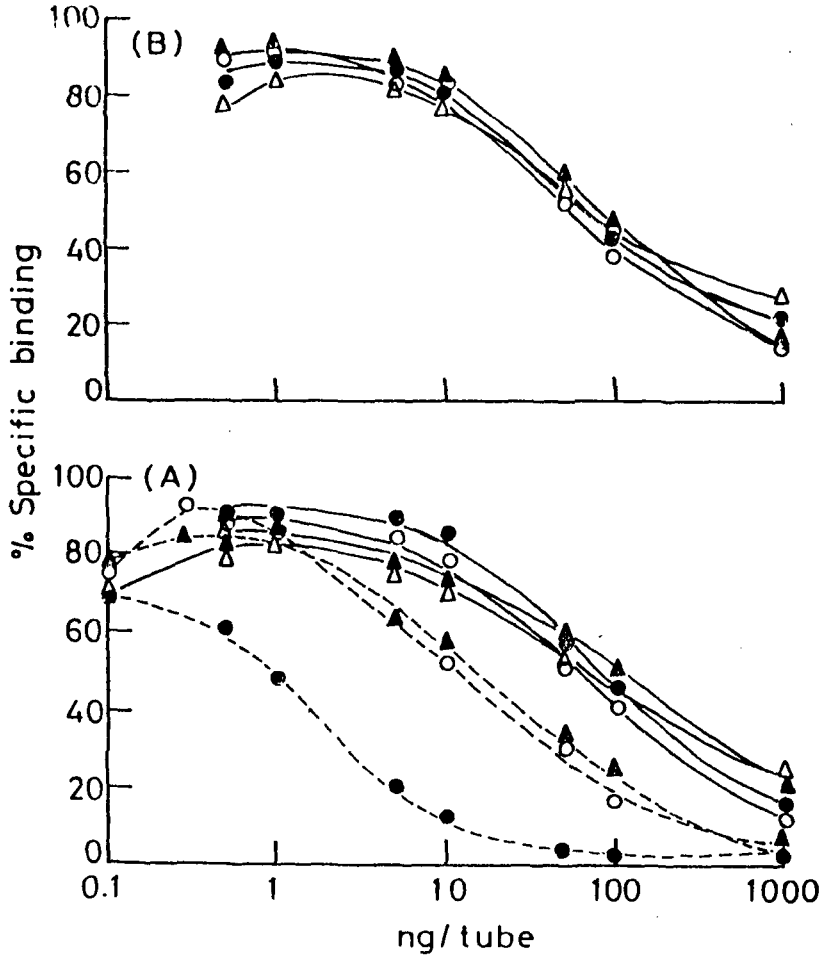


Figure 3.25: Receptor binding activity of oLH, its SPDP and LC-SPDP modified derivatives and their conjugates to gelonin. ^{125}I-oLH and rat testicular homogenate were used as a source of receptor to determine receptor binding activity in the competitive displacement analysis Lower panel A shows the competitive displacement curves of oLH (●--●), oLH-SPDP (O--O), oLH-LC-SPDP (Δ---Δ), C200A (●—●), C200ARA (O—O), C220A (Δ——Δ) and C220ARA (▲—▲). Upper panel B shows the competitive displacement curves of C210A (●—●), C210ARA (O—O) , C230A (Δ——Δ)and C230ARA (▲——▲).

TABLE 3.17: Receptor binding and steroidogenic activities of oLH-S-S-gelonin conjugates*

Hormone-cross-link-gelonin	Receptor binding activity			Steroidogenic activity (%)
	% activity			
	ED_{50} (ng)	A	B	
oLH	0.95	100	--	100
oLH-SPDP	11.20	8.5	100	110-120
oLH-LC-SPDP	13.60	7.0	100	120-130
oLH-S-S-gelonin				
C200A	79.10	1.20 (2.40)	14.16 (28.32)	3.4 (6.8)
C200ARA	59.40	1.60 (3.20)	18.85 (37.70)	3.2 (6.4)
C210A	68.00	1.40 (2.80)	16.48 (32.96)	4.0 (8.0)
C210ARA	61.00	1.56 (3.12)	18.36 (36.72)	3.6 (7.2)
C220A	105.00	0.90 (1.80)	12.95 (25.95)	3.0 (6.0)
C220ARA	79.20	1.20 (2.40)	17.20 (34.40)	2.6 (5.2)
C230A	79.20	1.20 (2.40)	17.20 (34.40)	3.5 (7.0)
C230ARA	63.40	1.50 (3.00)	21.45 (42.90)	2.5 (5.0)

The receptor binding is expressed as the percentage with respect to native oLH (column A) and oLH-SPDP/LC-SPDP (column B) which were taken as 100%. Numbers in parantheses show values when corrected for the molecular weight of the conjugate assuming a 1:1 complex formation.

molecular weight, the relative activity of the conjugate varied from 25-43% (TABLE 3.17). The rechromatographically purified conjugates (designated as C200ARA, C210ARA, C220ARA and C230ARA) showed relatively higher receptor recognition than the C200A, C210A, C220A and C230A (TABLE 3.17). The receptor binding ability of the conjugates prepared with the use of 2-IT is shown in the Figure 3.26. The quantitative data based on this observation are recorded in TABLE 3.18.

3.10.5: Steroidogenic activity :

conjugates prepared with the use of SPDP/LC-SPDP/2-IT :

All the conjugates stimulated progesterone production in rat granulosa cells, with a tendency to enhance steroidogenesis to the same extent as native oLH (Figure 3.27 and 3.28). Their relative potencies, when corrected for the presence of gelonin in the complex, varied from 5-8% in SPDP or LC-SPDP conjugates (TABLE 3.17) but 14-30% was observed in 2-IT conjugates (TABLE 3.18).

3.10.6: *In-Vitro* cell free translation assay :

In-vitro cell-free translation experiments were carried out on native gelonin, SPDP or LC-SPDP modified gelonin and their conjugates with oLH in order to determine their ability to inhibit protein synthesis. Figure 3.29 shows the inhibition of protein synthesis, and the quantitative data

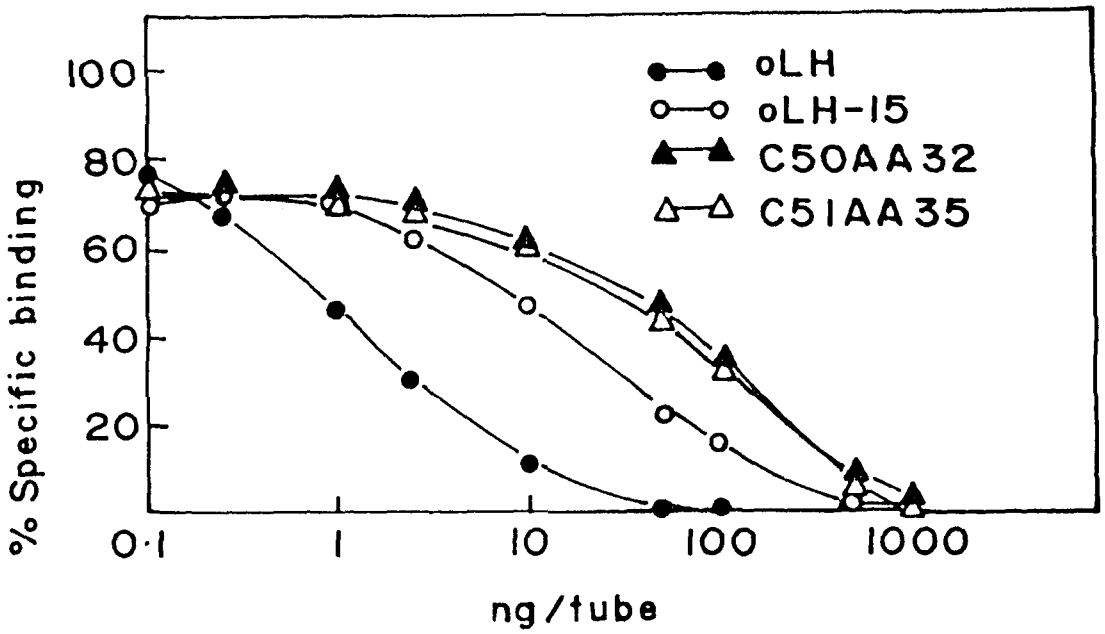


Figure 3.26: Receptor binding activity of oLH, oLH-15 and its conjugates to gelonin prepared with the use of 2-IT (C50AA32 and C51AA35). Rat testicular homogenate was used as a source of receptors and ^{125}I-oLH to determine the receptor binding activity of oLH. Panel shows the receptor binding activity of oLH (●——●), oLH-15 (O——O), C50AA32 (▲——▲) and C51AA35 (△——△). oLH-15 is the code of oLH modified by 2-IT.

TABLE 3.18: Receptor binding and steroidogenic activities of oLH, oLH-15 and their conjugates. The activities were determined as mentioned in SECTION 2.9.

Code	Receptor binding activity		Steroidogenic activity	
	ED_{50}	% Activity	ED_{50}	% Activity
oLH	0.90	100	1.40	100
oLH-15	8.50	10.58	1.27	110
oLH^{+}-S-S-Gelonin conjugates				
C50AA32	37.50	2.40 4.80[a] (22.60) (45.33)[a]	8.60	16.28 32.56[b] (14.76) (29.53)[b]
C51AA35	32.1	2.80 5.60[a] (26.48) (52.96)[a]	9.65	14.50 29.00[b] (13.16) (26.32)[b]

(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 and 5-12% deviation was observed in receptor binding and 10-15% deviation in steroidogenic activity.

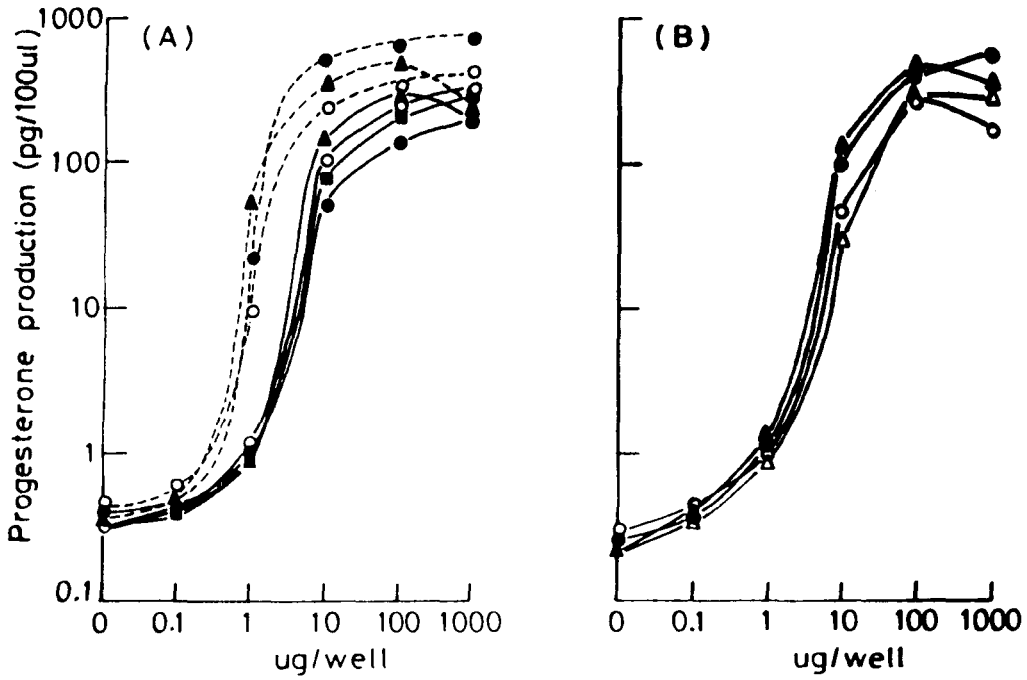


Figure 3.27: Steroidogenic activity of native oLH, SPDP and LC-SPDP modified oLH and their conjugates with gelonin. The progesterone induction ability was determined in Leydig tumor cells as described in Materials and Methods. Left panel A shows the progesterone induction ability of oLH (●--●), oLH-SPDP (O--O), oLH-LC-SPDP (▲---▲), C200A (●—●), C200ARA (O—O), C210A (▲——▲) and C220ARA (■--■). Left panel B shows the profile of C220A (● --●), C220ARA (O—O) , C230A (▲——▲) and C230ARA (△——△). Progesterone produced in 4 hr incubation was quantitated by specific RIA by using specific anti-progesterone antibodies. The quantitative data are recorded in TABLE 3.17.

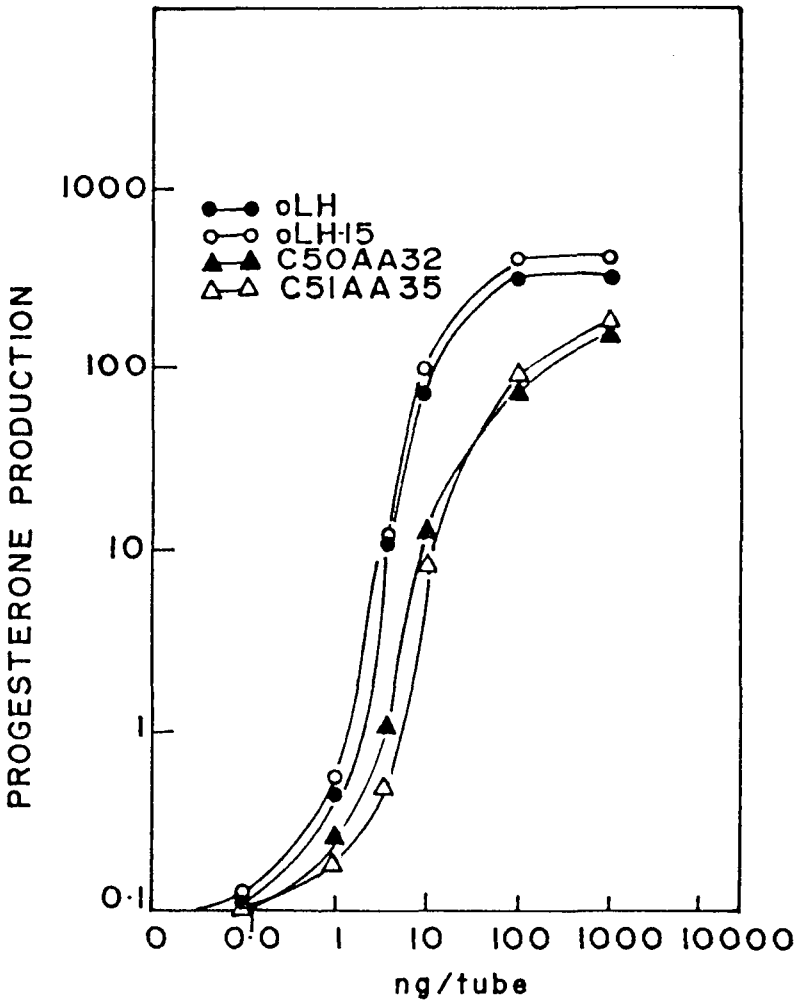


Figure 3.28: Steroidogenic activity of oLH, oLH-15 and its conjugates to gelonin prepared with the use of 2-IT (C50AA32 and C51AA35). The progesterone induction ability of oLH (●——●), oLH-15 (○——○), C50AA32 (▲——▲) and C51AA35 (△——△) are shown. oLH-15 is the code of oLH modified by 2-IT.

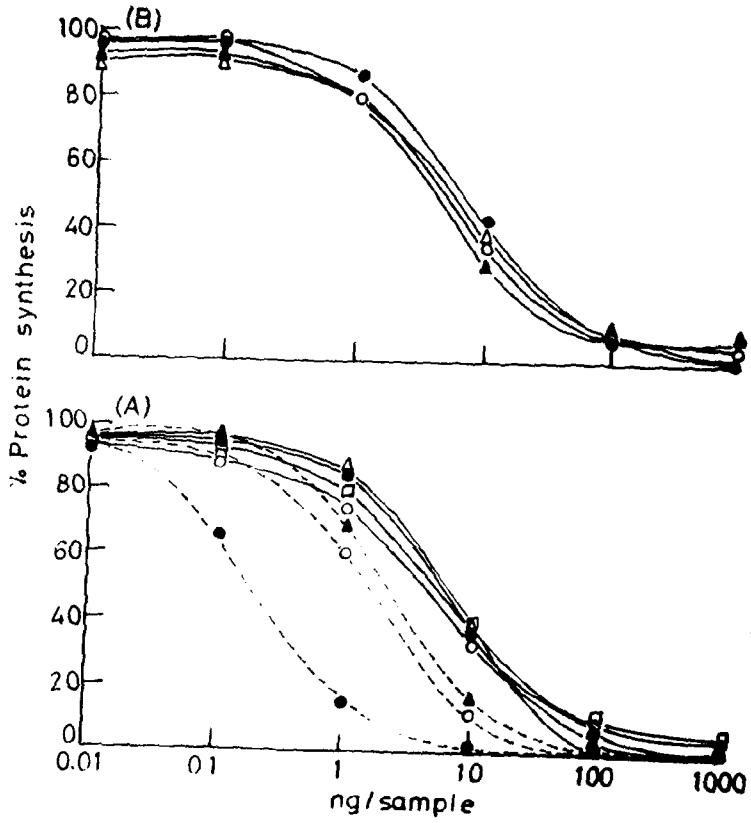


Figure 3.29: Inhibition of protein synthesis by gelonin, gelonin-SPDP, gelonin-LC-SPDP and their conjugates to oLH in a cell free translational system. Protein synthesis was measured by incorporation of ^{3}H-luecine into protein precipitable with TCA as described in the Material and Methods section. Percentage protein synthesis was calculated by measuring IC_{50} of control (native gelonin) and test samples. Lower Panel A shows the protein synthesis ability of gelonin (●--●), gelonin-SPDP (O--O), gelonin-LC-SPDP (▲--▲), C200A (●——●), C200ARA (O——O), C210A (Δ——Δ), and C210ARA (□——□). Upper panel B shows the protein synthesis ability of C220A (Δ——Δ), C220ARA (▲——▲), C230A (O——O), and C230ARA (●——●).

based on these observations are recorded in TABLE 3.19. The percentage bioactivity was determined after measuring IC_{50} ie, the dose of the test sample required for 50% inhibition of protein synthesis. A single amino group modification resulted in more than 85% loss in the ribosome-inactivating property (TABLE 3.19). Upon conjugation with oLH, the *in-vitro* protein synthesis inhibition activity was further affected (TABLE 3.19). By taking gelonin-SPDP or gelonin-LC-SPDP activity as 100%, and correcting for oLH contribution in a 1:1 molar ratio in the conjugate, the activity varied from 61-80% (TABLE 3.19).

Inhibition of protein synthesis in MLTC-1 tumor cells by a representative oLH-gelonin conjugate is shown in Figures 3.30 and 3.31. Incorporation of ^{3}H-Leucine (220,000 ± 20,000 cpm/well) by control untreated wells was taken as 100% protein synthesis. The extent (%) of inhibition in treated wells are expressed relative to controls. The molecular weight of oLH-gelonin conjugate in the calculation was taken as 57,300 Da (27,300 for oLH and 30,000 for gelonin) assuming the presence of a 1:1 combination. Percentage of protein synthesis was calculated on the basis of radioactivity incorporated in the precipitate (as described in SECTION 2.10.6).

TABLE 3.19: Ribosome inactivating property of gelonin, gelonin-SPDP, gelonin-LC-SPDP and oLH-S-S-gelonin conjugates*

Hormone-cross-link-gelonin	IC_{50} (ng)	protein-synthesis-inhibitor activity % activity	
		A	B
gelonin	0.25	100	
gelonin-SPDP	1.67	15.0	100
gelonin-LC-SPDP	2.08	12.0	100
oLH-S-S-gelonin			
C200A	5.43	4.6	30.75
		(9.2)	(61.50)
C200ARA	4.80	5.2	34.80
		(10.4)	(69.60)
C210A	6.25	4.0	33.30
		(8.0)	(66.60)
C210ARA	5.70	4.4	36.50
		(8.8)	(73.00)
C220A	4.50	5.5	37.10
		(11.0)	(74.20)
C220ARA	5.00	5.0	33.40
		(10.0)	(66.80)
C230A	5.20	4.8	40.00
		(9.6)	(80.00)
C230ARA	6.25	4.0	33.30

The ribosome inactivating property is expressed as the percentage with respect to gelonin (column A), gelonin-SPDP/gelonin-LC-SPDP (column B) which were taken as 100%.
The percentage protein synthesis was calculated on the basis of radioactivity incorporated in the precipitate. IC_{50} is the dose required for 50% inhibition of protein synthesis. Numbers in parantheses show the value when corrected for the molecular weight of the conjugate assuming a 1:1 complex formation.

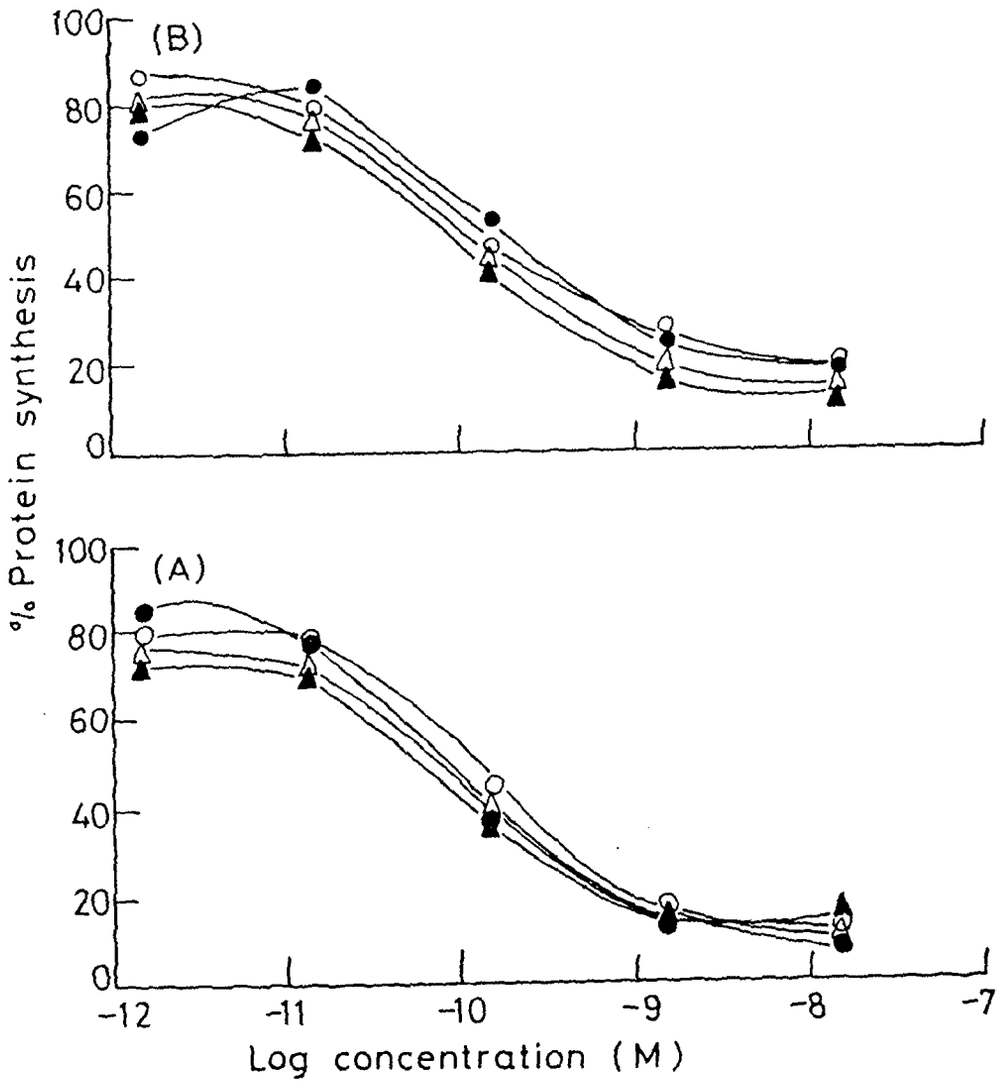


Figure 3.30 : Cytotoxic evaluation of oLH-gelonin conjugates prepared with the use of SPDP and LC-SPDP in mouse leydig tumor cells by leucine incorporation assay. The radioactivity incorporated in the control wells was taken as 100% and then the percentage inhibition in the treated wells are expressed. Lower panel A shows the percentage protein synthesis inhibition of C200A (●——●), C200ARA (O——O), C210A (Δ——Δ), and C210ARA (▲——▲). Upper panel B shows the protein synthesis inhibition curves of C230A (●——●), C230ARA (O——O), C220A (Δ——Δ), and C220ARA (▲——▲).

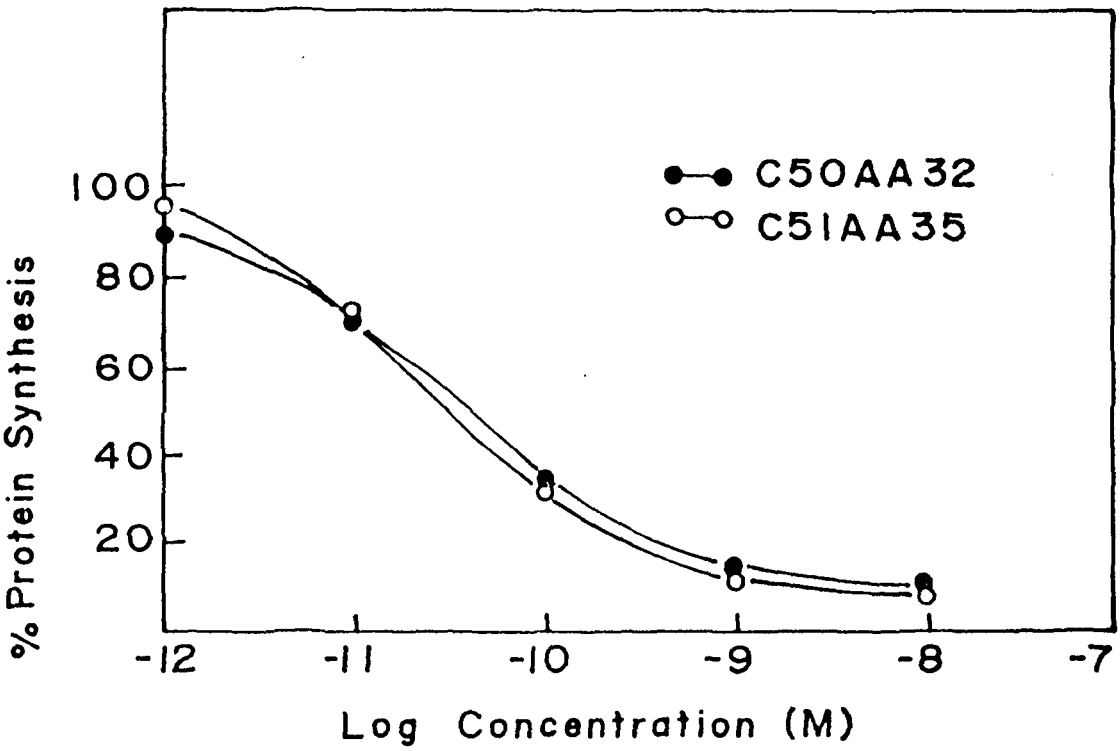


Figure 3.31: Cytotoxic evaluation of oLH-gelonin conjugates prepared with the use of 2-IT in mouse leydig tumor cells (MLTC-1) by leucine incorporation assay. The radioactivity incorporated in the control wells was taken as 100% and the percentage inhibition of protein synthesis in the treated wells was determined. The percentage inhibition of protein synthesis of C50AA32 (●——●) and C51AA35 (O——O) is shown in the figure. C50AA32 and C51AA35 are the codes of conjugates prepared with the use of 2-IT.

3.10.7: Binding and cytotoxicity to Leydig cells :

conjugates prepared with the use of SPDP/LC-SPDP :

The binding of the conjugates to the Leydig tumor cells was demonstrated with the help of gelonin antibody and by using ^{125}I-Protein-A as the probe. The binding of C200A, C200ARA, C210A, C210ARA conjugates are shown in the left panel of Figure 3.32 whereas, C220A, C220ARA, C230A and C230ARA conjugates are shown in right panel of Figure 3.32. Similarly, the binding is shown in Figures 3.32 and 3.33 (left). The non-specfic binding was determined by incubation of appropriate quantity of gelonin (as shown by dotted line in Figures 3.32 and 3.33 left).

All the conjugates saturate the receptors at a concentration of ~10μg/ml (Figures 3.32 and 3.33). The non-specific binding was also increased in the presence of half of the concentration of gelonin used but this was always less than 10%. This was in agreement with the earlier observations (Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991). On the basis of the present observation, a concentration of 10μg/ml of the conjugate was used for a competitive displacement analysis. The binding and displacement experiments are shown in Figures 3.33 (right) and 3.34. Open bar indicate the binding of untreated cells, cells

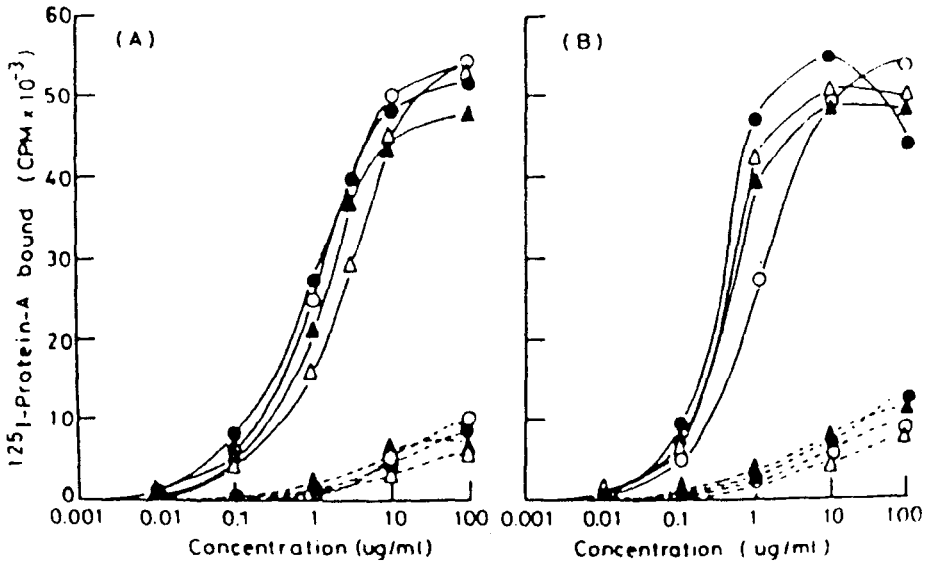


Figure 3.32: Saturation binding and competition ability of hormonotoxin to the leydig tumor cells. Saturation binding was determined as described in the text is shown by solid line while the non-specific binding is indicated by the dashed line. The cells were grown for 48 hr and subsequently washed with serum free medium were reacted with the indicated test materials. Following incubation and washing to remove unbound proteins, the cells were challenged with the anti-gelonin antibodies. The amount of antibody bound to the 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 site in the conjugate and detected by the antibody directed against gelonin. Left panel shows the saturation analysis of C200A (●—●), C200ARA (O—O), C210A (△——△) and C210ARA (▲——▲). Right panel shows the analysis of C220A (●—●), C220ARA (O—O), C230A (△——△) and C230ARA (▲——▲). The radioactivity incorporated in each set is a direct reflection of hormonotoxin binding via the gonadotropin site of the conjugate as described by gelonin antibodies.

treated with gelonin as well as with the conjugates (Figures 3.33 and 3.34). Binding and competition experiments of C200 and C210 are shown in the lower panel of Figure 3.34. The upper panel of this figure shows the data for C220 and C230 hormonotoxin. The solid bars show similar binding except the last one which indicates the competitive ability of the native oLH with the conjugates. It is evident from the data that the presence of native oLH in the binding reaction, clearly inhibited the conjugates to bind to the gonadotropin receptors of the tumor cells.

The cytotoxicity of the hormonotoxins, was evaluated using MLTC-1 cells which possess receptors for gonadotropin hormone and their action is mediated by internalization. The cytotoxicity was determined by leucine incorporation assay (SECTION 2.10.) (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991; Singh and Das, 1991). All the conjugates were toxic to the cells and the IC_{50} for C200A, C200ARA, C210A, C210ARA, C220A, C220ARA, C230A and C230ARA was $8.2x10^{-11}M$, $1.2x10^{-10}M$, $5.5x10^{-11}M$, $7.5x10^{-11}M$, $8.0x10^{-11}M$, $1.0x10^{-10}M$, $1.7x10^{-10}M$ and $1.3x10^{-10}M$ respectively. The cytotoxicity of the conjugates gradually decreased in the order of C200A > C220A > C210A > C230A. After rechromatography the order was C210ARA > C220ARA > C200ARA > C230ARA. In the parallel set,

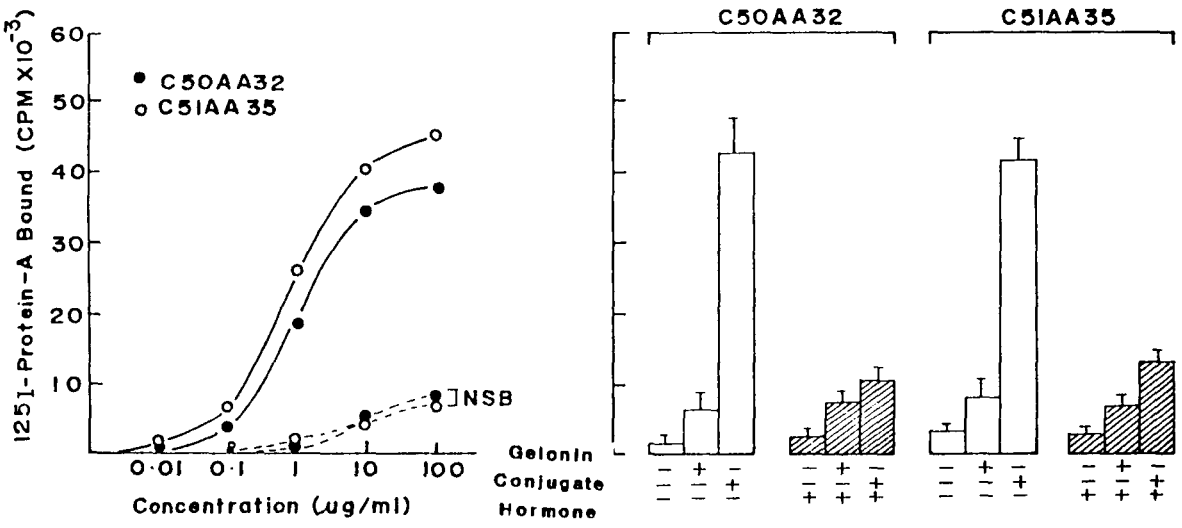


Figure 3.33: Saturation binding and competition ability of hormonotoxin to the mouse leydig tumor cells. Left panel shows the saturation analysis of oLH-gelonin [C50AA32 (●——●) and C51AA35 (O——O)] conjugates. Cells (~ 100,000/well) grown in RPMI-1640 media supplemented by 15% fetal calf serum for 48 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 unbound proteins, the cells were challenged with the anti-gelonin antibodies. The amount of antibody bound to the 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 site in the conjugate and detected by the antibody directed against gelonin. The non-specific binding (NSB) is shown by broken lines and open symbols obtained by using the gelonin fraction in each fractionation. Right panel 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 SECTION 2.10.7. 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 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 competitor (i.e., oLH) the bound radioactivity was significantly inhibited (as shown by hatched bar).

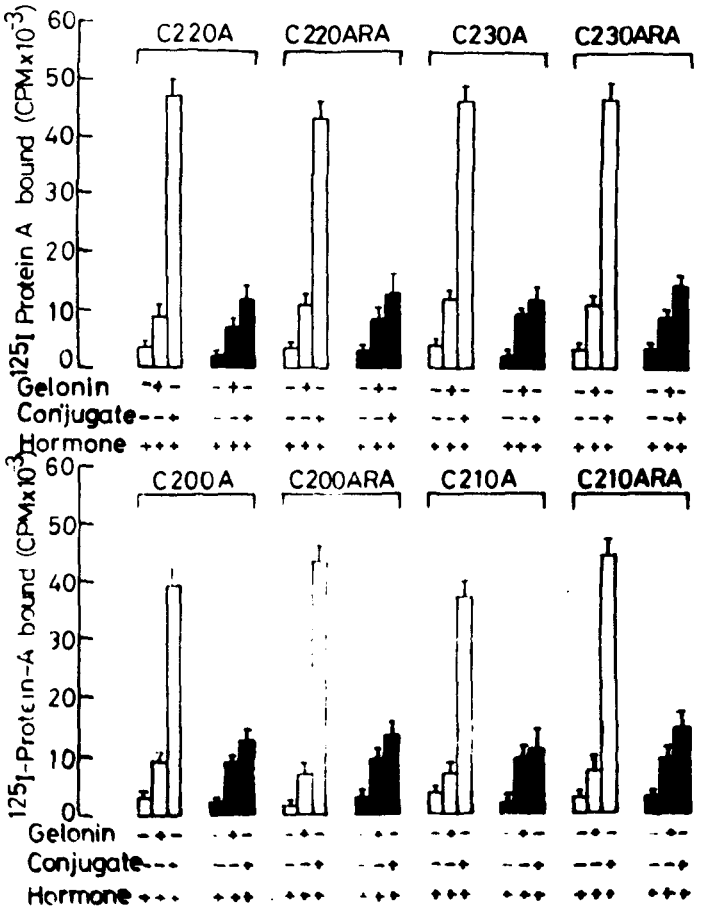


Figure 3.34: Competition ability of native oLH to the hormonotoxins for the binding to the Leydig tumor cells as described in the Method section. The binding assay was performed as described in Figure 3.33. The binding ability of the hormonotoxin (as shown by open bar) was drastically inhibited (as shown by closed bar) in the presence of competitor. (ie, native oLH) indicating that the binding occurs via oLH.

the conjugates incubated in the presence of native oLH did not show cytotoxicity.

While comparing carefully the cytotoxicity of SPDP/LC-SPDP conjugates with that of 2-IT conjugates, it is clearly shown that the cytotoxicity of the 2-IT conjugates to the cells was essentially higher than that of the conjugates prepared with the SPDP/LC-SPDP (Singh *et al*, 1989). The 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 cytotoxicity to the leydig tumor cells (Singh *et al*, 1989). 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 βoLH-gelonin conjugate failed to recombine with the native α-oLH and thus the bioeffectivity could not be recovered (Singh and Sairam, 1989).

PART - TWO

DISCUSSION

3.11: Modification and characterization of oLH :

3.11.1: Modification of oLH by SPDP :

Ovine LH contains 12 ε-NH_2 groups, 10 of which are in αoLH and 2 in βoLH. Based on three types of modification studies, introducing an acidic group, such as maleyl and citraconyl (Sairam and Li, 1975; Liu *et al*, 1974) or neutral groups like alkylation or carbamylation (Liu *et al*, 1977) or basic groups like guanidination or acetimidination (Liu *et al*, 1975), it has become clear that the most critical amino acids are in αoLH. The lack of ε-NH_2 groups in the β-subunit in the pig and rat further justifies the importance of -NH_2 groups in the α-subunit (Sairam and Li, 1975). The increasing use of heterobifunctional cross linking agents in the design and synthesis of hormone-carrier conjugates for selectively targeting to the specific cells (Singh *et al*, 1989; Singh and Sairam, 1989; Singh, 1991 a&b; Singh and Das, 1991; Oeltman and Heath, 1979; Bacha *et al*, 1983) or inducing anti-hormone antibody response (Talwar *et al*, 1983; 1986a,b&c; Singh, 1988, 1986; Gupta and Singh, 1988) prompted us to study the effect of -NH_2 group modification in the isolated αoLH and βoLH subunits on their recombination, immunoreactivity, receptor binding and biological activity. A modification of 0.70 to 5.7 (TABLE 3.9) amino groups by SPDP resulted in the loss of

immunoreactivity in the range of 22-2% and receptor binding (TABLE 3.10) in the range of 14.5-0.1%. Modification of 6 or more $-NH_2$ groups in the isolated αoLH although recombine fully with the native βoLH (TABLE 3.10), but failed to react with the oLH antibody (TABLE 3.9), and to LH receptors (TABLE 3.10). Introduction of SPDP in the initial four $-NH_2$ groups in the α-subunit compromised immunological and biological activities but further introduction of hydrophobic SPDP groups in the α-subunit abolished both immunological and biological activities (TABLES 3.9 and 3.10), therefore indicating the importance of later two $-NH_2$ groups in receptor recognition and steroidogenic activity. A single amino group modification resulted in a loss of 80% immunoreactivity and 90% receptor binding ability (TABLES 3.9 and 3.10). Receptor binding (3.10) and immunoreactivity (3.9) were compromised upto two-three $-NH_2$ modifications. Although, subsequent modifications upto 5 $-NH_2$ groups retained immunoreactivity upto 2%, receptor binding was drastically reduced upto 0.1% (TABLES 3.9 and 3.10). Contrary to αoLH, βoLH-SPDP modified derivative upto two ε-NH_2 groups retained substantial immunoreactivity (TABLE 3.9) and receptor binding (TABLE 3.10), thereby indicating that the $-NH_2$ groups of lysine in the α-subunit are more crucial in the receptor binding than

-NH_2 groups in the βoLH. The steroidogenic activity was determined by rat testicular homogenate and ^{125}I-oLH as tracer was found to be unaffected in the low molar ratio modified derivatives (upto 1:3) in αoLH-SPDP.βoLH recombinants (Figure 3.11). Higher modification affected the steroidogenic activity in the αoLH-SPDP-βoLH recombinant (Figure 3.11). Interestingly, 1:1 modification enhanced the steroidogenic activity. This may be due to the introduction of hydrophobic group in the α-subunit which might have affected in the inhibition of receptor recognition but enhancement of steroidogenic activity. No appropriate explanation could be given regarding 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. Therefore, It would of interest to identify the lysine residues which will possibly indicate the receptor binding site. Once such site is identified, the induction of immune response to such epitopes will become an easier task to design a specific immunogen.

3.11.2: Modification of oLH by LC-SPDP :

This was a part of our attempt to optimise the conditions of $-NH_2$ group modification of oLH with LC-SPDP and to study its effect on immunoreactivity, receptor binding and steroidogenic activity. The conditions are to be used for covalently cross-linking a plant toxin to oLH in order to obtain a bioeffective hormonotoxin. Similar studies, carried out by using SPDP, which introduced a $6.8A^{o}$ carbon chain in the hormone are comparable. Due to increase in chain length in LC-SPDP that enhances its hydrophobicity, the $-NH_2$ group modification study was initiated to determine its effect on immunological and biological properties. These properties are also compared with SPDP-modified derivatives. Like SPDP, introducton of more hydrophobic LC-SPDP group in the α-subunit did not alter the quarternary structure of the hormone as no dissociation occurs. The comparison of immunoreactvity and receptor binding properties of SPDP modified oLH with LC-SPDP modified derivatives of oLH clearly reveal that the introduction of LC-SPDP into oLH affected more drastically as immunoreactivity (TABLE 3.8) and receptor binding (TABLE 3.11) activities were further inhibited. However, the steroidogenic activity was relatively little affected although the recptor binding activity was drastically inhibited in such

preparations (TABLE 3.11). We have no rational explanation at the present time for this puzzling observation but others in previous studies also have noted higher potency for some derivatives of oLH in which ε-NH_2 groups of lysine were modified. It is possible that the increase in the hydrophobicity might have induced an orientation of the hormone-receptor complex in the cells which induces higher progesterone production in the cells. In some cases of alkyl substitution in the -NH_2 groups of oLH, some differences in adenylate cyclase activation and hormone-receptor interaction have been reported (Sairam, 1983). The report that 1% receptor occupancy is required for full steroidogenic response (Mendelson *et al*, 1975) may be another likely explanation for the retention of steroidogenic response in the SPDP/LC-SPDP modified derivatives (TABLE 3.11). A comparison on the immunoreactivity and receptor binding properties of SPDP modified oLH and LC-SPDP modified oLH derivatives clearly reveal the following observations. *(i) One or two NH_2 group modification with SPDP or LC-SPDP show comparable immunological* (TABLE 3.8) *and biological properties* (TABLE 3.11). *(ii) The immunological and biological properties of LC-SPDP modified oLH derivatives (3-4 NH_2 groups) were significantly hampered compared to the SPDP modified*

derivatives **(TABLES 3.8 and 3.11)**. *Upon further modification, with LC-SPDP, the immunoreactivity and receptor binding properties were virtually lost. However, similar SPDP modified derivatives did show some residual activity* **(TABLE 3.8 and 3.11).**

In conclusion, our efforts clearly demonstrate that a molar ratio of hormone:SPDP/LC-SPDP of 1:2 ± 0.2 could generate the site(s) in the α-subunit of oLH that retained some immunological and biological activity of the hormone (TABLES 3.8 and 3.11). This molar ratio was subsequently used in the design and synthesis of boeffective hormonotoxins.

3.12: Modification and Characterization of gelonin :

The determination of molar ratio of gelonin and its carrier oLH becomes very important for the construction of the carrier conjugate with respect to the cytotoxicity to the test system. This is because the antigen-antibody affinity is not drastically altered by the modification, but the hormone-receptor interaction is very sensitive and the affinity was reduced after SPDP modification of the hormone. As SPDP is usually employed in coupling gelonin to its carrier proteins, it was considered important to evaluate its impact on ε-NH_2 group modification on the immunoreactivity of gelonin. It might be mentioned that, in order to determine the

gelonin content in the conjugates, it is essential that the immunoreactivity of gelonin be not altered after conjugation to hormone. An RIA of oLH could not be used, since the extent of the decrease in immunoreactivity after conjugation is not consistent and uniform among the conjugates.

Each molecule of gelonin has 20 lysine residues as determined by amino acid analysis (Singh *et al*, 1989) and their modification by succinic anhydride did not hamper the ribosome-inactivating property of gelonin (Sreenivasan *et al*, 1985). Modification of up to 70% of the ε-NH_2 groups preserved 80% of the protein synthesis inhibiting ability as tested in a cell free translation assay (Singh, 1991). This observation strengthens the possibility of development of conjugates using ε-NH_2 groups. However, minor modifications resulted in the loss of 64-70% of immunoreactivity of gelonin (TABLE 3.15). The relationship between the number of amino group modified and the immunoreactivity may be divided into four categories based on the degree of loss. The modification of about one or two ε-NH_2 groups induced a loss of about 75-80% ; that of three ε-NH_2 groups gave a loss of up to 84-88% ; that of three to seven ε-NH_2 groups gave a loss of up to 97-99% and further modification up to twelve ε-NH_2 groups led to almost complete loss of activity. These data suggest that the ε-NH_2 groups are

somehow involved in the gelonin-anti-gelonin interactions. The molar ratio of oLH and gelonin in their conjugates as obtained by gelonin immunoreactivity clearly demonstrate the utilization of the gelonin immunoreactivity in the determination of molar ratio. These molar ratio data were quite comparable with earlier specctrophotmetric and SDS-PAGE analysis. As immunoreactivity of gelonin tends to stabilize at around ~ 30% when one or two amino groups participate in the reaction, and this is not subsequently reduced after conjugation, it seems reasonable to suggest that such derivatives would be suitable in the routine experiments on gelonin based hormonotoxin.

3.13: Conjugation of oLH with gelonin :

3.13.1: Conjugation by SPDP/LC-SPDP :

Based on the structure function data that the ε-NH_2 groups of α-subunit were more sensitive to chemical modification while the amino groups of β-subunit were resistant to such modifications, an attempt was made to cross-link β-subunit to the toxin, prior to recombination with α-subunit. But its failure of recomibination with native α-subunit, forced us to use native oLH to cross-link to the toxin by using SPDP as a cross-linking agent. oLH and gelonin have been modified by SPDP to introduce one potential site for

conjugation. Later SPDP modified gelonin was reduced with DTT to generate-SH group which was subsequently used to obtain oLH-S-S-gelonin conjugate. Similar to SPDP which introduces 8.6A° spacer arm , a long-chain SPDP has been used to generate a 15.6A° long spacer arm between oLH and gelonin. The hypothesis that the introduction of long spacer arm can effectively overcome the steric hinderence of oLH which may help in increased receptor binding and cytotoxicity. Therefore, the hormonotoxins in this study were synthesized with the help of SPDP alone, LC-SPDP alone and by using both SPDP and LC-SPDP (TABLE 3.7) in order to obtain conjugates of intermediate spacer arm and a comparative study on their immunoreactivity, receptor binding, biological activity and cytotoxicity was carried out. As observed in case of SPDP, the primary modification of ε-NH_2 group occurs in the α-subunit of oLH. But RP-HPLC analysis clearly demonstrated that the LC-SPDP modified α-oLH was more hydrophobic in nature compared to that of SPDP modified derivative. The α-subunit had 11 NH_2 groups (10 ε-NH_2 group and one α-NH_2) while βoLH possessed only 3 NH_2 groups (2 ε-NH_2 and one α-NH_2). The observations indicated that the amino groups of α-subunit are more accessible to SPDP/LC-SPDP modification than that of the β-subunit. The molar ratio of oLH and gelonin was found to be

1:1 as determined by three different methods such as spectrophotometric, amino acid and SDS-PAGE analysis (Singh *et al*, 1993).

3.13.2: Conjugation by 2-IT :

It has been found that the retention of positive charge on the ε-NH_2 groups of oLH plays an important role in the receptor recognition and the biological activity of the hormone. The SPDP conjugation lead to reduction of a positive charge. To construct a bioeffective oLH-gelonin conjugate, where the positive charge is preserved on the ε-NH_2 groups of oLH, 2-IT was used as the heterobifunctional cross-linking agent to modify oLH, on the basis of the observation that the 2-IT can retain the positive charge on the lysine residues of oLH during the conjugation process. (TABLE 3.16 and 3.18).

3.14: Characterization of Hormonotoxins :

The oLH-gelonin conjugates prepared with the use of SPDP/LC-SPDP and 2-IT were subjected to SDS-PAGE and RP-HPLC for its physico-chemical characterization. SDS-PAGE clearly revealed that the conjugate did not contain unreacted SPDP/LC-SPDP, 2-IT, oLH or gelonin as a contaminant (Figure 3.21). It is important to measure at this stage as oLH if present as contaminant will compete with the hormonotoxin and eventually affect the cytotoxicity of the hormonotoxin.

The physico-chemically analysed hormonotoxins were subjected to immunological and biological characterization. The immunoreactivity, receptor binding, steroidogenic activity and cytotoxicity of oLH-gelonin conjugates prepared with the use of LC-SPDP were comparable with that of SPDP (TABLES 3.14-3.19). The experiments further demonstrated that in case of hormonotoxin, the cytotoxicity is not affected with the use of long chain spacer arm. The oLH-gelonin conjugates prepared with the use of 2-IT, showed higher receptor binding and cytotoxicity comparative to oLH-gelonin conjugates prepared with the use of SPDP/LC-SPDP, which was due to the retention of positive charge on the lysine residues of oLH in the oLH-gelonin conjugates. The charge was retained during the conjugation reaction.

CHAPTER - FOUR

CONCLUSION

4.1: Purification and characterization of oLH :

oLH was purified from the lyophilized sheep pituitary powder according to the standard published procedures and was stored at 4°C. Purified oLH was subjected to characterization studies in order to determine its purity, homogeneity and biological activity prior to its application in experiments. The apparent molecular weight was determined as 37kDa. Purified oLH retained its immunoreactivity, receptor binding activity and steroidogenic potential as compared to standard oLH.

4.2: Purification and characterization of gelonin :

Gelonin, purified from the dry seeds of *Gelonium multiflorum* by three different methods. The ammonium sulfate fractionation method (Method-II) although yielded less amount of gelonin, but was completely devoid of non-proteinous material which absorbs at 280nm. Gelonin was further subjected to extensive physico-chemical, immunological and ribosome inactivating property studies. The molecular weight of gelonin was 30kDa and was homogeneous in nature. Purified gelonin showed high degree of reactivity against specific anti-gelonin antibodies. The ribosome inactivating property of gelonin was fully preserved after purification as shown by a cell-free translation assay. An attempt has also been made to study the

effect of liposome encapsulated gelonin on its immunoreactivity and ribosome inactivating property. Studies clearly demonstrate that the encapsulated gelonin fully preserves its immunological property but ribosome inactivating property was slightly hampered. The liposome encapsulation method involved mild conditions and was highly reproducible.

4.3: Modification of oLH :

The oLH which has 12 ε-NH_2 groups (10 in α and 2 in β) was subjected to SPDP/LC-SPDP modification in order to synthesize its conjugate to gelonin. The ε-NH_2 groups of αoLH and βoLH subunits were modified using SPDP/LC-SPDP. The modified αoLH derivatives recombined fully with the βoLH to generate a complete hormone. Sequential modification of α- and β-subunits led to progressive reduction in the immunoreactivity and receptor binding but the steroidogenic ability of βoLH-SPDP.αoLH was relatively comparable. Introduction upto four SPDP groups in αoLH compromised immunological and biological activities but further addition of two or more SPDP groups completely abolished antibody reactivity, receptor binding receptor binding and steroidogenic activity indicating the importance of later two -NH_2 groups in the receptor recognition and steroidogenic potential.

4.4: Modification of gelonin :

Nine different molar ratio of gelonin:SPDP were used to modify $-NH_2$ groups of gelonin. Thiolation of two $-NH_2$ groups resulted in the loss of 70-84% immunoreactivity against anti-gelonin antibodies. When 3 $-NH_2$ groups were modified, it resulted in a loss of 92% immunoreactivity and modification of 6-7 amino groups resulted 96-97% loss in immunoreactivity. Further modification of amino groups of gelonin completely abolished immunoreactivity property of gelonin. When the ribosome inactivating property was determined in a cell-free translation assay, like immunoreactivity, modification of a single $-NH_2$ group amounted to a loss of 90% protein synthesis inhibition activity. Further modification upto 2-3 amino groups resulted in 95-99.9% loss in RIP activity.

4.5: Conjugation of oLH and gelonin :

The conjugates of oLH to gelonin were prepared with the use of three types of heterobifunctional cross-linking agents, SPDP, LC-SPDP and 2-IT. Using SPDP/LC-SPDP four conjugates were synthesized having spacer arms of variable lengths ranging from 13.6A°-31.2A°. The conjugates were subjected to extensive characterization in order to determine purity, homogeneity and bioefficacy. The gel-filtration chromatography showed that the oLH-gelonin conjugates were devoid of any

ingredients. SDS-PAGE analysis both under reduced and unreduced conditions using purified conjugates further confirmed that the conjugates were devoid of unreacted oLH or gelonin. Upon reduction of the conjugates, the disulfide bond between the oLH and gelonin was reduced and the oLH and gelonin were seen on the gel. RP-HPLC analysis performed on the conjugates clearly demonstrated that the coupling occurs via the α-subunit of oLH. The conjugates retained hormone immunoreactivity (6-9%) and gelonin immunoreactivity (30-38%) compared to native oLH and gelonin respectively. The receptor binding ability of the conjugate ranged from 1-4% when compared to the native oLH. However, 4-7% steroidogenic activity was observed. The protein synthesis inhibitor activity of the conjugate was in the range of 60-80% as determined in a cell-free translation assay. The conjugates were shown to bind to the leydig tumor cells via the hormone leaving gelonin free as determined in a competitive displacement assay. In short, the hormonotoxins internalized to a sufficient degree to inhibit protein synthesis activity.

4.6: Role of positive charge on oLH :

Since, the positive charge on the lysine residues of oLH plays an important role in the affinity of oLH to its receptors and subsequently its biological activity, the

oLH-gelonin conjugates were prepared with the use of 2-IT. A total of 1:5 molar ratio of the oLH:2-IT was required to modify one ε-NH_2 to generate one -SH group. The conjugates prepared with the use of 2-IT were subjected to physico-chemical and biological characterization in order to assess their purity, homogeneity and bioefficacy. SDS-PAGE analysis under unreduced conditions clearly showed that the molecular weight of the conjugates were in the range of ~ 57kDa and were devoid of free oLH, gelonin, modified oLH or modified gelonin. When subjected to immunoreactivity studies, it is found that a single -NH_2 group modification of oLH by 2-IT resulted in a loss of 75% immunoreactivity, but after conjugation, the immunoreactivity was not much altered. Like immunoreactivity, receptor binding ability was also reduced to ~ 10% by a single -NH_2 group modification, which was further reduced after conjugation with gelonin to a level of 4-6%. But steroidogenic potential was preserved may be due to the fact that, even a 1% receptor binding is enough to elicit a response in terms of steroid hormone induction. Binding and cytotoxic studies using competitive displacement analysis showed that the hormonotoxin conjugates bind to the receptor cells via the oLH part, leaving gelonin free. When oLH-gelonin conjugate synthesized by using 2-IT was compared for

cytotoxicity with that of conjugates synthesized with SPDP/LC-SPDP, the former exhibited higher cytotoxic potential, possibly due to its ability to preserve the positive charge on the ε-NH_2 groups of ovine luteinizing hormone during the conjugation reaction.

REFERENCES

Abuchowsky, A., Van Es, T., Palczuk, N.C. and Davis, F.F., J. Biol. Chem., 252, 3578-3581, 1977.

Ampon, K. and Means, G.E., Biotechnol. Bioeng. 32, 689-697, 1988.

Bacha,P., Murphy, J.R. and Reichlin, S., J. Biol. Chem.,258, 1565-1570, 1983.

Barbieri, L and Stripe, F., Cancer Surveys, 1, 489-520, 1982.

Barthelemy, I., Martineau, D., Ong, M., Matusunami, R., Ling, N., Benatti, L., Cavallaro, U., Soria, M.R. and Lappi, D.A., J. Biol. Chem., 268, 6541-6548, 1993.

Bayley, H., In Photogenerated reagents in Biochemistry and Molecular biology, Elsevier, New York, 1983.

Blossey, H.C., Gayer, B. and Amiv-Zaltsman, Y., Mol.Cell. Endo. 46, 245-249, 1986.

Boime, I., Boothby, M., Hoshina, M., Daniels-McQueen, S. and Darnell, R., Biol. Reprod., 26, 73-91, 1982.

Boothy, M., Kukowska, J. and Boime, R., J. Biol. Chem., 258, 9250-9253, 1983.

Bousefield, G.R. and Ward, D.N., J. Biol. Chem., 259, 1911-1921, 1984.

Carlsson, J., Drevin, H. and Axen, R., Biochem. J., 173, 723-737, 1978.

Carr, E.E . and Chin, W.W., Endocrinology, 116, 1151-1157, 1985.

Cavallaro, U., Vecchio, A.D., Lappi, D.A. and Soria, M.R., J. Biol. Chem., 268, 23186-23190, 1993.

Cawley, D.R., Hershman, H.R., Gillisand, G.D. and Collier, R.J., Cell, 22, 563-570, 1980.

Choudhary, V.K., Mizukami, T., Furest, T.R., Fitzgerald, B.J., Moss, B., Pastan, I. and Berger, E.A., Nature, 335, 369-372, 1988.

Coleman R.F., Annu. Rev. Biochem., 52, 67-91, 1983.

Das, M and Fox, C.F., Annu. Rev. Biophys. Bioeng., 8, 165-193, 1979.

Dixon, H.B.F. and Perham, R.N., Biochem. J., 109, 312-314, 1968.

Ellman, G.L., Arch. Biochem. Biophys., 82, 70-77, 1959.

Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K., J. Biol. Chem., 262, 5908-5912, 1987.

Endo, Y. Tsurugi, K., J. Biol. Chem., 263, 8735-8739, 1988.

Fiddes, J.C. and Goodman, H.M, Nature, 281, 351-356, 1979.

Fiddes, J.C. and Goodman, H.M, Nature, 286, 684-687, 1980.

Fraker, P.J. and Speck, J.R., Biochem. Biophys. Res. Commun., 80, 849-857, 1978.

Frankel-Conrat, H. and Olcott, H.S., J. Am. Chem. Soc., 70, 2673-2684, 1948.

Ghose, T. and Blair, A.H., CRC Crit. Rev. Ther. Drug carrier systems, 3, 263-359, 1987.

Glenn, S.D., Liu, W-K., and Ward, D.N., Biol. Reprod., 25, 1027-1033, 1981.

Goldstein, L., Leven, Y. and Katchalsky,E., Biochemistry, 3, 1913-1919, 1964.

Gordon, W.L. and Ward, D.N. in Luteinizing Hormone Action and Receptors editor: M.Ascoli, CRC Press, Boca Raton, FL. 1985, pp 173-197,

Greenfield, L., Groetsema, G., Bell, D., Young, J., Chan, T., Auckerman, L., Nitecki, D. and Moreland, M., II[nd] International sypmposium on Immunotoxins, Lake Buena Vista, USA, 1990.

Greenfield, L., Jhonson, V.G. and Youle, R.J., Science, 238, 13203-13207, 1987.

Griffin, T.W., Richardson, C, and LePage, D., Cancer Res., 47, 3328-3336, 1987.

Han, K.K., Richard,C. and Delacorte, A., Int. J. Biochem., 16, 129-145, 1984.

Herriot, R.M., Adv. Protein Cem., 3, 161-225, 1947.

Hudson, E.N. and Weber, G., Biochemistry, 12, 4154-41, 1973.

Hunter, J.M. and Ludwig, M.L., J. Am. Chem. Soc., 84, 3491-3504, 1962.

Huston, E.E., Grammer, J.C. and Yount, R.G., Biochemistry, 17, 8945-8952, 1988.

Jameson, J.L., Jaffe, R.C., Gleason, S.L. and Habener, F., Endocrinology, 119, 2560-2567, 1986.

Ji, T.H., Methodol. Enzymol., 91, 580-609, 1983.

Jue, R., Lambert, J.M., Pierce, L.R. and Traut, R.R., Biochemistry, 17, 5399-5406, 1978.

Kitagawa, T. and Aikawa, T., J. Biochem. (Tokyo), 79, 233-236, 1976.

Knowles, J. R., Acc. Chem. Res. 5, 155-160, 1972.

Korodi, I., Asboth, B. and Polgar, L., Biochemistry, 25, 6895-6900, 1986.

Lacowics, J.R., In Principles of fluorescent spectroscopy, Plenum press, New York, 1983.

Laemmli, U.K., Nature, 227, 680-685, 1969.

Lambert, J.M., Senter, P.D., Yau-Young, A., Blatter, W.A. and Goldmacher, V.S., J. Biol. Chem., 260, 12035-12041, 1985.

Liu, W.K., Young, K.P., Nakagama and Ward, D.N., J. Biol. Chem., 249, 5544-5550, 1974.

Liu, W.K. and Ward, D.N., Bioche. Biophys. Acta., 405, 522-526, 1975.

Liu, W.K., Esfahani, M. and Ward, D.N., Endocrine Res. Commun. 2, 47-63, 1975.

Liu, W.K., Furlong, N.B. and Ward, D.N., J. Biol. Chem., 252, 522-527, 1977.

Marsh, J.M., In Advances of cyclic nucleotide research, Vol: 6, Ed: P. Grungard and A.G. Robinson, 137, Raven Press, New York.

Matzuk, M.M. and Boime, I., J. Cell Biology, 106, 1049-1059, 1988.

Matzuk, M.M., Spangler, M.M., Camel, M., Saganuma, N. and Boime, I., J. Cell Biol., 109, 1429-1438, 1989.

Matzuk, M.M. and Boime, I., Biol. Reprod., 40, 40-53, 1989.

Matzuk, M.M., Kriger, M., Corless, C.L. and Boime, I., Proc. Natl. Acad. Sci. USA., 84, 6354-6358, 1987.

Matzuk, M.M., Hsuch, A.J.W., Labolt, P., Tsafiri, A., Keene, J.L. and Boime, I., Endocrinology, 126, 376-383, 1990.

Means, G.E., and Feeney, R.E., Biochemistry, 7, 1366-1371, 1968.

Milsted, A., Cox, R.P. and Nilson, J.H., DNA, 6, 213-219, 1986.

Neville, D.M. Jr., Ann. NY. Acad. Sci., 507, 507-511, 1987.

Nishikawa, A.H., Morita, R.Y. and Becker, R.R., Biochemistry, 7, 1506-1513, 1968.

O'Keefe, D.O. and Draper, R.K., J. Biol. Chem., 260, 932-937, 1985.

Oeltman and Heath, E.C., J. Biol. Chem., 254, 1022-1027, 1979a.

Oeltman and Heath, E.C., J. Biol. Chem., 254, 1028-1032, 1979b.

Olcott, H.S. and Frankel-Conrat, H., Chem. Rev., 41, 151-197, 1947.

Otani, T., Otani, F., Krych, M., Chaplin, D.D. and Boime, I., J. Biol. Chem., 263, 7322-7329, 1988.

Parkinson, D. and Redshaw, J.D., Anal. Biochem., 141, 121-136, 1984.

Pastan, I., Willingham, M.C. and Fitzgerald, D.J., Cell, 47, 641-648, 1986.

Pierce, J.G. and Parson, T.F., Ann. rev. Biochem., 50, 465-495, 1981.

Raftery, M.A. and Cole, R.D., J. Biol. Chem., 241, 3457-3461, 1966.

Ramakrishnan, S. and Houston, L.L., Cancer Res., 44, 201-208, 1984a.

Raso, V. and Basala, M., J. Biol. Chem., 259, 1143-1149, 1984.

Rebois, R.V., J. Cell. Biol., 94, 70-76, 1982.

Rice, R.H., Means, G.E. and Brown, W.D., Biochem. Biophys. Acta, 492, 316-321, 1977.

Sairam, M.R., In Gonadotropins Vol.XI, Hormonal peptides and proteins, Editor: C.H. Li., Academic Press, New York, 1-79, 1983.

Sairam, M.R. and Bhargavi., Science, 229, 65-67, 1985.

Schoellmann, G. and Shaw, E., Biochemistry, 2, 252-255, 1963.

Simpson, D.L., Cawley, D.B. and Herschman, H.R., Cell, 29, 469-473, 1982.

Singh, V. and Sairam, M.R., Int. J. Peptide & Protein Res., 33, 22-28, 1989.

Singh, V., Sairam, M.R., Bhargavi, G.N. and Akhras, R.G., J. Biol. Chem., 264, 3089-3095, 1989.

Singh, V. and Sairam, M.R., Mol. Cell. Endocrinol., 67, 255-262, 1989.

Singh, V. and Sairam, M.R., Biochem. J., 263, 417-423, 1989.

Singh, V. and Sairam, M.R., Mol. Cell. Endocrinol., 67, 217-229, 1989.

Singh, V. and Sairam, M.R., Int. J. Peptide & Protein Res., 35, 46-51, 1990.

Singh, V. and Das, C., Biochemistry International, 24, 689-699, 1991.

Singh, V., Biochemistry International 15, 153-162, 1987.

Singh, V., Indian J. Exp. Biol., 26, 252-254, 1988.

Singh, V., Indian J. Exp. Biol., 26, 905-906, 1988.

Singh, V., Indian J. Exp. Biol., 27, 1-4, 1989.

Singh, V., Indian J. Exp. Biol., 27, 5-9, 1989.

Singh, V., Biochemistry International, 24, 677-688, 1991.

Singh, V. and Curtiss, R., Biochemistry International, 25, 531-536, 1991.

Singh, V., Indian J. Exp. Biol., 29, 916-925, 1991.

Singh, V. and Kar, S.K., Indian J. Biochem. & Biophys., 29, 31-41, 1992.

Singh, V. and Curtiss, R., Mol. Cell. Biochem., 120, 95-102, 1993.

Singh, V. and Curtiss, R., Mol. Cell. Biochem., 130, 91-101, 1994.

Singh, V., Mavila, A.K. and Kar, S.K., Bioconjugate Chemistry, 4, 473-482, 1993.

Sreenivasan, Y., Ramprasad, M.P. and Surolia, A., FEBS Lett., 192, 113-118, 1985.

Staros, J.V., Biochemistry, 21, 3940-3955, 1982.

Staros, J.V., Acc. Chem. Res., 21, 435-441, 1988.

Stripe, F. and Barbieri, L., FEBS Lett., 195, 1-8, 1986.

Talmadge, K., Vamvakopoulos, N.C. and Fiddes, J.C., Nature, 307, 37-40, 1984.

Talwar, G.P., Gupta, S.K., Singh, V., Sahal, D., Iyer, K.S.N. and Singh, O., Proc. Natl. Acad. Sci. USA, 82, 1228-1231, 1985.

Talwar,G.P., Singh, O. and Singh, V., In "Fertility Regulation Today and Tomorrow", Editor : E Diczfalusy and E Bygdmen, Serono Symposia series; vol.36 Raven Press, New York, pp 43-54, 1987.

Thorpe, P.E., Brown A.N.F., Bremner, J.A.G. Jr., Foxwell, B.M.J. and Stripe, F., J. Natl. Cancer Inst.,75, 151-159, 1985.

Uy, R. and Wold, F., Adv. Exp. Med. Biol., 86A, 169-186, 1977.

Varga, J.M., Asato, N., Lande, S.R. and Lerner, A.B., Nature, 267, 56-58, 1977.

Veronese, F.M., Largalolli, R., Boccu, E., Benagassi, C.A. and Schiavon, O., Appl. Biochem. Biotechnol., 11, 141-152, 1985.

Vitetta, E.S., Foulton, R.J., May, R.D., Till, M. and Uhr, J.W., Science, 238, 1098-1104, 1987.

Vitetta, E.S. and Uhr, J.W., Ann. Rev. Immunol. 3, 197-212, 1985.

Ward, D.N., Adams-Mayne, M, Ray, N., Balke, D.E., Coffey, J.A. and Showalter, A.M., Gen. Comp. Endocrinol., 8, 44-53, 1967.

Wawrzynczak, E.J. and Thorpe, P.E., In **Immunoconjugates : antibody conjugates in radioimaging and therapy of cancer**, Ed. Vogel, C-W., Oxford University Press, 28-55, 1987.

Weare, J.A. and Reichert, I.E., **J. Biol. Chem.**, **254**, 6964-6971, 1979.

Waldmeyer B. and Bosshard, H.R., **J. Biol. Chem.**, **260**, 5184-5190, 1985.

Willing, A.H., Gregoriadis, M.M., Rees, D.C. and Howard, J.B., J. Biol. Chem., 264, 8499-8503, 1989.

Wofsy, L., Metzger, H. and Singer, S.J., **Biochemistry**, **1**, 1031-1039, 1962.

Wold, F., **Methods. Enzymol.**, 25, 623-651, 1972.

Wang, K. and Richards, F.M., J. Biol. Chem., 249, 8005-8018, 1974.

Yoshitake, S., Yamada, Y., Ishikawa, E. and Massayef, R., Eur. J. Biochem., 101, 395-399, 1979.

Youle, R.J. and Neville, D.M., Proc. Natl. Acad. Sci. U.S.A., 77, 5483-5486, 1980.

Youle, R.J. and Colombatti, M., J. Biol. Chem., 262, 4676-4682, 1987.

Youle, R.J. and Colombatti, M., In Monoclonal antibodies in cancer: Advances in diagnosis and treatment, Editor. Roth, J.A., 173-213, Futura publishing, 1986.

Zalman, L.S. and Wisinisky, B.J., Proc. Natl. Acad. Sci. USA, 81, 3341-3345, 1984.

List of Publications :

1. Design of liposome to improve encapsulation efficiency of gelonin and its effect on immunoreactivity and ribosome inactivating property.

   Anis Alam, S.R.K. Bhuri, Anil K. Mavila and Vinod Singh

   Mol. Cell. Biochem., 112, 97-107, 1992.

2. Effect of lysine residue modification of ovine luteinizing hormone by heterobifunctional cross-linking reagent SPDP on subunit-subunit association, receptor binding and biological activity.

   Vinod Singh Anil K. Mavila and Anis Alam
   Indian J. Exp. Biol., 30, 1093-1100, 1992.

3. Comparison of cytotoxic effect of hormonotoxins prepared with the use of heterobifunctional cross-linking agents N-Succinimidyl 3-(2-Pyridyldithio) propionate and N-Succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexaonate

   Vinod Singh, Anil K. Mavila and S. K. Kar
   Bioconjugate Chemistry, 4, 473-482, 1993.

4. Biochemical and Cytotoxic properties of Hormonotoxins

   Vinod Singh, Anil K. Mavila and Anis Alam

   XIIth International Pharmacology Congress,
   July 24-29, 1994, Montreal, Canada.