Effects of thiolation on the immunoreactivity of the ribosome-inactivating protein gelonin

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Gelonin purified from the seeds of *Gelonium multiflorum* using cation-exchange and gel-filtration chromatography was characterized for its purity, homogeneity and M_r by reverse-phase h.p.l.c. and SDS/polyacrylamide-gel electrophoresis analysis and judged to be 98 % pure. As the cross-linking agent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) has been used for linking gelonin via its ϵ -NH₂ group to its carrier antibodies or hormones for immunotoxin or hormonotoxin respectively, an attempt was made to study the effect of this modification of gelonin on its immunoreactivity. A radioimmunoassay was developed for this purpose. By sequential modification, four categories of amino group modifications on immunoreactivity were observed. Even one or two modifications, representing one-twentieth to one-tenth of available ϵ -NH₂ groups in the protein caused about 75 % loss in immunoreactivity, with additional reactions contributing to further deteriorations. By using a gelonin radioimmunoassay, the immunoreactivity of gelonin in three hormonotoxins was determined with gelonin and modified gelonin as standards. The gelonin equivalent in our hormonotoxins was in agreement with the values determined by spectrophotometric and gel-electrophoresis methods. As the immunoreactivity of gelonin-SPDP was not further altered after conjugation to its carrier protein ovine lutropin, a specific radioimmunoassay of gelonin could be used to evaluate the molar ratio of the conjugates prepared by using SPDP as cross-linker and gelonin-SPDP as a standard.

INTRODUCTION

In recent years many ribosome-inactivating proteins (RIPs) have been extensively used in linkage with numerous monoclonal antibodies directed against the cell-surface antigens of cancer cells, in order to develop potential immunotoxins which could selectively destroy malignant cells (for review see [1–3]). Many investigators have employed proteins such as ricin or diphtheria toxin, which are good examples of two-chain proteins, the two chains being designated A and B and joined by a disulphide bond [1–3]. These proteins are bound to the cell surface via the B-chains, with the A-chain causing catalytic inactivation of ribosomes [4]. In order to overcome problems of non-specific effects, the use of either A chain alone, representing the catalytic unit of the toxin, or a recombinant version of the whole toxin, eliminating the cell-binding units of the B chain, have been considered [3]. Alternatively there are many single-chain RIPs, many of which are also of plant origin and could serve the purpose of constructing conjugates with biologically active proteins. Included in this category are examples such as pokeweed antiviral protein [5], saporin [6] and gelonin [7], because they are innocuous to cells in the free form, but can inactivate ribosomes once they are provided with an entry into cells by a receptor-mediated

process. Gelonin, isolated from the Indian plant *Gelonium multiflorum*, has been used to prepare specific immunotoxins exhibiting toxicity *in vitro* and *in vivo* to specific cancerous cells [8,9]. In an analogous manner we have now begun a series of studies to prepare and study the potential of 'homonotoxins' [10]. In such conjugates the hormones, which are glycoproteins, are two-chain proteins (α - and β -subunits), whereas the toxin, gelonin, consists of a single polypeptide. In an ideal situation, which may be difficult to achieve, the activities of the respective constituents should be maintained to the maximum extent possible to yield conjugates of therapeutic utility. We have thus analysed the effects of modification of lysine amino groups in both the hormone [11] and gelonin; the results of the latter are reported herein. The cross-linking agent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) has been used to generate the sites for conjugation of the two proteins.

MATERIALS AND METHODS

Materials

SPDP and DTT were obtained from Pierce Chemicals (Rockford, IL, U.S.A.). Chromatographic materials, Sephadex G-25 (medium grade), G-100 (medium grade)

Abbreviations used: BSA, bovine serum albumin; CMC, carboxymethylcellulose; DTT, dithiothreitol; oLH, ovine luteinizing hormone (lutropin); r.i.a., radioimmunoassay; RIP, ribosome-inactivating protein; RP-h.p.l.c. reverse-phase h.p.l.c.; PAP, pokeweed antiviral protein; PAGE, polyacrylamide-gel electrophoresis; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate.

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and carboxymethylcellulose (CMC-23) were purchased from Pharmacia Fine Chemicals Canada (Dorval, Quebec, Canada) and Whatman Co. (Mandel Scientific, Montreal, Canada). Na¹²⁵I (IMS-30) was from Amersham (Chicago, IL, U.S.A.). Bovine serum albumin (BSA, Cohn fraction V), Freund's complete and incomplete adjuvants were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The seeds of *Gelonium multiflorum* were obtained from United Chemicals and Allied Products (Calcutta, India). Other chemicals used in the investigation were of the highest purity.

Methods

Isolation of gelonin. Gelonin was isolated from the seeds by following the published procedure [7] with minor modifications. Briefly, the deshelled seeds were soaked overnight in the extraction buffer (0.05 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl). The soaked seeds were ground with 10 vol. of the extraction buffer and left stirring overnight at 4 °C. The mixture was centrifuged in the cold, and the pellet was further extracted with the same buffer. The supernatants of both extractions were dialysed extensively against 0.05 M-sodium phosphate buffer, pH 6.5, and passed through a CMC-23 column (3.2 cm × 40 cm) pre-equilibrated with the same buffer. The column was washed extensively with 0.05 M-sodium phosphate buffer, pH 6.5, until the eluate showed an absorbance of less than 0.05 at 280 nm. The bound material was eluted with a linear 0–0.3 M-NaCl gradient in the same buffer, at a flow rate of 30 ml/h at 4 °C. The fractions collected were read at 280 nm. The protein eluted at 0.15 M-NaCl was concentrated by ultra-filtration on a YM10 filter (Amicon). The concentrated protein solution was subjected to gel-filtration chromatography on Sephadex G-100 in 0.05 M-ammonium bicarbonate. The protein fractions were pooled and freeze-dried.

Characterization of gelonin. The gelonin obtained after gel-filtration chromatography was further characterized by reverse-phase (RP-) h.p.l.c., SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) and amino acid analysis. The RP-h.p.l.c. was performed on a Waters μ Bondapak phenyl column (4 mm × 250 mm) using a continuous gradient. The SDS/PAGE was done in gel slabs (20 cm × 12 cm) cast with an acrylamide gradient (5–15% w/v), as described by Laemmli [12], under both reducing and non-reducing conditions. The gels were stained with Coomassie Blue dye. Low- and high- M_r reference standards from Bio-Rad (Richmond, CA, U.S.A.) were used as markers. The amino acid analysis [13] was performed on a Beckman model 120 MB instrument.

Production of anti-gelonin antibodies. The antibodies against gelonin were produced in rabbits (about 3 kg body weight; New Zealand strain) by immunizing them first with 1 mg of gelonin dissolved in saline and emulsified with Freund's complete adjuvant. The subsequent two injections were given at fortnightly intervals with Freund's incomplete adjuvant. The animals were bled after 1 week, and the sera separated were stored frozen.

Development of radioimmunoassay (r.i.a.) of gelonin

Radioiodination. The radioiodination of gelonin was performed by the lactoperoxidase method, following the

protocol described for gonadotropins [14]. Briefly, 5 μ g of gelonin was iodinated with 0.5 mCi of Na¹²⁵I, and the iodinated gelonin was purified on a Bio-Gel P6DG disposable column. The specific radioactivity of the ¹²⁵I-gelonin, calculated by the incorporation efficiency, was 80 μ Ci/ μ g. The immunoreactivity of the labelled gelonin was estimated by measuring the binding ability (> 85%) in presence of excess anti-gelonin antibodies.

Antibody binding. The antibody-binding assay was carried out in 12 mm × 75 mm polystyrene tubes in triplicate. The antibodies were initially screened by using a series of dilutions made in 0.05 M-sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃, 1% BSA and 1% normal rabbit serum, at a single concentration of ¹²⁵I-gelonin ($\approx$ 30000 c.p.m./tube). The assay system consisted of 0.1 ml each of ¹²⁵I-gelonin and different dilutions (1:10–1:10⁸ dilutions) of antibodies. The tubes were incubated at 37 °C overnight. Bound and free ¹²⁵I-gelonin was separated by adding 0.2 ml of Pansorbin (Calbiochem, La Jolla, CA, U.S.A.) to each tube.

Competitive binding assay. Cross-reaction of anti-gelonin antibodies with SPDP-modified gelonin (gelonin–SPDP) and its conjugates to ovine luteinizing hormone (oLH) were determined by a competitive-displacement method. A dilution of antibody giving 30–50% binding in the assay buffer was allowed to compete with the gelonin–SPDP or oLH–gelonin conjugates in the radioimmunoassay as described above. The assay system consisted of the following: 0.1 ml each of ¹²⁵I-gelonin, anti-gelonin antibody and various concentrations of competitor. The inhibition lines were obtained by plotting $(B/B_0) \times 100$ against the logarithm of the dose of gelonin or competitor (where B_0 represents the binding of ¹²⁵I-gelonin in the absence of unlabelled gelonin and B in the presence of the competitor). The extent of cross-reactivity (as a percentage) was expressed as:

$$C = (L_{50}/U_{50}) \times 100$$

where L_{50} is the dose of competitor which shows 50% inhibition and U_{50} is the dose of gelonin which shows 50% inhibition [15].

Modification of gelonin with SPDP. The modification of gelonin with SPDP was carried out according to published procedures [16] and adapted for gonadotropins [10,11]. The increase in absorbance at 343 nm after reduction was used to calculate the number of thiol groups incorporated in the modified protein [16].

Preparation of oLH–gelonin conjugates. The conjugates of oLH with gelonin used in the present study were the same as those reported recently [10].

RESULTS

Purification and characterization of gelonin

The extract of the seeds after dialysis was applied to the CMC column as described in the Materials and methods section. After the unadsorbed fraction had been washed with 0.05 M-sodium phosphate buffer, pH 6.5, the adsorbed protein was eluted with a 0–0.3 M-NaCl gradient in the same buffer. The elution profile shown in Fig. 1(a) is slightly different from that reported by Stirpe *et al.* [7], who noted that several peaks were eluted before the

Fig. 1. Purification of gelonin (three batches) from dry seed of *Gelonium multiflorum*

Seed extracts, dialysed against 5 mM-sodium phosphate buffer, pH 6.5, was applied to a CMC-23 column (3.2 cm $\times$ 40 cm) equilibrated with the same buffer. The unbound protein was washed off the column and the bound material was eluted with 1000 ml of a linear 0–0.3 M-NaCl gradient in the same buffer at a flow rate of 22 ml/h at 4 °C. Fractions (4.5 ml each) were collected and protein was read at 280 nm. Of the two major peaks, peak A was broader than B. As indicated, peak B was concentrated and rechromatographed on Sephadex G-100 (3.4 cm $\times$ 100 cm) (b). The aggregated protein indicated as peak A was eluted at the void volume of the column. The symmetrical portion of peak B of (b) was pooled, concentrated, and re-run on the same G-100 column (c). The three batches are designated G1 (—), G2 (---) and G3 (----).

gradient reached 0.15 M-NaCl. However, in our hands all batches showed a similar profile (Fig. 1a). The protein eluted at 0.15 M-NaCl (as shown by 'B' in Fig. 1a) was concentrated by ultrafiltration before gel filtration on Sephadex G-100. Fig. 1(b) shows gel-filtration chromatography of three batches of gelonin. Each showed a major peak with a small hump before the main peak. The marked area of the peaks were pooled, again

Fig. 2. RP-h.p.l.c. of gelonin G1 (—); G2 (---) and G3 (----)

Samples were dissolved in solvent A (water containing 0.1% trifluoroacetic acid) and separation was performed on a Waters μ Bondapak column (4 mm $\times$ 250 mm) equilibrated with solvent A + 25% (v/v) acetonitrile and 0.1% trifluoroacetic acid. A gradient of 25–50% acetonitrile was run on over 30 min at a flow rate of 2 ml/min in a Beckman h.p.l.c. system (450 controller) and monitored at 280 nm. The RP-h.p.l.c. chromatogram was analysed between 3 and 30 min. Arrows show the presence of the presumed isoforms of the gelonin of differing M_r (see Fig. 3). Retention times of the peaks are shown.

concentrated, and run on the same column (Fig. 1c). It may be noted from this Figure that the protein shows a slight tendency to aggregate. The marked portion of the peaks were pooled, freeze-dried, and stored at 4 °C until further analysis.

Before further physicochemical characterization of gelonin, the purity of each batch was assessed by RP-h.p.l.c. using a continuous gradient (see Figure legends for details). Fig. 2 shows the h.p.l.c. analysis of gelonin as purified above. It is evident that gelonin binds to the μ Bondapak phenyl column rather tightly and is eluted at higher acetonitrile concentrations. The retention time of the gelonin was 21.1 min. A careful analysis of the peaks clearly reveals that a small hump is present at the ascending portion of the peak. This could not be separated even under a shallow gradient (results not shown).

The purity and M_r were further determined by SDS/PAGE analysis. The SDS/PAGE pattern of gelonin(s) under non-reducing and reducing conditions is shown in Fig. 3. The estimated M_r of about 30000 is in good agreement with the literature value [7]. In addition to this major band, two distinct bands migrating before and after the main component persisted in all batches. These may be variant(s) of gelonin that could not be separated under the conditions we have presently used. It is possible that the hump of RP-h.p.l.c. patterns may correspond to one or both of these components. Under reducing conditions there was no change either in the position or in the intensity of the bands (Fig. 3), indicating that they are all single polypeptide chains.

The amino-acid-analysis data for the gelonin (native) and performic acid-oxidized products [10] are in agreement with those reported in the literature [17].

Fig. 3. SDS/PAGE (non-reducing) of three different batches of gelonin

A gel consisting of 5–15% (w/v) acrylamide was used. After the run the gel was fixed in methanol/acetic acid (9:1, v/v) and stained for 15 min with Coomassie Blue, followed by destaining in methanol/acetic acid (2:1, v/v) for 24 h. Lane 1, G1B (50 μ g); lane 2, G1B peak tube (50 μ g); lane 3, G2B (30 μ g); lane 4, G2B peak tube (30 μ g); lane 5, G2 tube no. 95; lane 6, G3B (5 μ g); lane 7, G3B peak tube (30 μ g); lane 8, G3B peak tube (30 μ g); lane 9, G3B tube no. 95. G1, G2 and G3 indicate batch number and B shows the peak from Fig. 1(b). G1B, G2B, G3B peak tubes show the peak tube of all batches (Fig. 1b). Similarly, tube no. 95 of peak B shows a minor ascending peak of the G2 and G3 (as shown by the arrow in Fig. 1b).

Modification by SPDP

The amino groups were modified by SPDP as described in the Materials and methods section. The u.v.-visible spectra of gelonin modified using different molar ratios of SPDP and then treated with DTT showed clear concentration-dependent increases in modified SPDP-gelonin. The relationship between the number of $-NH_2$ groups modified versus the molar concentration of SPDP added is shown in Fig. 4. Higher modification ratios (over 6 or 7) usually caused precipitation of the protein during the reaction. Therefore, in these cases, the reaction was terminated as soon as the solution appeared to be cloudy. After the desalting on a Bio-Gel P6DG column, the highly modified derivatives did not completely dissolve in 0.1 M-sodium phosphate buffer, pH 6.0, even after adding excess DTT. Thus we concluded that the protein must have been denatured after modification of many of the accessible ϵ -lysine residues. In view of this, the values shown for $-NH_2$ group modification for higher SPDP-gelonin derivatives should be considered tentative.

Immunoreactivity of gelonin and hormonotoxins

The anti-gelonin antibodies raised in two rabbits by the procedure described have similar binding properties

Fig. 4. Relationship between the SPDP/gelonin ratio and the number of amino groups modified

and titres. Indeed, both animals started producing antibodies even after first injection (results not shown). On the basis of the dilution curve, a 1:50000 dilution, which gives about 40% binding of the iodinated gelonin, was used in immunoreactivity experiments. A standard displacement curve obtained by using different concentrations of native gelonin showed that the assay is extremely sensitive, detecting about 0.7 ng/tube. The competitive binding ability of SPDP-gelonin(s) in the r.i.a. system is shown in Fig. 5. It is clear that even a small modification of one or two amino groups results in a decrease in immunoreactivity by about 75%. Further, the higher modifications show a progressive decrease in immunoreactivity (Fig. 5; Table 1). From Table 1 it is evident that the modification above seven groups produced a virtually complete loss of immunoreactivity. All the three oLH-gelonin conjugates showed immunoreactivity in the gelonin immunoassay (Fig. 6; Table 2). Hormonotoxins I and II showed 57–71% immunoreactivity, whereas hormonotoxin III was fully reactive in this respect.

DISCUSSION

Gelonin from the dry seeds of *Gelonium multiflorum* was isolated by cation-exchange and gel-filtration chromatography of the aqueous extracts. In preparing more than ten batches of gelonin by cation-exchange chromatography, our profiles were somewhat different (Fig. 1a) from those obtained hitherto [7]. Two distinct broad peaks were obtained on the CMC-23 column when a gradient of 0–0.3 M-NaCl in 5 mM-sodium phosphate buffer, pH 6.5, was applied (Fig. 1a). Stirpe *et al.* [7] observed four protein peaks, the last of which had ribosome-inactivation properties. In our hands peak B turned out to be gelonin, as judged on the basis of several biochemical parameters. This difference could be due to differences in the gel used for cation-exchange chromatography or in the batch of seeds selected for extraction. Further purification of gelonin was done by passing peak B of the CMC-23 column through a Sephadex G-100 column. After each gel-filtration run, a small proportion of the gelonin protein aggregated and was eluted earlier on the Sephadex G-100 column (Figs. 1b and 1c). These aggregated gelonin fractions reacted with anti-gelonin antibodies (results not shown).

Fig. 5. Immunological cross-reactivity of gelonin (○), G-SP1 (●), G-SP2 (□), G-SP3 (△), G-SP4 (▲), G-SP5 (■), G-SP6 (—○—), G-SP7 (—●—), G-SP8 (—▲—) and G-SP9 (—△—) as determined by competitive r.i.a. as described in the Materials and methods section

The quantitative data are recorded in Table 1. B and B_0 are defined in the text. G-SP1–9 are various derivatives prepared as described in Fig. 1.

Freeze-dried powders of gelonin, when dissolved in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, always had a fraction of protein which did not dissolve. This small portion, which may represent forms denatured during freeze-drying, or aggregates, were separated before subsequent modifications. The dissolved protein retains both immunoreactivity and ribosome-inactivating properties [10]. Quantitative assessment by RP-h.p.l.c. revealed 98 % purity. A careful

analysis of the profile clearly indicates a small hump at the ascending portion of the chromatogram (Fig. 2). Our attempts to separate this by applying more shallow gradients have been unsuccessful. It is possible that a low percentage of the gelonin is present as an isoform of the protein. A minor alteration in amino acid sequence/composition might have resulted in a hump in the chromatogram. SDS/PAGE revealed a homogeneous protein of $M_r \approx 30000$, which was in agreement with reported values [7]. When a high concentration of protein

Table 1. Immunoreactivity of different SPDP-modified gelonins with the anti-gelonin antibody

The immunoreactivity (cross-reactivity) is expressed as a percentage with respect to that of native gelonin, which was taken as 100 %. The method of calculating percentage activity is given in the Materials and methods section and values were derived from Fig. 5. ED_{50} is the dose causing a 50 % displacement. G-SP1–G-SP9 represent the various derivatives prepared as described in Fig. 1 and tested in Fig. 5.

Gelonin	ED_{50} (ng)	Cross-reactivity (%)
Gelonin	1.2	100.00
G-SP1	5.0	24.00
G-SP2	4.4	27.00
G-SP3	7.8	15.40
G-SP4	10.1	11.80
G-SP5	34.5	3.47
G-SP6	55.0	2.18
G-SP7	94.0	1.28
G-SP8	370.0	0.32
G-SP9	500.0	0.24

Fig. 6. Immunoreactivity of gelonin in G-SP3 (●), standard gelonin (○) and three hormonotoxins: I (□), II (△) and III (■)

The quantitative data are recorded in Table 2. B and B_0 are defined in the text. In modified gelonin preparation G-SP3, 1.2 ± 0.3 mol of ϵ -NH₂ groups were modified

Table 2. Immunoreactivity of different oLH-gelonin conjugates with anti-gelonin antibody

In column A, under 'Cross-reactivity (%)' the immunoreactivity of native gelonin was set as 100%, whereas in column B that of gelonin bearing one SPDP group was taken as 100% for comparing the gelonin immunoreactivity of the hormonotoxin preparation (see Fig. 6). The three hormonotoxin preparations of oLH conjugated to gelonin are those described in [10]. ED₅₀ is defined in Table 1. G-SP3 is SPDP-modified gelonin containing an average of one ϵ -NH₂ group modification.

Preparation	ED ₅₀ (ng)	Cross-reactivity (%)	
		A	B
Gelonin	1.6	100	
G-SP3	5.8	28	100
Homonotoxin			
I	10.2	16	57
II	8.2	20	71
III	4.8	33	117

was electrophoresed, two minor bands of M_r higher and lower than that of gelonin were obtained (Fig. 3, lanes 1, 2, 3, 4, 7 and 8), but these were unaffected after treatment with mercaptoethanol/DTT. Thus it may be concluded that each of these components represented a single polypeptide chain. The minor humps observed in RP-h.p.l.c. could be the source of these minor bands. However, these predictions demand detailed structural investigations for confirmation. As gelonin is a glycoprotein containing 2–3% carbohydrate [7], it is likely that differences in glycosylation may have resulted in a slight variation in M_r and interaction with the matrix in the RP-h.p.l.c. analysis.

The determination of the molar ratio of gelonin and its carrier (either monoclonal antibody or hormone) is important for the immunotoxin, but very crucial for the hormonotoxins as regards their cytotoxicity to the test system. This is because the antigen–antibody affinity is not drastically altered after either SPDP modification or coupling of antibody to gelonin. However, the hormone–receptor interaction was extremely sensitive, and affinity was decreased after SPDP modification of the hormone [11]. Generally, in immunotoxins, the gelonin–antibody molar ratio has been determined on the basis of an

Table 3. Molar ratio of the hormonotoxins determined by different methods

The values reported for spectrophotometry, gel electrophoresis and amino acid composition were taken from [10].

Hormono-toxin	Method...	Molar ratio (hormone/toxin)		
		Spectro- photo- metric	Gel electro- phoresis	Amino acid com- position
I		1:1.40	1:1.30	1:1
II		1:1.20	1:1.27	1:1
III		1:1.80	1:2.20	1:1
				Gelonin r.i.a.
I				1:1.14
II				1:1.42
III				1:2.30

increase in the M_r of the conjugate [8]. This is estimated either by using gel-filtration chromatography or by SDS/PAGE analysis. Both these techniques could only give the correct molar ratio if both components of the conjugate behave ideally in gel-filtration and SDS/PAGE analysis. Although monoclonal-antibody–gelonin conjugates could be analysed by these methods, similar application to gonadotropin–gelonin conjugates may lead to 'artifactual' values, because glycoprotein hormones behave abnormally in such analyses. Since gelonin does not cross-react with other RIP antibodies, we attempted to develop a specific radioimmunoassay of gelonin to determine gelonin contents in our hormone–toxin conjugates [10]. A survey of the literature revealed that no attempt has yet been made to determine molar ratios in conjugates using a specific r.i.a. of gelonin. As SPDP is generally employed in coupling of gelonin to its carrier proteins, it was considered important to evaluate its impact on ϵ -NH₂ group modification on its immunoreactivity. It might be mentioned that, in order to determine the gelonin contents in the conjugates, it is essential that the gelonin immunoreactivity be not altered after conjugation to the antibody and hormone. An r.i.a. of hormone (e.g. oLH) could not be used, since the extent of the decrease in immunoreactivity after conjugation is not consistent and uniform among the conjugates [10].

Each molecule of gelonin has 20 lysine residues, as determined by amino acid analysis [10], and their chemical modification by succinic anhydride or citraconic anhydride did not drastically hamper the ribosome-inactivating property of gelonin [18]. Modification of up to 70% of the ϵ -NH₂ groups preserved 80% of the protein-synthesis-inhibition ability as tested in a rabbit reticulocyte-lysate system [18]. This observation strengthens the possibility of development of conjugate using ϵ -NH₂ groups, and indeed our hormonotoxin (oLH coupled to gelonin) is cytotoxic to mouse Leydig-tumour cells, which bear receptors to this hormone [10]. However, as we have noted, minor modifications resulted in the loss of up to 75% of immunoreactivity of gelonin (Table 1). The relationship between the number of amino groups modified and the immunoreactivity may be divided into four categories, based on the degree of loss. The modification of about one or two ϵ -NH₂ groups (as in G-SP1–G-SP3) induced a loss of about 75–80%; that of three ϵ -NH₂ groups gave a loss of up to 84–88%; that of three to seven ϵ -NH₂ groups gave losses up to 97–99%; and further modification up to 12 ϵ -NH₂ groups (G-SP8 and G-SP9) led to almost complete loss of activity, although displacement could be seen at high concentrations (Fig. 5). These data would suggest that the ϵ -NH₂ groups are somehow involved in gelonin–anti-gelonin interactions. Ramakrishnan *et al.* [19] used a specific r.i.a. of pokeweed antiviral protein (PAP) to determine the antibody/PAP molar ratio of the conjugate [20]. The concentration of PAP determined was subtracted from the total concentration of the protein and the contribution of IgG in the conjugate was thus deduced [20]. Similarly our assessments of the molar ratio of the conjugates using gelonin r.i.a. (Table 3) demonstrate the utility of gelonin radioimmunoassay. These data were quite comparable with our earlier spectrophotometric and SDS/PAGE analyses. Consistent with our previous experience [10], hormonotoxin III also showed higher gelonin content by r.i.a. We have attributed this to the

use of a slightly different procedure for purification of the conjugate. It may therefore be concluded that the r.i.a. of gelonin could be used to evaluate the concentration of gelonin in the conjugates. As immuno-reactivity of SPDP-modified gelonin tends to stabilize at about 25 % when one to 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 as standards in routine analytical experiments on hormonotoxins/immuno-toxins involving gelonin.

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