Purification and characterisation of gelonin from seeds of *Gelonium multiflorum*

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Gelonin, a ribosome-inactivating protein has been isolated from the seeds of *Gelonium multiflorum* of Euphorbiaceae family by two methods and the results are compared. In method-I conventional aqueous extraction, cation-exchange and gel-filtration chromatography has been used. In method-II S-Sepharose fast flow gel has been used to purify the proteins from the seed extract, followed by ammonium sulfate fractionation, cation-exchange and gel-filtration chromatography. Extensive physico-chemical and immunological characterizations show that molecular weight of gelonin as determined by gel-filtration chromatography and SDS-PAGE is \( \approx 30 \) kDa. The non-proteinous material which binds to CMC-gel in association with gelonin in method-I is substantially removed when gelonin is purified by method-II. Cation exchange, G-100 chromatography, RP-HPLC and SDS-PAGE show that method-II yields 50% more purified gelonin when compared to the yield by method-I. The immunoreactivity of gelonin obtained by methods I and II vary from 22-26% and 50-66% respectively and the ribosome-inactivating property vary from 46-56% and 70-87% respectively.

Ribosome-inactivating proteins (RIP), a group of proteins that catalytically inactivate eukaryotic ribosomes, have been identified in the extract of a wide variety of plants\(^7\). These proteins are RNA N-glycosidases that depurinate the major rRNA, thus damaging ribosome and eventually arrest protein synthesis\(^7\). These have been classified into two groups: single chain proteins, type 1 RIPs and two chain proteins, type 2 RIPs. The type 1 RIPs and A chain of type 2 RIPs possess the same RNA N-glycosidase activity.

Due to the basic nature of gelonin, the protein was isolated following cation exchange and gel-filtration chromatography\(^5,12\). The other method involves the dye binding and ammonium sulfate fractionation prior to CMC-52 or gel-filtration chromatography\(^13\). The gelonin purified by conventional methods contain substantial contamination of non-proteinous substance which also binds to CMC column and co-elutes with gelonin\(^13\). The present communication describes the purification of gelonin by an improved method which involves acid precipitation followed by S-Sepharose fast flow gel binding chromatography as a first step of purification of proteins from the seed extract. This was followed by ammonium sulfate fractionation, CMC-52 and G-100 chromatography. The purified gelonin preparation was subjected to physico-chemical, immunological and ribosome-inactivating studies and compared with that of gelonin isolated by the conventional method.

Materials and Methods

Bovine serum albumin (Cohn fraction IV), Freund's complete and incomplete adjuvants were obtained from Sigma Chemical Co., St. Louis, MO, USA. Chromatographic materials such as G-100 (medium grade) and carboxymethyl cellulose (CMC-
52) were purchased from Pharmacia Fine Chemicals and Whatman Co. USA. IODOGEN™ was obtained from Pierce Chemicals, Rockford, IL, USA. Carrier free NaI (IMS-30) was from Amersham International Ltd., UK. The seeds of Gelonium multiflorum were from United Chemicals and Allied Products, Calcutta. Other chemicals used in the investigation were of highest purity.

**Purification of gelonin**

Gelonin was isolated from the dry seeds of Gelonium multiflorum by using two different methods which are briefly described below:

**Method I**

Gelonin was isolated by the method originally described by Stripe et al. with minor modification as described. The deshelled seeds were blended with ten volumes of the extraction buffer (50 mM sodium phosphate buffer, pH 7.2 containing 100 mM NaCl). The smooth slurry was stirred overnight at 4°C and centrifuged in cold. The precipitate was re-extracted with the same buffer and again centrifuged. The supernatants of both the extractions were pooled and dialyzed extensively against dialyzing buffer (50 mM sodium phosphate, pH 6.0, without NaCl). The dialyzed supernatant was again centrifuged to remove the precipitate and then passed through a CMC-52 column, pre-equilibrated with the dialyzing buffer. The column was extensively washed with the equilibration buffer until the eluate showed absorbance less than 0.05 at 280 nm. The material bound to the column was eluted by applying a linear gradient of 0 mM-300 mM NaCl in the same buffer at a flow rate of 30 ml/hr at 4°C. Fractions were read at 280 nm. The protein eluting at around 150 mM-200 mM NaCl gradient was pooled, concentrated by Centricon™ (Amicon) and subjected to gel-filtration chromatography on a pre-calibrated G-100 (3.4×110 cm) column. The column was developed with 50 mM ammonium bicarbonate. The protein eluting at ε 30 kDa was collected, concentrated and re-chromatographed on the same G-100 column. The 30 kDa protein peak was collected and lyophilized.

**Method II**

The deshelled seeds were homogenized with ten volumes of sodium phosphate buffered saline (10 mM sodium phosphate buffer, pH 7.2 containing 100 mM NaCl). The extraction was carried out at 4°C for 24 hr under constant stirring. The extract was centrifuged at 10,000 xg for 60 min at 4°C in order to remove particulate material and fat. The pH of the supernatant was then brought down to 4.0 by addition of glacial acetic acid. The solution was again stirred for 4 hr at 4°C and centrifuged at 10,000 xg for 30 min to remove the precipitate. The supernatant was passed through a S-Sepharose fast flow column, previously equilibrated with 10 mM sodium acetate buffer, pH 6.5. The column was washed with the same buffer and the bound material was eluted with 10 mM sodium phosphate buffer, pH 7.0 containing 1000 mM NaCl. The protein containing peak was collected and precipitated by addition of solid ammonium sulfate to 100% saturation. The precipitate was resuspended in the smallest possible volume of 5 mM sodium phosphate buffer pH 6.5, containing 10 mM NaCl and dialyzed extensively against the same buffer. The material precipitated during dialysis was removed by centrifugation and the supernatant was applied to CMC-52 column. After thorough washing of the column, the bound protein was eluted by applying a gradient of 10 mM-300 mM NaCl in the same buffer. The protein eluting ~150 mM NaCl gradient was concentrated and further purified by gel-filtration chromatography on Sephadex G-100 column as described in the above methods.

**Characterization of gelonin**

**Molecular weight and homogeneity determination**

The molecular weight of gelonin was determined by using different techniques. Gel permeation chromatography on Sephadex G-100 was used to determine the molecular weight of the purified gelonin. All gel permeation columns were calibrated with the calibration kit using known molecular weight proteins. Chromatography was carried out using HPLC system and monitored at 280 nm. A plot of Ve/Vo (where Ve and Vo are elution and void volume respectively) versus log MW was plotted and Ve of gelonin was used to calculate molecular weight (MW).

Homogeneity and molecular weight was also determined by SDS-PAGE analysis. The gel slab (20×12 cm) was cast with acrylamide gradient (5-15% w/v) according to Laemmli under reducing and non-reducing conditions. The gel was stained with Commassie blue. Low and high molecular weight reference standard from Bio-Rad served as markers.
RP-HPLC was used for further analysis of homogeneity and purity of gelonin obtained from different methods as described earlier. RP-HPLC was performed on a Waters µBondapak phenyl column (4×250 mm) using a continuous gradient. The samples dissolved in water containing 0.1% TFA (solvent A) was run on the above column in solvent A+25% acetonitrile containing 0.1% TFA. A gradient of 25-50% was run over 30 min at flow rate of 2 ml/min in a HPLC system equipped with a computer for the analysis of the data.

Amino acid analysis

The samples were hydrolyzed in vacuo at 110°C in 6 N HCl for 22 hr and analyzed by ninhydrin method in a Beckman amino acid analyzer.

Radioiodination of gelonin

The radioiodination of gelonin was performed by IODOGEN method following the protocol described earlier for GnRH. The specific activity of [125I]-gelonin was 80 µCi/µg as determined by autodisplacement method. The immunoreactivity of [125I]-gelonin was >85% as estimated by measuring the binding ability of [125I]-gelonin in the presence of excess of anti-gelonin antibodies.

Antibody binding assay

The antibody binding assay was carried out by following the procedure described. Briefly, the antibodies were initially screened using a series of dilutions made in the assay buffer (50 mM sodium phosphate buffer, pH 7.5, containing 0.1% each sodium azide and BSA and 1% normal rabbit serum) at a single concentration of [125I]-gelonin (~30,000 cpm/tube). The assay system consisted of 0.1 ml each [125I]-gelonin and different dilutions (1:10 to 1:10^6) of antibodies. The tubes were incubated overnight at 4°C. The bound and free [125I]-gelonin were separated by adding 0.2 ml of Pansorbin™ (Calbiochem, LaJolla, CA, USA). The tubes were further incubated for 30 min at 25°C and centrifuged in cold for 20 min at 3,000 x g. The supernatant was assayed and the pellet was counted in a LKB-Rackminigama counter (~70% efficiency).

Competitive binding assay

Cross-reaction of anti-gelonin antibodies with purified gelonin was determined by competitive displacement method. The dilution of antibody giving 30-50% binding in the absence of a competitor was allowed to compete with the different gelonin preparations in the radioimmunoassay as described above. The assay system consisted of 0.1 ml each of [125I]-gelonin, gelonin antibody and various concentrations of competitors. The incubation, precipitation and other assay procedures were the same as described above. The inhibition lines were obtained by plotting (B/Bo)×100 where Bo represents the binding of [125I]-gelonin in absence of cold gelonin and B indicates the binding of the competitor. The extent of cross-reactivity (in percentage) was expressed as: C=(L60/U30)×100, where L60 is the dose of gelonin which shows 50% inhibition and U30 is the dose of competitor which shows 50% inhibition.

In-vitro cell free translation assay

The inhibitory activity of purified gelonin preparations 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 [3H]-leucine into the TCA insoluble protein fraction. 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 tritiated leucine, 0.5 µl each 500 mM potassium acetate and 200 mM magnesium acetate, 7 µl nuclease treated rabbit reticulocyte lysate. The positive control was incubated with mRNA (no test sample) while negative control received equal volume of RNAsafe free water. After incubation at 30°C for 60 min, 5 µl reaction mixture was spotted at 10-15 places on a 3 mm Whatman filter paper and dipped in cold 5% TCA containing 0.2% leucine. Three more washing of two minutes each was carried out with 5% cold TCA. The paper was heated at 90°C in 5% TCA for 2 min 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 inhibition of protein synthesis (in percentage) was expressed as: IC50=(B/Bo)×100 where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and B0 is in absence of any inhibitor. IC50 is the dose of inhibitor required for 50% inhibition of protein synthesis.
ge lo nin a nd th ree ba tc he s o f ge lo nin we r e purifi e d by this m eth od and th e ir eluti on pr of ile s a r e sh ow n in Fig. 1A. T he m aj or peak eluti ng at ~150 mM NaCl gradient (as marked) was concen tra ted and fur th er purifi ed on Sephadex G-100 column (Fig. 1A and 1B). T he chromato graphy pr of ile s s l i g ht ly d i f fer fr om that of re port ed by Str ipe et al. 12 w here se ver al peaks we re ob tained. T he protein eluting between 150 mM-200 mM NaCl gradient showed inhib ition of pr otein synthesis in a cell-free tr anslation ass y us ing r abbit ret ic ul o c yte ass y (data no t s how n). T he v a ri a ti on in the pr of ile m ay be du e to the use of d i f fer ent ca ti on ex ch a n ge CMC-g el. M or eov er, th e se ed ba tc hes m ay a l so a t t rib u te to s u ch v a ri a ti on. T he a p propri ate peak was pool ed, concen tra ted and subj ec ted to ge l-fi ltr ati on chro ma tography on a pr ec a lib r a ted Sephadex G-100 column and re fra cti on ate d on th e sa me column. It we re n oted th a t ge lo nin s how s a ten dency to ag g reg ate duri ng concen tra ti on and eluted in th e void v olume of th e column. Th is aggreg ated pr otein was immunologically a ctive. T he m aj or peak of ~30 kDa wa s pool ed, lyo phil i z ed di rect ly a nd store d at 4°C until fu rther a nal y s i s.

S-S eph aro se fa st f low ge l w as u sed in m eth od-II as a f irst step of pu rif ic a ti on d u e to th e f act th a t th e ba sic pr otein from th e se ed extr act inter ac t w ith it. T h ree d if fer ent ba tc hes we re pr e pa red a nd th e ir elution pr of ile s a r e sh ow n in Fig. 1B. T he m aj or peak eluting at ~150 mM NaCl gradient (as marked) wa s concen trat ed and fur th er pu rif i ed by ge l-fi l tra ti on chro ma tography on Sephadex G-100 column (Figs. 2C and D).

T he di ffer ent ba tc hes of ge lo nin pu rif i ed by b o th m eth ods we re subj e cted to fu rther c har a cte r i za tio n fo r ho mog e neity, m olecu la r we ig h t d e te rmin a ti on, a m in o a c id comp os itio n, imm unore a c tiv ity , a n d pr o tein s ynth es is inhib itio n a c ti v it y. T he m olecu la r we ig h t d e ter min e d by ge l-fi ltr a ti on chro ma tography a n d S D S-P A GE a nal y sis w a s ~30 kDa fo r a ll p r e pa rar tio ns of ge lo nin. T he m ole cu la r we ig h t a n d ho mo geneity wa s al so d e te r min e d by HPLC us ing gel-pe r me a tio n c ol um n. O n th e ba sis of d ry w e ig h t, an e qu a l a m o u nt of ge lo nin d is so lve d in 100 mM so di um ph os pha te bu ff er p H 7.5 co nta in ing 100 mM NaCl a n d 0.1% so di um az id e we re run on a pre ca lib r a ted Sephadex G-100 column us ing HPLC sy st e m pumps. A l l b at ch es of ge lo nin w ere ru n on th e sa me column. T he peak a p pear ing in th e vo id v olume w as du e to th e ag g reg ated pr otein w hich w as c on firme d by imm unore a c tiv ity a nd S D S-P A GE a nal y sis wh ile th e m inor p eaks w ere th e co m m u nta ted pr oteins w hich di d no t re ac t to a nti -ge lo nin a nti b o d ie s (da ta no t s how n). Fig. 1 sh ows th e c ompar ison of b oth CMC-

Results and Discussion

Method I involved two steps for the purification of gelonin and three batches of gelonin were purified by this method and their elution profiles are shown in Fig. 1A. The major peak eluting at ~150 mM NaCl gradient (as marked) was concentrated and further purified on Sephadex G-100 column (Fig. 1A and 1B). The chromatography profiles slightly differ from that of reported by Stripe et al. 12 where several peaks were obtained. The protein eluting between 150 mM-200 mM NaCl gradient showed inhibition of protein synthesis in a cell-free translation assay using rabbit reticulocyte assay (data not shown). The variation in the profile may be due to the use of different cation exchange CMC-gel. Moreover, the seed batches may also attribute to such variation. The appropriate peak was pooled, concentrated and subjected to gel-filtration chromatography on a pre-calibrated Sephadex G-100 column and refractionated on the same column. It was noted that gelonin shows a tendency to aggregate during concentration and eluted in the void volume of the column. This aggregated protein was immunologically active. The major peak of ~30 kDa was pooled, lyophilized directly and stored at 4°C until further analysis.

S-Sephrose fast flow gel was used in method-II as a first step of purification due to the fact that the basic protein from the seed extract interact with it. Three different batches were prepared and their elution profiles are shown in Fig. 1B. The major peak eluting at ~150 mM NaCl gradient (as marked) was concentrated and further purified by gel-filtration chromatography on Sephadex G-100 column (Figs. 2C and D).

The different batches of gelonin purified by both methods were subjected to further characterization for homogeneity, molecular weight determination, amino acid composition, immunoreactivity, and protein synthesis inhibition activity. The molecular weight determined by gel-filtration chromatography and SDS-PAGE analysis was ~30 kDa for all preparations of gelonin. The molecular weight and homogeneity was also determined by HPLC using gel-permeation column. On the basis of dry weight, an equal amount of gelonin dissolved in 100 mM sodium phosphate buffer pH 7.5 containing 100 mM NaCl and 0.1% sodium azide was run on a precalibrated Sephadex G-100 column using HPLC system pumps. All batches of gelonin were run on the same column. The peak appearing in the void volume was due to the aggregated protein which was confirmed by immunoreactivity and SDS-PAGE analysis while the minor peaks were the contaminated proteins which did not react to anti-gelonin antibodies (data not shown). Fig. 1 shows the comparison of both CMC-
52 gel-filtration chromatograms of the crude gelonin obtained from method-I and method-II. The major peak was concentrated and refractionated on G-100 column (Fig. 2). A minor peak in the void volume was due to the aggregation of protein (Fig. 2). The major peak was concentrated and the total protein contents was determined. In order to compare, an equal quantity was again fractionated on the same G-100 column and the elution profiles are shown in Fig. 2B and 2C. Comparison of these profiles with that of Fig 2A and 2B clearly reveal that gelonin purified by method I contain more impurities absorbing at 280 nm than that of method-II. Method-II yielded relatively more purified material and devoid of non-proteinous substances although the yield was lesser.

The RP-HPLC chromatograms of gelonin purified from both the methods-I and -II are shown in Fig. 3A and 3B respectively. It is evident that gelonin binds to column tightly to μBondapack phenyl column and is eluted at higher acetonitrile concentration. The elution patterns clearly reveal a hump at the

![Diagram](image-url)
ascending portion of the peak (Fig. 3) which could not be resolved even after applying a shallow gradient. This may be due to variation in the glycosylation of gelonin. To compare the RP-HPLC chromatograms, an equal quantity of protein was subjected to RP-HPLC analysis. Upon comparison of the total area of the peak, it was confirmed that method-II yielded more purified gelonin than method-I.

The purity and molecular weight, was further determined by SDS-PAGE analysis (Fig. 4). In addition to a major band of ~30 kDa, two minor bands of ~32 kDa and ~28 kDa were also obtained. As predicted earlier, these may be the variants of gelonin that could not be resolved under the conditions described above. Possibly the hump of RP-HPLC may correspond to one of or both these components. No change either in the position or intensity of the bands was observed when SDS-PAGE was run under reducing conditions. This also indicates that gelonin obtained is a single polypeptide chain. Gelonin from both methods (40 μg each) were analyzed on SDS-PAGE gel (Fig. 4; lane 1 and 2). The densitometric scanning of the bands clearly showed that gelonin obtained from method-II contained about twice as much of gelonin as that of method-I.

The amino acid analysis on all batches of gelonin and their performic acid oxidized products are reported in Table 1. The amino acid data were in agreement with those reported earlier.

**Immunoreactivity studies**

The immunoreactivity of gelonin isolated by method-I and method-II was compared by competitive displacement method. In both the cases, native gelonin (obtained after G-100 rechromatography) and HPLC purified gelonin were used as standard and ED₅₀ for them was used to calculate the percentage activity. Fig. 5A and 5B show the displacement curves of different gelonin preparations obtained from method-I and II respectively and Table 2 shows their corresponding quantitative data. The data show that the immunoreactivity was less in gelonin from method-I than that of gelonin from method-II. All the
Table I—Amino acid composition of gelonin obtained by method-I and method-II.

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Number in parentheses indicate the values obtained from the literature.

Fig. 5—Immunological cross-reactivity of gelonin antibodies to HPLC purified gelonin (Gelonin-HPLC) and gelonin purified from two different methods as obtained by competitive displacement method. (A): Displacement curves of gelonin-HPLC (— — —) and 3 batches of gelonin (— — —), (— — —), (— — —) by method-I. (B): Displacement curves of Gelonin-HPLC (— — —) and 3 batches of gelonin (— — —), (— — —), (— — —) by method-II. The quantitative immunoactivity data as determined from these displacement curves are recorded in Table 2.

Fig. 6—Inhibition of protein synthesis in the cell free translation system from rabbit reticulocyte lysate assay by (A): Gelonin-HPLC (— — —) and gelonin from 3 batches (— — —), (— — —), (— — —) by method-I. (B): gelonin from 3 batches (— — —), (— — —), (— — —) by method-II. [Protein synthesis was measured in a cell-free system by the incorporation of [3H]-leucine into protein. The percentage protein synthesis was calculated by measuring IC<sub>50</sub> of control (gelonin) and test system. The quantitative data are presented in Table 2].
three batches of gelonin obtained from method-I were less active than that of the HPLC purified gelonin (Table 2). On the other hand, the activity of the three batches of gelonin obtained from method-II was 1.5-2.0 times less active than that of HPLC purified gelonin (Table 2). This may be due to the presence of non-gelonin material which absorbs at 280 nm in the preparations from method-I. This clearly reveals that the S-Sepharose fast flow prepurification followed by ammonium sulfate precipitation (method-II) is a useful step in the purification of gelonin. Possibly the heavy contamination was removed at 0-45% ammonium sulfate concentration during purification.

The results of the cell-free translation experiments using gelonin purified by methods-I and -II (Fig. 6) show a relationship between the percentage protein synthesis with the dose of different preparations. Their quantitative data are shown in Table 2. The percentage ribosome-inactivating activity was determined after measuring IC₅₀ (test sample required for 50% inhibition of protein synthesis).

The present investigation shows that the non-proteinous material which binds to CMC-gel in association with gelonin is removed when gelonin is purified by method-II. Cation exchange, G-100 chromatography, RP-HPLC and SDS-PAGE clearly indicate that although method-II yields less protein, about 50% more purified gelonin is obtained when compared to method-I. The immunoreactivity of gelonin obtained from methods I and II vary from 22-26% and 50-66% respectively and the ribosome-inactivating property vary from 46-56% and 70-87% respectively.

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**References**