Chemical modification studies of gelonin

Involvement of arginine residues in biological activity

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Gelonin inhibits protein synthesis by inactivating the eukaryotic 60 S ribosomal subunit by an unknown mechanism. The protein was purified in high yield by a new method using Cibacron blue F_3GA-Sepharose. Chemical modification studies reveal that arginine residues are essential for biological activity.

Ribosome inactivating protein *Protein synthesis* *Toxin* *Chemical modification*

1. INTRODUCTION

Proteins which inactivate eukaryotic ribosomes in a manner similar to ricin have been isolated and characterized from several plants [1]. These ribosomal inactivating proteins (RIPs) inhibit protein synthesis by enzymatically inactivating the 60 S ribosomal subunit. Despite considerable efforts, toxin-induced changes in the RNA or protein molecules of the 60 S ribosomal subunit have not been identified and their exact mechanism of action is unknown.

Much interest in the study of these proteins derives from their potential use in the synthesis of immunotoxins [2]. Gelonin, a prototype of the A chain toxin, has been purified earlier and some of its properties have been reported [3]. It has been chemically coupled to lectins and antibodies to generate cytotoxic molecules [4]. However, very little information is available on the structural features essential for biological activity. Detailed structure-activity correlation studies of these proteins are essential, not only for understanding the nature of their combining site, but also for the design and synthesis of more potent toxin conjugates. Chemical modification of various amino acid side chains is one of the very useful methods for identifying residues involved at the active site.

Here, gelonin has been purified in high yields by a new method, the amino acid residues modified using specific reagents and the effects of these chemical modifications on its biological activity investigated. Such studies indicate that arginine residues are essential for manifestation of the biological activity of gelonin.

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Abbreviations: CHD, cyclohexane-1,2-dione; CD, circular dichroism; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Materials*

Seeds of *Gelonium multiflorum* were obtained from United Chemicals, Calcutta. [^{3}H]Leucine of specific activity 182 Ci/mmol was obtained from Amersham Radiochemical Centre. Succinic anhydride, CHD and 2-hydroxy-5-nitrobenzyl bromide were obtained from Pierce. Ethoxyformic anhydride and *N*-bromosuccinimide were from Sigma. SDS-PAGE molecular mass markers were from Pharmacia. Synthesis of *p*-nitrophenylglyoxal was as described [5].

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2.2. *Purification of gelonin*

Gelonin was purified by chromatography on Cibacron blue F_3GA-Sepharose columns. The dye was coupled to Sepharose as described in [6] and the details of purification are described in fig.1A.

2.3. *In vitro protein synthesis*

The biological activity of gelonin was assayed by its ability to inhibit protein synthesis in the rabbit reticulocyte lysate assay system as described [7].

2.4. *Amino acid analysis*

Gelonin was hydrolysed in evacuated sealed ampoules with 6 N HCl at 100°C for 24 h. Amino acid analyses were performed in duplicate on an LKB 4400 amino acid analyser.

2.5. *Circular dichroism studies*

CD spectra were recorded on a Jasco J-20 automatic recording spectropolarimeter. Protein concentration was 200 μg/ml. Solutions were made in 0.1 M NaCl, 10 mM sodium phosphate buffer, pH 7.2 (PBS), and a 5 mm path length cell was used.

2.6. *Protein estimation*

Protein estimations were performed by the method of Lowry et al. [8] or by Bradford's dye binding procedure [9].

2.7. *Preparation of antisera*

Rabbits were immunized at biweekly intervals with 1 mg each of gelonin in complete Freund's adjuvant. Booster injections were 100 μg protein in incomplete Freund's adjuvant. Immune sera were collected and IgG prepared as described [10].

2.8. *Chemical modification studies*

The various amino acid side chains were modified as described below. After modification, excess reagent was removed by gel filtration on Sephadex G-25 columns (20 × 1 cm) equilibrated in PBS.

2.8.1. Modification of lysine residues

The lysine side chains were modified using succinic anhydride [11] or citraconic anhydride [12]. To the protein (2 mg/ml in 50% saturated $NaHCO_3$ solution, pH 8.0) was added a 300 M excess of either reagent. The solution was stirred at 4°C for 1 h and the pH maintained at 8.0. Reductive methylation was carried out as described [13]. The percentage of modification of the lysine ϵ-amino groups was assessed using 2,4,6-trinitrobenzenesulphonic acid [14].

2.8.2. Modification of arginine residues

Gelonin (2 mg) in 3 ml of 0.1 M sodium pyrophosphate, 0.15 M sodium ascorbate, pH 9.0, was reacted with 25 μl of a 10% methanol solution of *p*-nitrophenylglyoxal for 30 min at 30°C. The extent of modification was determined as described [15]. Alternatively the arginine residues were modified with CHD as described [16]. Protein (2 mg/ml) in 0.2 M borate buffer, pH 9.0, was reacted with a 200 M excess of a methanol solution of CHD. The reaction was carried out in the dark under N_2 for 18 h. The number of unreacted arginine residues remaining after modification with CHD was estimated using *p*-nitrophenylglyoxal. The reaction with CHD was reversed with hydroxylamine at pH 7.0 as described [16].

2.8.3. Modification of tyrosine, tryptophan, histidine and carboxyl residues

For modification of tyrosine residues 2 mg/ml of gelonin in 0.01 M phosphate buffer, pH 7.5, was treated with 300 M excess of *N*-acetylimidazole at 5°C for 1 h. The extent of modification was estimated as described [17].

Modification of tryptophan residues was carried out with 2 mg/ml of protein in 0.1 M sodium acetate buffer, pH 4.0, as described using *N*-bromosuccinimide in the absence of urea [18] or with 2-hydroxy-5-nitrobenzyl bromide in the presence of 8 M urea [19].

Modification of histidine residues was carried out as described using ethoxyformic anhydride [20]. Carboxyl groups were modified using [^{14}C]glycine methyl ester [21].

3. RESULTS

3.1. *Purification of gelonin*

Gelonin eluted on Cibacron blue F_3GA-Sepharose columns at 0.7 M NaCl (fig.1A). The protein thus obtained was pure and had an apparent molecular mass of 30 kDa on SDS-PAGE gels (fig.1B). Yields of gelonin were high; 60 mg

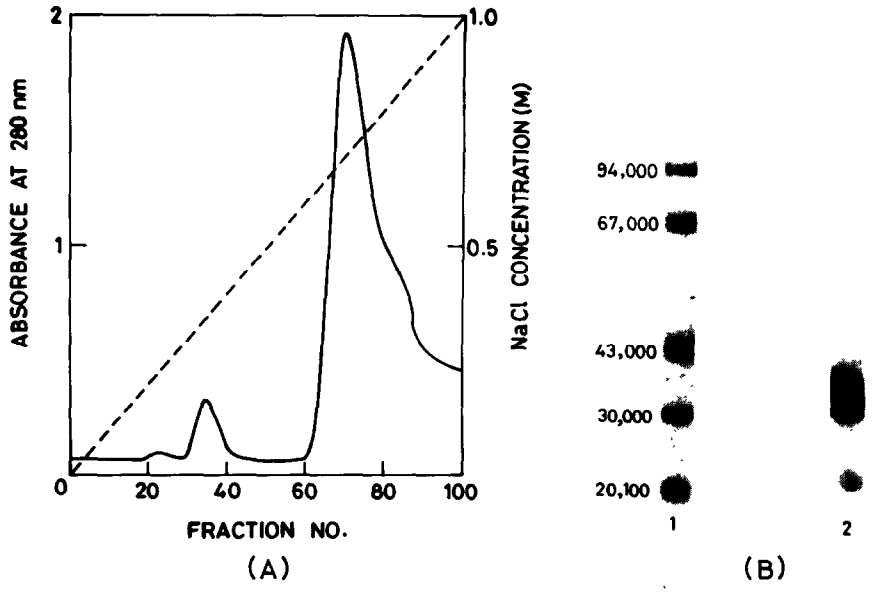

Fig.1. (A) Chromatography of gelonin on Cibacron blue F_3GA-Sepharose. About 150 ml (780 mg protein) of a crude homogenate of *Gelonium multiflorum* seeds in 0.1 M NaCl, 10 mM phosphate buffer, pH 7.2 (PBS) was loaded on a 100 ml column of gel. The column was washed with PBS and eluted with a gradient of NaCl in PBS (0.1–1.0 M), 250 ml each. Fractions containing protein were pooled and lyophilized. (B) SDS-PAGE of purified protein. Gel 1: molecular mass markers (in kDa): phosphorylase *b* (94), BSA (67), ovalbumin (43), carbonic anhydrase (30), β-lactalbumin (21). Gelonin (20 μg) was loaded in gel 2. Electrophoresis was according to the procedure of Laemmli [24].

pure protein were obtained from 10 g seeds. Purified samples can be stored indefinitely at −20°C without any loss of activity.

3.2. *Modification studies*

The results of the chemical modification of various amino acid side chains of gelonin are shown in table 1. It is apparent that the biological activity of the protein is unaffected by a variety of chemical treatments. Extensive modification of the ϵ-amino groups of lysine or histidine side chains did not result in any significant decrease in its biological activity. Modification of either the tyrosine or carboxyl residues also did not affect the activity.

Modification of the single tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide in the presence of urea did not affect the enzymatic activity of gelonin. This single tryptophan residue was not oxidised with *N*-bromosuccinimide suggesting that it is buried in the hydrophobic interior of the protein molecule, inaccessible to the reagent.

Modification of the arginine residues with either *p*-nitrophenylglyoxal or CHD, reagents specific for these residues, led to 80 and 70% loss in biological activity, respectively. All 10 arginine residues of the protein were modified with *p*-nitrophenylglyoxal, and 9 residues with CHD, indicating that most of the arginine residues are exposed on the hydrophilic surface of the molecule. Reversal of the modification with CHD using hydroxylamine led to a complete recovery of activity. Thus the observed loss of biological activity is exclusively due to modification of arginine residues.

Fig.2 shows the inhibition of protein synthesis by gelonin as a function of the number of arginine residues modified with CHD. Gelonin was reacted with CHD and at the different time intervals indicated, samples were estimated for both the number of arginines modified and their ability to inhibit protein synthesis. Two of the 10 arginine residues could be modified without loss of activity. Subsequent modification, however, led to a dramatic loss in activity. Thus from 1–2 h of reac-

Table 1
Chemical modifications of gelonin

Reagent	Residue modified[a]	% modification (mol/mol protein)	Protein synthesis (% control)
Succinic anhydride	Lys (18)	70	80
Citraconic anhydride	Lys	60	80
Formaldehyde + sodium borohydride	Lys	55	90
p-Nitrophenylglyoxal	Arg (10)	100	20
Cyclohexanedione	Arg	90	25
Cyclohexanedione + NH_2OH (pH 7.0)	Arg	0	90
N-Acetylimidazole	Tyr (8)	60	80
2-Hydroxy-5-nitrobenzyl bromide	Trp (1)	100	90
N-Bromosuccinimide	Trp	–	90
Ethoxyformic anhydride	His (2)	100	95
[^{14}C]Glycine methyl ester	Glu,Asp (48)	30	93

[a] Numbers in brackets indicate number of amino acid residues present in native gelonin as determined by amino acid analysis

Gelonin, 2 mg (60 μM), was reacted with the various reagents essentially as described. After the reaction, samples were chromatographed on Sephadex G-25 columns to remove free reagent. Control samples were treated similarly except no modifying reagent was added. Modified and control samples were tested for their ability to inhibit protein synthesis using the rabbit reticulocyte lysate assay described [7]. The same final concentration (160 nM) of control or modified gelonin was used in these assays

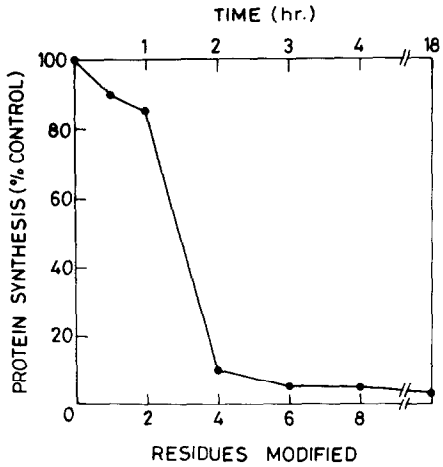

Fig.2. Gelonin (2 mg) in 1 ml of 0.2 M borate buffer, pH 9.0, was treated with 200 M excess of CHD in methanol. At the different time intervals indicated, samples were chromatographed on Sephadex G-25 superfine columns in PBS, dialysed and lyophilized. The extent of modification and the activity of control and modified samples were determined as described in the text.

tion with CHD there was a 90% loss of activity when a total of 4 arginine residues were modified. There was no further change in activity when the remaining 6 residues were modified.

The observed loss of activity could be the result of a conformational change in the molecule following modification. CD measurements and immunodiffusion studies were carried out to investigate the conformational changes, if any, resulting from chemical modification of the arginine residues.

The far-UV CD spectra (fig.3A) of native gelonin and gelonin wherein all the arginine residues had been modified with CHD (18 h reaction) are almost superimposable. Thus the observed loss of activity following modification is a direct result of the modifying reaction and not due to any gross conformational change.

Fig.3B shows that antibodies raised to native gelonin cross-reacted with the arginine modified protein giving lines of total immunological identity. This also rules out the possibility that the observed loss of biological activity is due to a

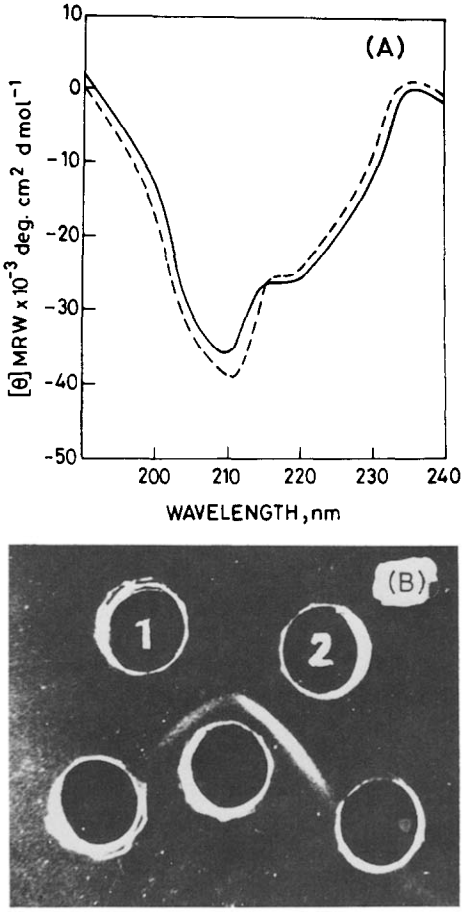

Fig.3. (A) CD spectra of arginine-modified (– – –) and native (——) gelonin. Modified protein sample had been treated with CHD for 18 h. (B) Double immunodiffusion of native and arginine modified gelonin. Plates were made in 1% agarose in PBS. Central well contained purified anti-gelonin IgG (100 μg); wells 1 and 2 contained 20 μg each of native and modified protein, respectively.

change in the conformation of the protein following modification.

4. DISCUSSION

Using visible difference absorption spectroscopy it has been shown that ricin A chain binds specifically to the dye Cibacron blue F_3GA [22]. We have found that gelonin, an A chain toxin, also interacts specifically with the dye molecules immobilized on Sepharose. The protein can be purified in a single step using Cibacron blue F_3GA-Sepharose columns. The yields of gelonin by this method are 6-fold higher than that reported by Stirpe et al. [3] using CM-cellulose columns. The tremendous interest generated recently in the synthesis and use of immunotoxins should make dye-Sepharose affinity chromatography a very useful method to purify other A chain toxins.

Chemical modification studies of gelonin were undertaken to determine the amino acid residues essential for its biological activity. Gelonin is particularly amenable to such studies because of its ease of purification and extreme stability to a variety of chemical treatments. Modification of lysine, tyrosine, tryptophan, histidine and carboxyl groups had no effect on the biological activity of gelonin. Modification of the arginine residues alone led to a drastic loss of activity. This loss of activity was fully restored on treatment with hydroxylamine which reverses the modification. The arginine residues of gelonin fall into 3 classes. Modification of the first 2 residues had no effect on activity, whereas modification of the second set of 2 led to a dramatic loss of activity. The remaining 6 residues could be modified with practically no change in activity.

Phenylglyoxal, 2,3-butanedione and CHD have been used for modification of arginine residues, the latter 2 being even more specific than phenylglyoxal. We have obtained comparable results using both sets of reagents, though the reaction with *p*-nitrophenylglyoxal is much more rapid than the dicarbonyl reagents. The unreacted arginine residues after CHD treatment can be easily monitored using *p*-nitrophenylglyoxal instead of the more tedious procedure of amino acid analysis. Both CD data and immunodiffusion studies indicate that the loss of activity following arginine modification was not due to a drastic conformational change. Thus the loss of activity on modification of arginine residues was due to modification per se.

In a similar study, the involvement of histidine and possibly arginine residues in the biological activity of another A chain toxin from pokeweed has been suggested [23]. However, no conclusive evidence for the involvement of these residues in biological activity was presented. The possibility of the involvement of arginine residues in the biological activity of a variety of A chain plant toxins remains to be investigated.

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