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Role of positive charge of lysine residue on ribosome-inactivating property of gelonin

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The report that gelonin cross-linked with monoclonal antibodies with the use of 2-iminothiolane (2-IT) exhibited higher cytotoxicity than the conjugates prepared with the use of N-succinimidyl-3-(2-pyridylthio) propionate (SPDP) alone, has prompted us to investigate the effect of ε-NH_2 group modification with 2-IT on the ribosome-inactivating property (RIP) of gelonin. The purified gelonin was modified with 2-IT at a different molar ratio and their effects on immunoreactivity and ribosome-inactivating property were compared with those of N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (long chain-SPDP) and SPDP modified gelonin derivatives. Modification of single amino group with 2-IT results in about 25-50% inhibition of immunoreactivity and 60-70% loss of protein synthesis inhibition activity. Modification of 2-3 amino groups further hampers both immunoreactivity and protein synthesis inhibition property of gelonin. Both the long chain-SPDP with SPDP modifications showed more pronounced effects on immunoreactivity and RIP activity as compared to the similar ratio of 2-IT modification(s). It may, therefore, be concluded that the positive charge plays an important role in the immunological as well as the protein synthesis inhibitory effect of gelonin.

The plant ribosome-inactivating proteins (RIPs) which catalytically inactivate 60S ribosomal unit have been used in the design of specific hybrid molecules, for selective targeting to the specific tumor cells, having both functions of specific recognition and cytotoxicity. These molecules were synthesized either by covalently cross-linking monoclonal antibodies specific to the tumor cell to the highly cytotoxic molecules or by genetically fusing both the antibody or toxin genes to produce recombinant immunotoxins. RIPs have been divided into two groups. One group of RIPs (type 2) is composed of two non-identical and disulfide linked polypeptide chains, A and B. The B-chain binds to galactose residues on the cell surface and helps the A-chain to translocate in the cell and arrest protein synthesis machinery by enzymatically inactivating the 60S ribosomes. The non-specific toxicity due to B-chain was avoided either by using immunotoxins in the presence of excess galactose or by completely depleting B-chain from the toxin part. Due to the non-specific cytotoxicity of type 2 RIP's (such as ricin), the intact ricin, either ricin-A-chain alone representing catalytic unit of the toxin or a recombinant version of the whole toxin have been used in the design of immunotoxins. In order to avoid non-specific cytotoxicity, single chain RIPs (type 1) have covalently been linked to monoclonal antibodies to synthesize immunotoxins. These are innocuous to cells in their free form, but can inactivate ribosomes once provided entry into the cell by a receptor mediated process. Gelonin has been used to covalently cross-link with different cross-linking agents to prepare specific cytotoxic hybrid molecules to target tumor cells[1-6]. The extent of cytotoxicity of these conjugates is comparable to that of ricin-A chain based immunotoxins. Gelonin has been cross-linked with different cross-linking agents such as SPDP, and LC-SPDP and, these agents deplete the positive charge on the toxin. It has been observed that gelonin modified with 2-IT and later linked with the monoclonal antibodies exhibited higher toxicity to the target cells than the conjugates prepared with SPDP alone[7]. This has prompted us to investigate the role of positive charge on the *in vitro* RIP activity of gelonin.

Materials and Methods

Gelonin used in the present investigation was purified by the method described earlier[8]. This method yielded gelonin that was devoid of non-proteinaceous substance that absorbs at 280 nm. Purified gelonin was extensively characterised as described earlier prior to use for RIP activity.

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Modification of gelonin by SPDP and LC-SPDP

The amino group(s) of gelonin were modified by SPDP and LC-SPDP as described earlier[9].

Modification of ε-NH_2 group of gelonin with SPDP/ LC-SPDP and 2-IT

Gelonin was dissolved in 50 m*M* triethanolamine-HCl containing 100 m*M* NaCl, 1 m*M* EDTA and the *p*H was adjusted to 8.0 with 0.2 *M* NaH_2PO_4. Gelonin was reacted with 2-IT in the molar ratios varying from 1:5, 1:10, 1:20, 1:30, 1:40, 1:50 1:100, 1:200 and 1:250 (mole/mole) in the initial reaction. The reaction was stopped by adding 100 µl of borated buffer-saline containing 2.2 *M* glycine. The derivatized gelonin was then treated for 1 hr at room temperature with 2 m*M* DTNB (Ellman's reagent). The mixture was passed through Sephadex G-25 column equilibrated with nitrogen flushed buffer consisting of 100 m*M* sodium phosphate buffer (*p*H 7.4), 100 m*M* NaCl and 1 m*M* EDTA. The number of activated disulfide groups introduced into the ovine luteinizing hormone (oLH) was determined by reducing the DTNB treated sample with DTT and measuring the absorption of released 3-carboxylate 4-nitrothiophenolate ion which has molar absorptivity of 1.36×10^4 M^{-1} cm^{-1} at 412 nm[10].

In vitro cell free translation assay

The inhibitory activity of gelonin, their SPDP, LC-SPDP and 2-IT modified derivatives on cell-free protein synthesis was determined using a system consisting of nuclease treated rabbit reticulocyte lysate. The extent of protein synthesis was determined by measuring incorporation of ^{3}H-leucine into the TCA insoluble protein fraction as described earlier[8,9]. Briefly, to a total of 15 µl reaction mixture, 2 µl of test sample was incubated with 1 µl BMV, mRNA (0.5 µg/l), 2 µl each amino acid mixture (minus leucine) and ^{3}H-leucine, 0.5 µl each 500 m*M* potassium acetate and 200 m*M* magnesium acetate, and 7 µl 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°C for 60 min, 5 µl reaction mixture was spotted at least 10-15 places on the 3 mm Whatman filter paper and dipped in cold 5% TCA containing 0.2% leucine. Three more washings of two minutes each were carried out with 5% cold TCA. The paper was heated at 90°C in 5% TCA for 2 min and 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 8 ml scintillation cocktail. Percentage of protein synthesis was calculated on the basis of the radioactivity incorporated in the precipitate. The protein synthesis inhibition (in percentage) was expressed as: $IC_{50} = (B/B_o) \times 100$ where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and Bo is in absence of any inhibitor. IC_{50} is the dose of inhibitor required for 50% inhibition of protein synthesis[8,9].

Result and Discussion

The present investigation is an attempt to understand the role of retention of positive charge on the lysine residues of gelonin on the immunological and protein synthesis inhibitor activity. This is based on the fact that gelonin cross-linked with monoclonal antibodies using 2-IT that preserves positive charge, exhibited higher cytotoxicity to the target cells than the conjugates prepared with the use of SPDP alone. The sequential modification of the ε-NH_2 group(s) of HPLC-purified gelonin with 2-IT was carried out following the standard procedure and its effect on immunological and on the RIP activity were determined. These properties were also compared with long chain-SPDP and SPDP modified gelonin derivatives. The structures of different modified derivatives are shown in Fig.1. The cell-free translation experiments were carried out on 2-IT-modified gelonin and compared with that of SPDP and LC-SPDP modified derivatives. Table 1 summarises immunological and protein synthesis inhibition activity in percentage of different preparations. The percentage of RIP activity was determined after measuring IC_{50}. Modification of single amino group with 2-IT results in about 25-50% inhibition of immunoreactivity and 60-70% loss of protein synthesis inhibition activity (Table 1). Modification of 2-3 amino groups further hampers both immunoreactivity and protein synthesis inhibition property of gelonin (Table 1). Both the long chain-SPDP and SPDP modifications showed more pronounced effect on immunoreactivity and RIP activity as compared to the similar ratio of 2-IT modification(s) - (Table 1). It may, therefore, be concluded that the positive charge plays an important role in the immunological as well as the protein synthesis inhibitory effect of gelonin. These observations are of great importance as eventually this would be used *in vivo* cytotoxicity after cross-linking with the carrier proteins.

Table 1—Modification of -NH_2 groups of gelonin by LC-SPDP/SPDP and 2-IT and its effect on immunological and RIP

[The RIP activity was determined *in vitro* in a cell free translational system using rabbit reticulocyte lysate assay as described in the text. GSP-0.5 to GSP-40, GLC-SP-0.5 to GLC-SP-40 and G-IT-5 to G-IT-250 represent various SPDP, LC-SPDP and 2-IT modified derivatives respectively. The SPDP and LC-SPDP modified derivatives preparations were the same as reported earlier[9], but the immunological and protein synthesis inhibiting activity were evaluated again in separate experiments. The cross-reactivity is expressed as percentage with respect to that of native gelonin which was taken as 100% as reported earlier[9]. The immunological and protein synthesis inhibiting activity are expressed as percentage with respect to native gelonin (Column A) and gelonin-HPLC (Column B) which were taken as 100%. ED_{50} and IC_{50} are the dose of inhibitor required for 50% inhibition of immunoreactivity and protein synthesis respectively. The experiments were carried out in triplicate and 10-15% deviation was observed in both the immunological and protein synthesis inhibiting activity]

Gelonin code	LC-SPDP/ SPDP/ 2-IT (mole/ mole)	No. of -NH_2 modified	Immunological activity			Protein synthesis inhibiting activity		
				% reactivity			% reactivity	
			ED_{50} (ng)	A	B	IC_{50} (ng)	A	B
Gelonin			0.56	100	-	0.24	100	-
Gelonin-HPLC			0.38	100	67.85	0.20	100	83.33
G-LCSP-0.5	0.5	-	1.01	55.0	37.62	0.80	30.0	25.00
G-SP-0.5	0.5	0.4 ± 0.1	0.80	70.0	47.50	0.63	38.0	31.74
G-IT-5.0	0.5	0.2 ± 0.1	0.51	109.80	74.50	0.30	80.0	66.66
G-LCSP-1.0	1.0	0.6 ± 0.1	2.0	28.0	19.00	2.26	10.6	8.84
G-SP-1.0	1.0	0.8 ± 0.2	1.21	46.0	31.40	1.09	22.0	18.34
G-IT-10	10	0.5 ± 0.2	0.65	85.0	58.46	0.40	60.0	50.00
G-LCSP-2.0	2.0	1.5 ± 0.2	2.15	26.0	17.67	4.00	6.0	5.00
G-SP-2.0	2.0	2.0 ± 0.2	1.16	48.0	32.75	3.00	8.0	6.66
G-IT-20	20	1.2 ± 0.2	0.74	75.0	51.35	0.60	40.0	33.33
G-LCSP-3.0	3.0	-	-	-	-	-	-	-
G-SP-3.0	3.0	2.6 ± 0.4	1.60	35.0	23.75	6.00	4.0	3.33
G-IT-30	30	2.2 ± 0.4	0.86	65.0	44.18	2.40	10.0	8.33
G-LCSP-4.0	4.0	3.4 ± 0.5	5.49	10.2	6.92	60.00	0.4	0.33
G-SP-4.0	4.0	3.2 ± 0.4	2.80	20.0	13.57	24.00	1.0	0.83
G-IT-40	40	2.9 ± 0.5	1.27	44.0	29.90	10.00	2.4	2.00
G-LCSP-5.0	5.0	-	-	-	-	-	-	-
G-SP-5.0	5.0	4.5 ± 0.8	3.11	18.0	12.21	240.0	0.10	0.08
G-IT-50	50	3.5 ± 0.5	1.86	30.0	20.43	10.0	2.40	2.00
G-LCSP-8.0	8.0	5.0 ± 0.5	5.60	10.0	6.78	-	-	-
G-SP-8.0	8.0	6.5 ± 0.8	3.68	15.2	10.32	-	-	-
G-IT-100	100	5.2 ± 0.8	2.54	22.0	14.96	20.0	1.2	1.00
G-LCSP-10	10	-	-	-	-	-	-	-
G-SP-10	10	6.0 ± 1.0	9.03	6.2	4.20	-	-	-
G-IT-150	150	5.6 ± 0.8	2.80	20.0	13.57	120	0.2	0.16
G-LCSP-15	15	6.0 ± 1.0	-	-	-	-	-	-
G-SP-15	15	10 ± 1.0	17.5	3.2	2.17	-	-	-
G-IT-200	200	8.0 ± 1.0	5.60	10.0	6.78	240	0.1	0.08
G-LCSP-20	20	-	-	-	-	-	-	-
G-SP-20	20	12.2 ± 2.0	18.66	3.0	2.03	-	-	-
G-IT-250	250	10.6 ± 2.0	10.76	5.20	3.53	-	-	-
G-LCSP-25	25	-	-	-	-	-	-	-
G-SP-25	25	14.8 ± 2.0	70	0.80	0.54	-	-	-
G-LCSP-30	30	-	-	-	-	-	-	-
G-SP-30	30	14.6 ± 2.0	140	0.40	0.27	-	-	-
G-LCSP-35	35	-	-	-	-	-	-	-
G-SP-35	35	13.0 ± 2.0	700	0.08	0.05	-	-	-
G-LCSP-40	40	-	-	-	-	-	-	-
G-SP-40	40	13.2 ± 2.0	-	-	-	-	-	-

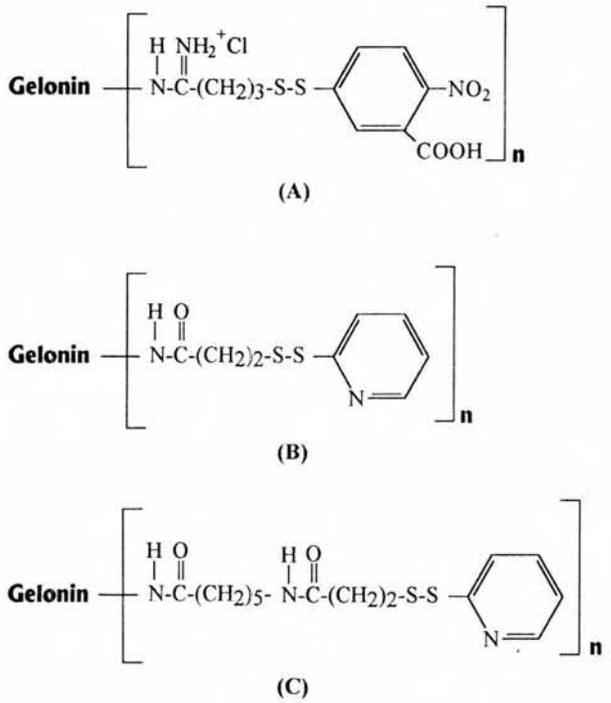

Fig. 1—Structure of gelonin modified with the use of 2-IT (A), SPDP (B) and LC-SPDP (C). The number *n* indicates the number of -NH_2 groups modified with the use of HBCLA as shown in Table 1. The -SH group(s) of 2-IT modified gelonin derivatives are blocked with DTNB as described in Methods section

Similar to gelonin, we studied earlier[11], the significance of positive charge of lysine residues of oLH on immunological and biological activity by sequentially modifying its ε-NH_2 group(s) with 2-IT that preserves the positive charge of the lysine while the overall charge of the hormone remains unchanged[11]. SPDP and LC-SPDP abolish positive charge of lysine residues. Sequential modification led to progressive reduction in receptor binding and immunological activities[11]. However, the steroidogenic activity was substantially retained[11]. The immunoreactivity and receptor binding properties of 2-IT modified oLH (oLH-2-IT) were less affected when compared to SPDP (oLH-SPDP) or LC-SPDP (oLH-LC-SPDP) modified derivatives suggesting that increase in hydrophobic carbon chain in oLH-LC-SPDP molecule resulted in drastic inhibition in immunological and biological properties. But the steroidogenic potential of oLH-2-IT, oLH-LC-SPDP or oLH-SPDP was relatively comparable. This suggests that a single -NH_2 group modification with 2-IT would generate the site in the hormone for conjugation to the toxin/carrier proteins that may retain better immunological and biological activity compared to that of SPDP or LC-SPDP modified oLH. But it will be difficult to use 2-IT in both hormone and gelonin for generating an effective hormone-gelonin conjugate that preserves the overall positive charge on the molecules.

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