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Characterization of the Enzymatic Mechanism of γ-Momorcharin, a Novel Ribosome-Inactivating Protein with Lower Molecular Weight of 11,500 Purified from the Seeds of Bitter Gourd (*Momordica charantia*)

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The enzymatic mechanism of a small ribosome-inactivating protein, γ-momorcharin, purified from the seeds of *Momordica charantia*, has been characterized. By SDS–polyacrylamide and electrospray ionization mass spectrometry, its molecular weight was measured to be 11,500 daltons which is much lower than other RIPs known to date. It can inhibit the protein synthesis in the rabbit reticulocyte cell-free system with ID_{50} of 55 nM. When rat liver ribosome was incubated with γ-momorcharin, a diagnostic RNA fragment appeared on the gel after rRNAs were treated with acid aniline. Sequencing of the RNA fragment indicates that the action site of γ-momorcharin in 28S ribosomal RNA of rat liver is at a specific adenosine (position 4324), which is in a highly conserved loop of 28S rRNA.

Ribosome-inactivating proteins (RIPs) from plants are toxins with RNA N-glycosidase activity that act on the largest rRNA of eukaryotic and prokaryotic ribosomes (1–3). The molecular mechanism of RNA N-glycosidase is to remove a specific adenine from a highly conserved rRNA loop (''R/S domain'') of the largest rRNA that is responsible for the interaction of both eukaryotic and prokaryotic elongation factors with the ribosomes (4, 5). They depurinate the 28S rRNA, and thus, inhibit the protein synthesis. The depurinating site of RNA N-glycosidase is at position 4324 (A_{4324}) in 28S rRNA of rat liver ribosomes. After treatment with aniline, the depurinated 28S rRNA releases a diagnostic RNA fragment from its 3′-terminus (2, 6, 7). According to their primary structures, RIPs can be classified into two types (3). Type I RIP is composed of a single peptide chain with molecular weights between 25 000–30 000 dalton, while type II RIP with the molecular weight around 60 000 dalton usually consists of two chains (A- and B-chain) connected by a disulfide bond.

The seeds of bitter gourd (*Momordica charantia*) have been used in China for a long time as a source of medicinal ingredients. Two RIPs, α- and β-momorcharins, had been purified from the seeds of *M. charantia* (8, 9). The molecular weights of α- and β-momorcharins are 29 000 and 28 000 dalton, respectively (10, 11). In addition, a small protein was also purified from the seeds of *M. charantia.* It was named γ-momorcharin. In the present paper, the enzymatic mechanism of γ-momorcharin was characterized to be a RIP with RNA N-glycosidase activity. It can also inhibit protein synthesis in a cell-free system.

MATERIALS AND METHODS

Materials. γ-Momorcharin was purified from the seeds of *Momordica charantia* in the laboratory of Professor Shan-wei Jin (Shanghai Institute of Organic Chemistry, Academia Sinica). The purification procedure will be published elsewhere. Ampholyte (pH 3.5–10) was purchased from Shanghai Lizhu-dongfeng Biotech. Co., Ltd. T_4 polynucleotide

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kinase, bacterial alkaline phosphatase, ribonuclease T_1 and ribonuclease U_2 were purchased from Promega Corp., USA. [γ-^{32}P] ATP (~3000 Ci/mmol) and L-[U-^{14}C] leucine (303mCi/mmol) are products of Amersham Corp., UK. The mass spectrometer is a product of VG Quattro GC/MS/MS (England) equipped with an electrospray ionization source (Analytic Company, USA). Other chemicals and reagents are all of analytical grade.

Determination of the molecular weight and isoelectric point (pI) of γ-momorcharin. To determinate the molecular weight of γ-momorcharin, 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous electrophoretic system for separating peptides was carried out (12). Measurement of the molecular weight of γ-momorcharin by electrospray ionization mass spectrometry (ESI-MS) was performed according to the method of Jardine (13). For determining the pI, isoelectric focusing gel electrophoresis using a native gel system with 10%T, 5%C and 2.5% ampholyte was employed as described by Bollag and Edelstein (14).

Protein synthesis in the cell-free system. Rabbit reticulocyte lysate was prepared as described by Sambrook et al (15). The reaction mixture of 1 ml contained 10 mM Tris·HCl, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 50 μg creatine phosphokinase, 50 μM amino acids (minus leucine), 0.5 ml lysate, 2.5 μCi L-[U-^{14}C] leucine and various amounts of γ-momorcharin. After incubation at 30°C for indicated time, aliquots of 20 or 30 μl were withdrawn and absorbed onto Whatmann 3MM filter paper, then washed with 10% trichloroacetic acid and ether (16). The acid insoluble radioactivity was measured.

Assay of the RNA N-glycosidase activity. Ribosomes were isolated from rat liver (17). The concentration of 80S ribosomes was calculated from the extinction at 260 nm (18). Incubation of ribosomes with RIPs was carried out in 100 μl of buffer (25 mM Tris·HCl, pH 7.6, 25 mM KCl, 5 mM MgCl) at 37°C for 15 min. The reaction was stopped by adding 20 μl of 10% SDS. Ribosomal RNAs were extracted with phenol-chloroform and precipitated with ethanol. After aniline treatment at acidic pH at 60°C for 15 min (1, 2), rRNAs were separated by 3.5% polyacrylamide gel electrophoresis.

Sequencing the RNA fragment produced by γ-momorcharin/aniline treatment on 28S rRNA. Rat liver ribosomes (200 pmol) were incubated with γ-momorcharin (100 ng) and treated with acid aniline as described above. After rRNAs were separated by 3.5% polyacrylamide gel electrophoresis, the new appeared RNA fragment released from the 3′-terminus of 28S rRNA was excised from the gel and eluted by electrophoresis. The 5′ terminal sequence of this RNA fragment was determined enzymatically (19). The RNA fragment was treated with bacterial alkaline phosphatase, labelled its 5′ end with [γ-^{32}P] ATP and T_4 polynucleotide kinase. The radioactive fragment was separated from contaminants by 5% polyacrylamide gel electrophoresis and localized by a brief exposure of the gel to X-ray film. The gel containing the RNA fragment was excised and the RNA fragment was eluted. The sequence at the 5′ terminus of this RNA fragment was then determined by partial digestion with either ribonuclease T_1 (G) or ribonuclease U_2 (A).

RESULTS

Some Physicochemical Properties of γ-Momorcharin

γ-Momorcharin displayed a single peak in its last purification procedure by high performance liquid chromatography (HPLC). Its purity was characterized by the SDS-polyacrylamide gel electrophoresis (Figure 1) and isoelectric focusing gel electrophoresis (Figure 3). Figure 1 shows that the molecular weight of γ-momorcharin is 11 500 dalton. This value is consistent with that obtained by electrospray ionization mass spectrometry (Figure 2). Like most of other RIPs (3), γ-momorcharin is also a strong basic protein. Its isoelectric point (pI) is 9.5 (Figure 3). However, unlike α- and β-momorcharins (10), γ-momorcharin contains no neutral sugar (data not shown).

Inhibition of Protein Synthesis by γ-Momorcharin

As shown in Figure 4, γ-momorcharin inhibited protein synthesis in rabbit reticulocyte lysate. In 1 ml of reaction mixture, 0.2 μg of γ-momorcharin can inhibit protein synthesis. When 0.8 μg of γ-momorcharin was added, the protein synthesis was strongly inhibited. After incubation at 30°C for 10 min, the protein synthesis decreased and virtually stopped. The protein synthesis was even entirely inhibited when 3 μg of γ-momorcharin was added. It can be seen from Figure 5 that the ID_{50} (the concentration of RIP giving 50% translation inhibition) of γ-momorcharin is approximately 600 ng/ml (55 nM).

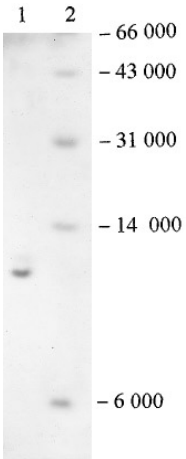

FIG. 1. Determination of the molecular weight of γ-momorcharin by 15% SDS–polyacrylamide gel electrophoresis. The electrophoresis was carried out as described in ''Materials and Methods''. The protein bands were stained with Coomassie blue. Lane 1, γ-momorcharin (10 μg); lane 2, protein molecular weight markers (dalton).

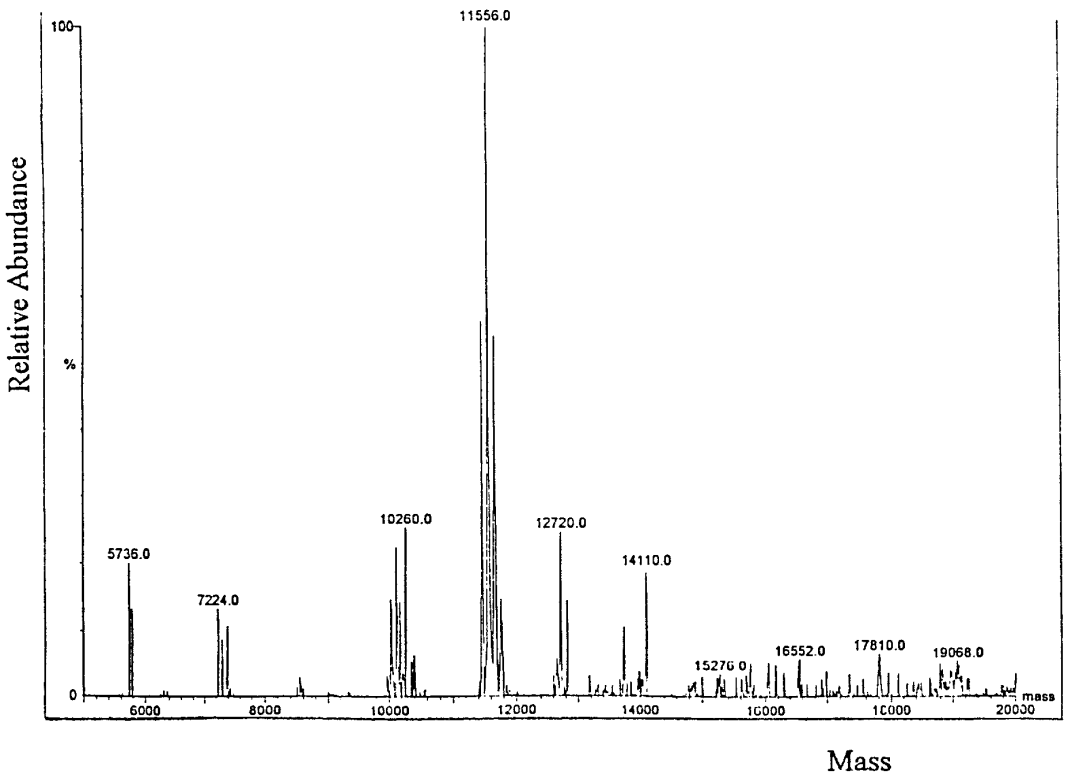

FIG. 2. The electrospray ionization mass spectrum of γ-momorcharin.

FIG. 3. Isoelectric focusing gel electrophoresis by 10% polyacrylamide gel with a pH range of 3.5–10. The electrophoresis was carried out as described in ''Materials and Methods''. The protein bands were stained with Coomassie blue. Lane 1, γ-momorcharin (10 μg); lane 2, trichosanthin (10 μg), which is a single-chain RIP with pI 9.3; lane 3, camphorin (10 μg), which is a single-chain RIP with pI 6.1.

RNA N-Glycosidase Activity of γ-Momorcharin

As shown in Figure 6, γ-momorcharin exhibited RNA N-glycosidase activity as ricin A-chain (1, 2) and trichosanthin (20) did. Two nanograms of γ-momorcharin could induce rat liver ribosomes (the molar ratio of protein/substrate is 1:220) to produce a diagnostic RNA fragment. As the concentration of γ-momorcharin increased, production of the RNA fragment also increased. Treatment without RIP or without aniline had no effect on ribosomes as compared with ribosomes treated without RIP nor aniline. These data demonstrate exclusively that γ-momorcharin exhibited RNA N-glycosidase activity.

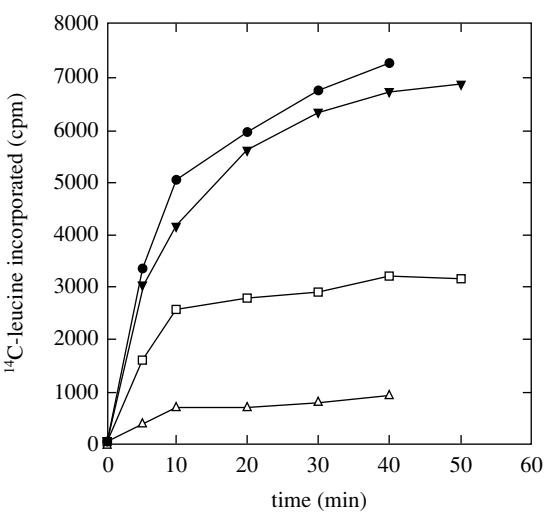

FIG. 4. Inhibition of protein synthesis by γ-momorcharin in the cell-free system of rabbit reticulocytes. Various amounts of γ-momorcharin were added in the protein synthesis system as described in ''Materials and Methods'' in a final volume of 1 ml. At the indicated time of incubation at 30°C, aliquots of 20 μl were withdrawn and the acid-insoluble radioactivity was measured. ●, control; ▼, 0.2 μg of γ-momorcharin; □, 0.8 μg of γ-momorcharin; △, 3 μg of γ-momorcharin.

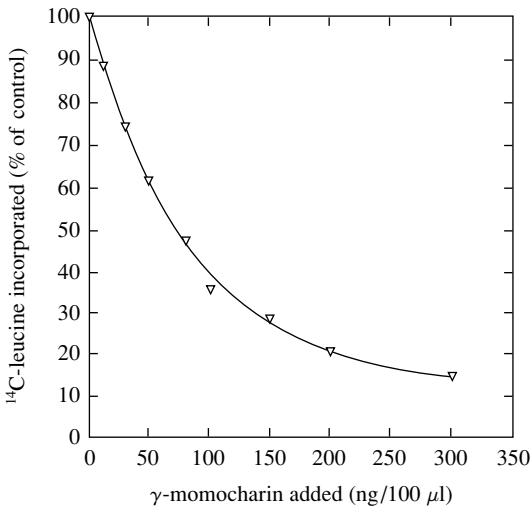

FIG. 5. Effect of γ-momorcharin on protein synthesis in the cell-free system of rabbit reticulocytes. The protein synthesis system as described in ''Materials and Methods'' contained the indicated amounts of γ-momorcharin in a final volume of 100 μl. After incubation at 30°C for 15 min, aliquots of 30 μl were withdrawn and the acid-insoluble radioactivity was measured. The control value is 15,700 cpm.

Action Site of γ-Momorcharin on 28S rRNA

In order to determine the action site of γ-momorcharin on 28S rRNA, the sequence of 5′ terminal nucleotides of the RNA fragment produced by γ-momorcharin/aniline treatment was analyzed. Since both the complete sequence of rat 28S rRNA and the 5′ terminal nucleotide sequence of the RNA fragment produced by ricin A-chain/aniline treatment are known (1, 2, 21), the 5′ terminal nucleotide sequence of the RNA fragment produced by γ-momorcharin/

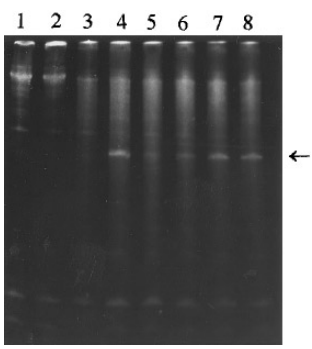

FIG. 6. RNA N-glycosidase activity of γ-momorcharin. Rat liver ribosomes (40 pmol) were incubated with various amounts of RIPs at 37°C for 15 min. Ribosomal RNA was extracted and treated with acid aniline at 60°C for 15 min. Electrophoresis was carried out by 3.5% polyacrylamide gel and the RNA bands were visualized with ethidium bromide. Each lane contained the amount of RNA corresponding to 10 pmol of ribosomes. Lane 1, ribosome without RIP and aniline treatment; lane 2, ribosome incubated with 50 ng of γ-momorcharin without aniline treatment; lane 3, ribosome treated with aniline without RIP incubation; lane 4, ribosome incubated with 50 ng of ricin A-chain and treated with aniline; lane 5, 6, 7, 8, ribosome incubated with 2, 5, 10, 50 ng of γ-momorcharin respectively and treated with aniline. The arrow indicates the RNA fragment released from 28S rRNA after aniline treatment.

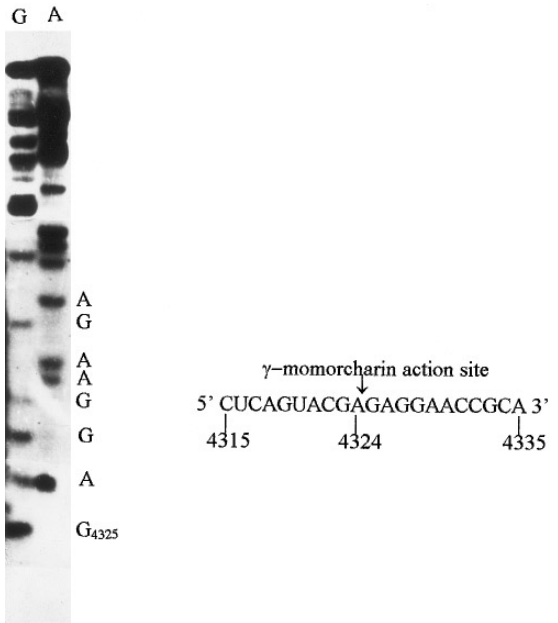

FIG. 7. Radioautograph of 20% polyacrylamide sequencing gel of the 5′ terminal region of the RNA fragment produced from 28S rRNA after treatment of rat liver ribosome with γ-momorcharin/aniline. The RNA fragment (shown with arrow in figure 6, lane 8) was recovered by 5% PAGE, treated with bacterial alkaline phosphatase, then labelled its 5′ end with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The labelled fragment was repurified by electrophoresis and partially digested with either ribonuclease T_1 (G) or ribonuclease U_2 (A).

aniline treatment can be deduced just from the G, A sequence by comparing with that of the RNA fragment from ricin A-chain/aniline treatment (1, 2) as well as the complete sequence of 28S rRNA (21). The RNA fragment shown with arrow in Figure 6 (lane 8) was isolated and its 5′ terminal sequence was determined enzymatically. As indicated in Figure 7 (left), the 5′ terminal nucleotide sequence of this RNA fragment was identical to that produced by ricin A-chain. Comparing with the complete sequence of 28S rRNA, it can be concluded that γ-momorcharin acts on the same action site in the 28S rRNA of rat liver ribosome as ricin A-chain does. Its depurinated site is at A_{4324} (Figure 7, right).

DISCUSSION

γ-Momorcharin is a small RIP with RNA N-glycosidase activity like ricin A-chain and trichosanthin. Its molecular weight is only 11 500. This is very interesting in the study of RIPs. It seems unlikely that the preparation of γ-momorcharin contaminated by other larger RIPs (i.e. α- and β-momorcharins) from the same seeds because its last purification procedure was by HPLC which gave a single peak and it displayed a monoband on both SDS-PAGE and isoelectric focusing gel electrophoresis (Figure 1 and Figure 3). The fact that γ-momorcharin contains no neutral sugar while both α- and β-momorcharins are glycoprotein with neutral sugar content of 1.6% and 1.3% respectively (10) excluded the possibility that the preparation of γ-momorcharin contaminated by the other two RIPs in the seeds.

The molecular weights of RIPs are all around either 25 000–30 000 (type I) or 60 000 (type II). The molecular weight of a RIP with as low as 11 500 has not been reported so far. This is highly significant in the study of the molecular evolution and the relationship of

structure and function of RIPs. Morris and Wool reported that among the 267 amino acids in ricin A-chain, 222 (83%) can be deleted without loss of its capacity to recognize a single nucleotide or to catalyze hydrolysis. Only 45 amino acids cannot be deleted without loss activity (22). RIPs such as ricin A-chain, abrin A-chain and α-trichosanthin have a very similar tertiary structure (23–25). This proposed that a small protein, such as γ-momorcharin, can also share a similar structure for RNA N-glycosidase activity. Although γ-momorcharin has a higher ID_{50} value (55 nM) in inhibiting protein synthesis in the cell-free system than most of other RIPs, it exhibits the RNA N-glycosidase activity at the molar ratio of enzyme/substrate as low as 1:220 and depurinates adequately (Figure 6, lane 5) as did by most of other RIPs. The higher ID_{50} maybe just reflect the small molecular feature of this RIP in inhibiting the protein synthesis with a lower activity.

Recently, it has been reported that many RIPs expressed a supercoil-dependent endonuclease activity to cleave supercoiled double-stranded circular form of DNA into the nicked circular form and linear form of DNA (26–28). More interesting, a single chain RIP, named camphorin, in addition having RNA N-glycosidase and endonuclease activities, was further found to express the activity of superoxide dismutase (SOD) (27, 29, 30). However, γ-momorcharin does not exhibit the SOD and endonuclease activities (unpublished data). This demonstrates that the limited amino acid residues of γ-momorcharin are not enough to construct other active sites.

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