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Active site of trichosanthin acting as a ribosome-inactivating protein[1]

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KEY WORDS trichosanthin; plant proteins; cell-free system; polyacrylamide gel electrophoresis; hydroxylamines; monoclonal antibodies; leucine; Western blotting; peptide fragments

AIM: To localize the active site of ribosome inactivation of trichosanthin (Tri), a Chinese herb protein. **METHODS**: Hydroxylamine was used to specifically cleave the unique Asn-Gly peptide bond of Tri. Preparative SDS-polyacrylamide gel electrophoresis was applied to get 2 cleaved fragments, HATf1 and HATf2. Western blotting was used to determine the different epitopes of Tri and screen the antibodies. A cell-free system, rabbit reticulocyte lysate, was introduced to quantitate the inhibitory activity of Tri and its fragments on protein biosynthesis. **RESULTS**: HATf1 and HATf2 were separated with the purity of 96.9 % and 80.5 % respectively. HATf1, like intact Tri, retained the inhibitory activity on protein biosynthesis. The mAb № 14 and № 16 against Tri showed different immunoreactivities with 2 fragments and were selected as representatives in further blocking tests. The mAb № 14 hindered the activities of Tri and HATf1, whereas the mAb № 16 did not. **CONCLUSION**: The active site of Tri responsible for inhibitory activity on protein biosynthesis was on the HATf1 side near the junction of two portions.

Trichosanthin (Tri) is a basic protein extracted from the root tuber of Chinese herb *Trichosanthes kirilowii* Maxim. It was first crystallized by Chinese 3 decades ago. In addition to terminating term pregnancy, it was also useful in treating ectopic pregnancy, hydatidiform mole and choriocarcinoma. Tri had an antiviral activity and blocked HIV replication *in vivo* and *in vitro*, and improved the symptoms of AIDS patients as well[1,2]. However, Tri might cause anaphylaxis[3], which limited its clinical use. Tri was a RNA *N*-glycosidase and depurinated at A4324 from 28S RNA[4]. This action could unstabilize the frame of the ribosomes and further induce the collapse of protein biosynthesis. So Tri was classified as a single-chain protein in the family of ribosome-inactivating proteins. On the other hand, Tri could elevate the serum complement factor 3 conversion, that was thought to be the cause of anaphylactoid effect in clinical application[5].

The aim of this study was to localize the enzymatic action of Tri.

MATERIALS AND METHODS

Materials Tri was crystallized by Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. Hydroxylamine hydrochloride was the product of Shanghai № 4 Reagent Factory. Reagents for Western blotting were purchased from Bio-Rad Co except 3,3′-diaminobenzidine (DAB) from BDH Co. Mouse-derived monoclonal antibodies against Tri were prepared in our Lab. Caprylic acid and ammonium sulfate were introduced into 2-step mAb purification. Hemin chloride, ATP, GTP, creatine phosphate, creatine phosphokinase, 19 kinds of amino acids except leucine (Leu) were purchased from Dongfen Reagent Factory affiliated to Chinese Academy of Sciences. [^{3}H] Leu (2.0 PBq · mol^{-1}) was the product of Shanghai Institute of Nuclear Research, Chinese Academy of Sciences.

Hydroxylamine cleavage A reactive solution including guanidine 6 mol · L^{-1} and hydroxylamine 2 mol · L^{-1} was prepared[6]. The pH was adjusted to 9.0 with LiOH 4 mol·L^{-1}. Tri 4 g·L^{-1} was added and incubated at 45 ℃ for 4 h. The reaction was terminated and then dialyzed at 4 ℃ for 2 d.

SDS-PAGE The discontinuous system of Laemmili was used with 12.5 % separating gel and 3 % stacking gel[7]. The efficiency of cleavage of Tri was calculated according to the area of gel scanning at 556 nm on the dual-wavelength flying-spot scanner (Shimadzu CS-9000).

Electrophoretic transfer The transfer was done on Bio-Rad Trans-Blot cell under 0.8 A for 1 h. The nitrocellulose

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membrane was cut into pieces in 5 mm width. The pieces were soaked in PBS 0.01 mol·L^{-1} (pH 7.2) and stored at 4 ℃.

Immunologic detection The nitrocellulose pieces were blocked with PBS 0.01 mol·L^{-1}, containing 0.05 % Tween 20 and 5 % BSA. Diluted mAb ascites (1∶200 – 1∶1000) were incubated with nitrocellulose pieces for 30 min while the ascite of SP2/0 was selected as control. Horseradish peroxidase-labeled antibody (sheep anti-mouse Ig) was used as second antibody with the dilution of 1∶4 000 followed by DAB as substrate.

Preparative electrophoresis The method of Laemmli was adopted and modified[8]. Electrophoresis was performed on Bio-Rad Protean™ I cell with the gel of 15 cm (width) × 12 cm (length) × 0.5 cm (thickness). Sample peptide mixture was processed in sample buffer at 20 ℃ overnight. On d 2, with a cooling system 6-mL sample was loaded on the surface of stacking gel and run under 100 mA for 4 h.

Visualization and localization The gel was put into a clean tray and 3 pieces were taken (1 from each end, 1 from the center). The gel pieces were stained with Coomassie brilliant blue R-250 at 60 ℃ for 30 min. After the peptides were localized in remainder of unstained gel according to the bands on the stained gel pieces, the gel strips with purposed peptides were cut out, crushed, and soaked into the eluting solution (Tris-HCl 0.05 mol·L^{-1} pH 6.8, containing 0.1 % SDS) at 20 ℃ overnight.

SDS removal and renaturation The supernatats were transferred to 15-mL tuber after the eluates were spun at 800 × *g* for 10 min. Four volumes of acetone (−20 ℃) were added, peptides were allowed to precipitate for 30 min in ice bath. The centrifugation at 15 000 × *g* for 10 min was made. The precipitates were allowed to dry for about 10 min and dissolved in 20 μL of dilution buffer with guanidine chloride 6 mol·L^{-1} at 20 ℃ for 20 min. The peptide samples were diluted 50-fold with dilution buffer (Tris-HCl 0.05 mol·L^{-1}, 20 % glycerol, NaCl 0.15 mol·L^{-1}, BSA 0.1g·L^{-1}, and DTT 1 mmol·L^{-1}). Then, they were renatured for 2 h. Renatured peptides were stored at 4 ℃.

Identification of purity and analysis of amino acid components The purity of peptides was identified by gel scanning at 556 nm. Components of amino acids were analyzed on LKB autoanalyzer model 4151.

Preparation of rabbit reticulocyte lysate Heparinized reticulocyte-rich whole blood was obtained and lysate was prepared according to the protocols[9].

Reticulocyte lysate assay A microassay method was used[10]. A 96-well plate was pre-immersed in 0.1 % diethyl pyrocarbonate overnight. [^{3}H]Leu 37 kBq were added. Lysate was determined in 18 μL to get the highest counts. The total volume was adjusted to 60 μL with Tris-HCl 10 mmol·L^{-1}. The [^{3}H]Leu incorporation was quantitated by scintillation counting. Blocking test was carried out by preincubating peptides with specific antibodies for 30 min beforehand.

RESULTS

Effect of cleavage On the SDS-PAGE diagram, 3 bands were mainly identified in the Tri mixture. Except for the band of uncleaved Tri, the other 2 bands HATf1 and HATf2 represented the high and low molecular weight peptides, respectively (Fig 1).

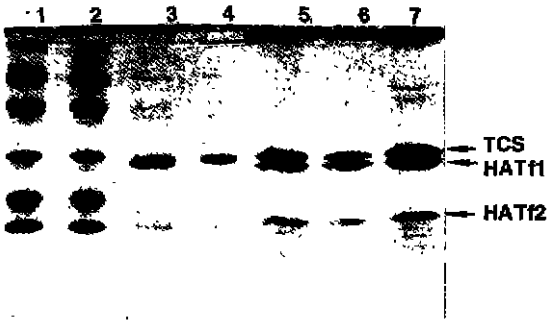

Fig 1. SDS-PAGE diagram. 1, 2: low molecular weight marker (Sigma); 3, 4: trichosanthin; 5, 6, 7: hydroxylamine cleaved fragments.

The efficiency of cleavage was near 70 % of Tri in several trials (Fig 2).

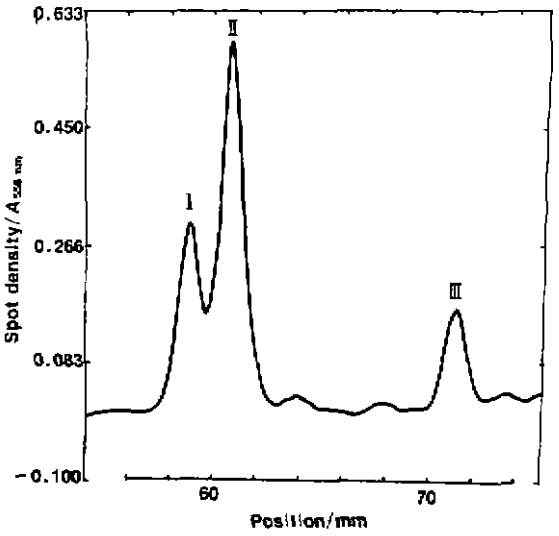

Fig 2. Cleaving efficiency of hydroxylamine on trichosanthin (by gel scanning at 556 nm). I: uncleaved trichosanthin 26.7 %; II: HATf1 57.1 %; III: HATf2 12.6 %.

Western blotting Two groups were

differentiated from the varieties of Western blotting patterns caused by 21 strain mAb. The mAb № 14 was selected as the representative of double positive antibodies against HATf1 and HATf2, while the mAb № 16 represented single positive ones against HATf1[11] (Fig 3).

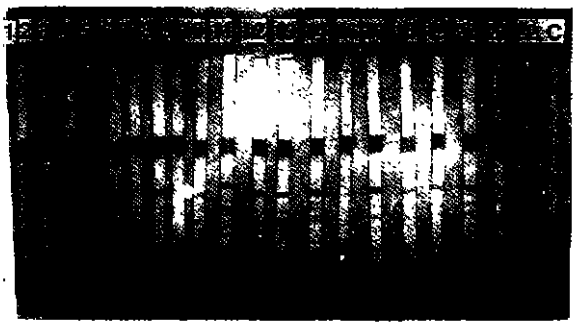

Fig 3. Western blotting patterns of peptide mixture reacted with 21 strains mAb against trichosanthin. 1 – 22: mAb number (lack of № 15 in experiment); c: Negative control (sp2/0).

Purity and identity of peptides Two purified peptides HATf1 and HATf2 were obtained by preparative SDS-PAGE with the purities of 96.6 % and 80.5 % respectively (Fig 4).

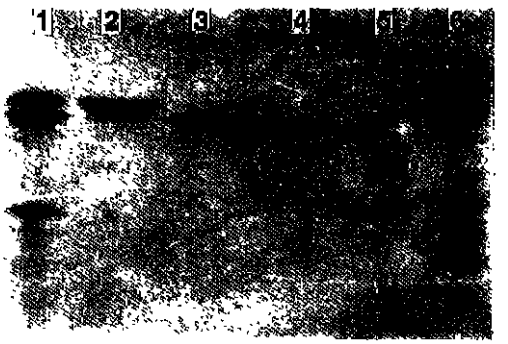

Fig 4. Purified fragments by preparative electrophoresis. 1, 6: mixture; 2: uncleaved trichosanthin; 3: HATf1; 4: unwanted band; 5: HATf2.

Amino acid analysis identified that HATf1 was the *N*-terminal part of Tri with 206 amino acid residuals while HATf2 was the *C*-terminal part with 41 residuals (Tab 1). The difference between the observed value and predicted one may be ascribed to the relatively low purity of HATf2.

Tab 1. Component analyses of HATf1 and HATf2.

Amino acid	HATf1		HATf2	
	Observed	Predicted*	Observed	Predicted*
Asp + Asn	20.3	20	6.1	8
Thr	11.5	12	2.0	3
Ser	22.1	24	1.8	2
Glu + Gln	8.2	15	0.7	4
Pro	7.8	7	0.6	1
Gly	10.0	10	2.0	2
Ala	23.8	24	3.7	4
Val	8.0	9	5.0	6
Met	2.4	3	0.3	1
Ile	13.7	16	1.8	3
Leu	18.4	21	3.2	4
Tyr	10.8	14	0.0	0
Phe	7.7	8	0.3	1
Lys	9.6	11	0.0	0
Arg	8.8	10	1.4	2
His	0.6	1	0.0	0

* According to the primary structure of trichosanthin[12].

Inhibition of protein synthesis Like intact Tri, HATf1 inhibited the protein biosynthesis. HATf2, however, had no such action (Fig 5).

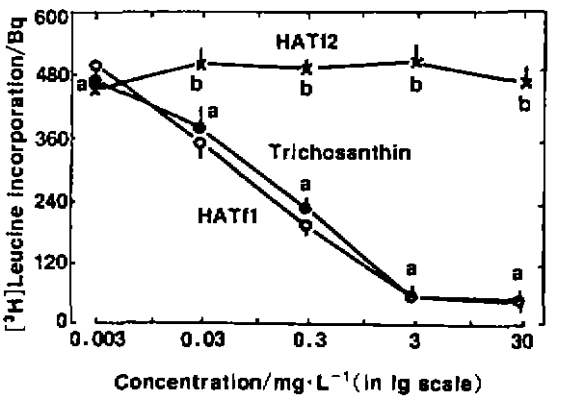

Fig 5. Effect of trichosanthin or its fragments on inhibition of protein synthesis in rabbit reticulocte lysate. $n = 3$ wells for 1 dose, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* Tri.

HATf1 also had similar behavior of Tri by mAb blocking test (Tab 2).

DISCUSSION

Result obtained in our study indicated that HATf1 retained the inhibitory activity of protein biosynthesis. The active site was on the HATf1 part, never related with the *C*-terminal of Tri. The mAb № 14 could

Tab 2. Effect of mAb on blocking inhibitor (3 mg·L^{-1})-induced protein synthesis inhibition expressed with ^{3}H incorporation (Bq). $n = 3$ wells for 1 dose, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* blank.

Inhibitor		Radioactivity/Bq
Control		
	Blank	487 ± 11
	№ 14	454 ± 11^a
	№ 16	481 ± 8^a
Trichosanthin		
	Blank	44 ± 5
	№ 14	482 ± 14^c
	№ 16	57 ± 11^a
HATf1		
	Blank	57 ± 7
	№ 14	359 ± 25^c
	№ 16	60 ± 10^a
HATf2		
	Blank	488 ± 11
	№ 14	491 ± 11^a
	№ 16	459 ± 19^a

block the inhibitory activity of *Trichosanthin*, but the mAb № 16 almost had no effect. This result at least meant that the mAb № 14-recognized epitope was somewhat near the active site, but the antibody № 16-recognized one was more aloof from the active site. According to the Western blotting pattern, mAb № 14 was directed against epitope on both sides near the joint site of 2 fragments. The active site of Tri was on the HATf1 part near the junction of 2 fragments.

Using the chemical agent, hydroxylamine, to cleave the unique peptide bond between Asn206 and Gly207, we obtained the *N*-terminal peptide with 206 amino acid residuals in length. We found that HATf1 possessed the inhibitory activity as Tri did. From the peptide length, it is five sixths of intact Tri. It may have lower antigenicity than Tri. Both HATf1 and HATf2 still had the ability of elevating C_3 conversion but in a lower way than Tri did[12]. From above results, HATf1 may act as a candidate of substitute for Tri. Since the results was from the cell-free system, further research must be focused on elucidating the way of Tri entering the sensitive cell and testing the effect on the animal model.

In conclusion, HATf1 is an effective portion for inhibiting protein synthesis in rabbit reticulocyte lysate.

REFERENCES

1 McGrath MS, Hwang KM, Caldwell SE, Gaston I, Luk KC, Wu P, *et al*. GLQ223: an inhibitor of human immunodeficiency virus replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage. Proc Natl Acad Sci USA 1989; **86**: 2844 – 8

2 Mayer RA, Sergios PA, Coonan K, O'Brien L. Trichosanthin treatment of HIV-induced immune dysregulation. Eur J Clin Invest 1992; **22**: 113 – 22.

3 Zhang JS, Liu WY. The mechanism of action of trichosanthin on eukaryotic ribosomes — RNA *N*-glycosidase activity of the cytotoxin. Nucleic Acids Res 1992; **20**: 1271 – 5.

4 Lu YL, Ma BL, Jin YC. Significance of several immunologic tests in diagnosis of trichosanthin-induced anaphylaxis. Shanghai J Immunol 1988; **8**: 86 – 90.

5 Chen X, Ma BL. Trichosanthin, an initiator of the alternative complement activation pathway. Clin Exp Immunol 1993; **93**: 248 – 52.

6 Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 1970; **227**: 680 – 5.

7 Bornstein P, Balian G. Cleavage at Asn-Gly bonds with hydroxylamine. Methods Enzymol 1977; **47**: 132 – 45.

8 Hager DA, Burgess RR. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal Biochem 1980; **109**: 76 – 86.

9 Woodward WR, Ivey JL, Herbert E. Protein synthesis with rabbit reticulocyte preparations. Methods Enzymol 1974; **30**: 724 – 31.

10 Press OW, Vitetta ES, Martin PJ. A simplified microassay for inhibition of protein synthesis in reticulocyte lysates by immunotoxins. Immunol Lett 1986/1987; **14**: 37 – 41.

11 Xi ZD, Ma BL, Wang M, Cao HN, Yang LM. Study on the immunoreactivity of trichosanthin's fragments cleaved with hydroxylamine. Shanghai J Immunol 1996; **16**: 278 – 82.

12 Collins EJ, Robertus JD, LoPresti M, Stone KL, Williams KR, Wu P, *et al*. Primary amino acid sequence of α-trichosanthin and molecular models for abrin A-chain and α-trichosanthin. J Biol Chem 1990; **265**: 8665 – 9.

13 Xi ZD, Ma BL, Cao HN, Wang M, Yang LM. Preparation of trichosanthin's fragments cleaved with hydroxylamine and observation of the effect on complement activation induced by these peptides. Chin J Immunol 1997; **13**: 45 – 8.

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天花粉蛋白的核糖体灭活活性部位

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关键词 天花粉蛋白；植物蛋白；无细胞系统；聚丙烯酰胺凝胶电泳；羟胺；单克隆抗体；亮氨酸；蛋白质印迹；肽断片 核糖体 灭活活性

目的：天花粉蛋白核糖体灭活活性部位的定位，

方法：羟胺特异裂解天花粉蛋白唯一 Asn-Gly 肽键．制备性凝胶电泳获 HATf1 和 HATf2 二片段．免疫印迹确定天花粉蛋白上不同表位并筛选抗体．兔网织红无细胞系统测定天花粉蛋白及片段对蛋白合成的抑制活性．**结果**：HATf1 和 HATf2 纯度各达 96.6 % 和 80.5 %．HATf1 保留完整天花粉蛋白的抑制活性．第 14 号和第 16 号抗天花粉蛋白单抗与二片段显示不同免疫反应性，并用于封闭试验．第 14 号单抗能封闭天花粉蛋白及 HATf1 活性，而第 16 号单抗则否．**结论**：天花粉蛋白抑制蛋白质生物合成的活性部位位于 HATf1 侧，近二部分交界．

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Detection of DNA damage in peripheral lymphocytes by 7 compounds using comet assay[1]

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KEY WORDS gel electrophoresis; single-stranded DNA; mutagenicity tests; hydrogen peroxide; ethyl methanesulfonate; dimethylnitrosamine; mitomycin C; benzo(a)pyrene; cyclophosphamide; 2-aminofluorene

AIM: To detect the DNA single strand breaks (SSB) in peripheral lymphocytes of mice, rats, and human induced by hydrogen peroxide (H_2O_2), ethyl methane sulphonate (EMS), dimethylnitrosamine (DMNA), mitomycin C (MMC), benzo (a) pyrene (BaP), cyclophosphamide (CP), and 2-aminofluorene (2-AF). **METHODS**: Alkaline single cell microgel electrophoresis assay *in vitro* (comet assay). **RESULTS**: All were positive with 2 exceptions: EMS (0.97 mmol·L^{-1}) in mice and MMC (30 μmol·L^{-1}) in mice and human. The lowest concentrations detectable were H_2O_2 (1 μmol·L^{-1}), EMS (0.48 mmol·L^{-1}), BaP (5.0 μmol·L^{-1}), CP (2.0 mmol·L^{-1}), MMC (10 μmol·L^{-1}), DMNA (27.3 mmol·L^{-1}), and 2-AF (62.5 μmol·L^{-1}). CP, BaP, and 2-AF were positive only in the presence of metabolic activation system. **CONCLUSION**: H_2O_2, DMNA, BaP, CP, and 2-AF induce SSB in peripheral lymphocytes of mice, rats, and human detected by comet assay, whereas MMC induces SSB only in rats, and EMS in rats and human lymphocytes.

The single cell microgel electrophoresis assay (SCGE, comet assay) can quantitatively detect DNA single strand breaks (SSB) at the individual cell level in virtually any eukaryote cell population both *in vivo* and *in vitro*. With the advantage of need for extremely small numbers of cells per sample without radiation labeling, this sensitive, simple and cost-effective novel technique is promising in fast screening the genotoxicity of drug candidates[1-7]. This study aimed to investigate 7 compounds with different genotoxic mechanisms for their potentialities to induce SSB in peripheral blood lymphocytes of mice, rats, and human using *in vitro* comet assay.

MATERIALS AND METHODS

Na_2-edetic acid (Shanghai № 1 Reagent Factory), Tris, Me_2SO, trypan-blue (EMK), Triton X-100 (Farco), normal melting point agarose (NMA, Sigma Type I-A, mp = 88 ℃), low melting point agarose (LMA, Promega, mp = 64 ℃), ethidium bromide (Fluka), lymphocyte seperation medium (LSM, Shanghai Huajing Biological Co), RPMI 1640 medium (Gibco BRL), fetal bovine sera (Sijiqing Institute of Biomaterials, Hangzhou).

Fully frosted slides (25 mm × 75 mm), coverglass (25 mm × 25 mm), horizontal electrophoresis unit (Shanghai DP

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