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# Purification and Primary Structure Determination of a Novel Polypeptide Isolated from Mistletoe *Viscum coloratum*

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**Abstract:** A novel polypeptide was isolated from mistletoe *Viscum coloratum*. The primary structure of the polypeptide ′named viscotoxin B2′ was determined to be KSCCKNTTGRNIYNT CRFAGGSRERCAKLSGCKIISASTCPSDYPK by Edman degradation. Viscotoxin B2 shared high sequence homology with viscotoxins isolated from *Viscum album*. Pharmacological experiments showed that viscotoxin B2 had distinct cytotoxic activity on tumor cells. Viscotoxin B2 could be used as a leading compound in cancer therapy.

**Keywords:** *Viscum coloratum*, isolation, primary structure, viscotoxin.

Mistletoe, which is a semiparasitic plant growing on deciduous trees, has been regarded as a medicinal plant not only in China but also in Europe for centuries. Various pharmacologically active components including lectin, viscotoxin and polysaccharide have been found in European mistletoe (*Viscum album* L.)[1-2]. Proprietary extracts of the mistletoe have been used in an adjuvant cancer therapy because of the evidence that they exhibit immunostimulatory capacity and simultaneously cytotoxic properties[3-4]. Viscotoxins are one important kind of active components in these extracts, which are highly basic polypeptides of molecular mass about 5kDa[5-6]. Mistletoe (*Viscum coloratum* (Komar.) Nakai) in Northeast China has been used for the treatment of rheumatism, quickening, hypertension, liver cancer, *etc*., in Chinese folk medicine. Some recent studies were mainly focused on the active small molecular compounds, such as amines, flavonoids, organic acids and terpenoids by the Chinese scientists[7-8]. However, few studied active polypeptides in *Viscum coloratum* (Komar.) Nakai and viscotoxins were thought to be only in *Viscum album* L. This paper reports the results of the isolation and determination of primary structure of one polypeptide that has been verified to have distinct cytotoxic effect against tumor cells from *Viscum coloratum* (Komar.) Nakai.

## Materials

Endoproteinase Glu-C (EC 3.4.21.19), iodoacetamide, trifluoroacetic acid and dithiothreitol were purchased from Sigma (St. Louis, USA). CM Sepharose F.F. and Sephadex

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G-25 Superfine were purchased from Amersham Biosciences. Other chemicals were at the grade of A.R.

**Isolation and Purification of Polypeptide from Mistletoe**

Leaves and stems form mistletoe (*Viscum coloratum* (Komar.) Nakai) growing on poplar were collected in Liuhe County, Jilin Province in December 2002. The dried plant parts were smashed into powder and then extracted twice with 0.01 mol/L NaOAc buffer (pH 5.0) by stirring for 24 h at room temperature. The combined filtrates were uploaded on CM Sepharose F.F. column (1.6 cm × 20 cm) previously equilibrated with the same buffer, which was also used for elution. When the UV absorption value monitored at 280 nm was less than 0.005 AU, the NaOAc buffer containing 0.4 mol/L NaCl was employed for further elution. The fraction corresponding to the one peak was pooled and concentrated *in vacuo* in rotavapor at a temperature not exceeding 35℃. The concentrated solution was added to a pre-equilibrated Sephadex G-25 Superfine column (2.6 cm × 100 cm) and eluted with 0.2 mol/L HOAc. UV absorption at 280 nm (curve a in **Figure 1**) and conductivity (curve b in **Figure 1**) were monitored at the same time on ÄKTAprime chromatograph. The fractions were collected at the flow rate of 0.5 mL/min. The peak B was pooled, concentrated and purified with a Vydac C18 semipreparative column (9.6 mm × 250 mm) on Agilent 1100 HPLC. The polypeptides were eluted with 0.1% trifluoroacetic acid in 25% acetonitrile. The fraction studied was located in peak 2 that was repurified under a gradient of acetonitrile with a Vydac C18 analytic column (4.6 mm × 250 mm) (**Figure 2**). About 20 mg white powder that was designated as viscotoxin B2 was obtained from 200 g mistletoe.

**Determination of Primary Structure of Viscotoxin B2**

About 200 μg of viscotoxin B2 was dissolved in 100 μL buffer (1 mol/L Tris·HCl, 6 mol/L guanidine hydrochloride, 1 mmol/L EDTA, pH 8.5) and reduced with 1.8 mg of dithiothreitol at 37℃ for 5 h. Then it was alkylated with 6 mg of iodoacetamide and kept in the dark for 1 h at room temperature. The modified polypeptide (RAM- viscotoxin B2) was purified by HPLC, and then used for automatic sequencing on an Applied Biosystems Procise 491 pulsed liquid sequencer. The N-terminal sequencing of RAM-viscotoxin B2 resulted in a sequence up to the $37^{th}$ residue. The partial N- terminal sequence was determined as follows: KSCCKNTTGRNIYNTCRFAGGSRERCA?LSGC?IISA……(? denoted the amino acid residue that was difficult to interpret).

Endoproteinase Glu-C was selected to digest viscotoxin B2 because there was only one Glu residue in the identified sequence. RAM-viscotoxin B2 and the enzyme were mixed at a ratio of 30:1 and incubated in 300 μL buffer (50 mmol/L ammonium dicarbonate, 2 mmol/L EDTA, pH 8.5) at 37℃ for 18 h. The cleaved fragments were separated and purified with a Vydac C18 analytic column (**Figure 3**).

Mass spectral analyses of viscotoxin B2, its modified form RAM-viscotoxin B2 and the enzyme digested fragments were performed on MALDI-TOF mass spectrometer (Brucker Reflex Ⅲ). The average mass of natural viscotoxin B2 was determined to be

**Figure 1** Gel filtration chromatogram

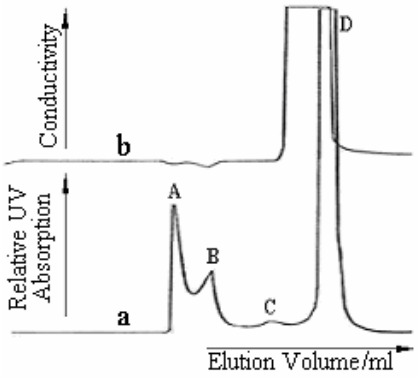



**Figure 2** Viscotoxin B2 repurified by RP- HPLC

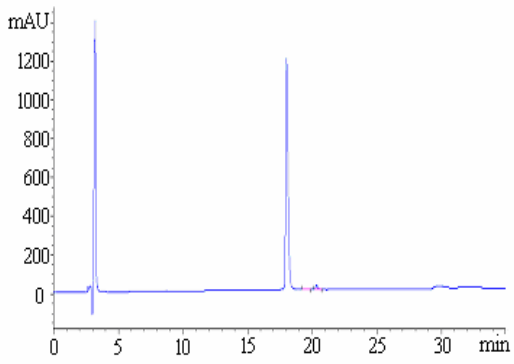



**Figure 3** Digested fragments separated by RP-HPLC

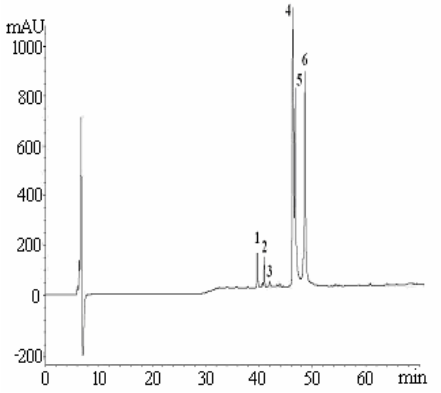
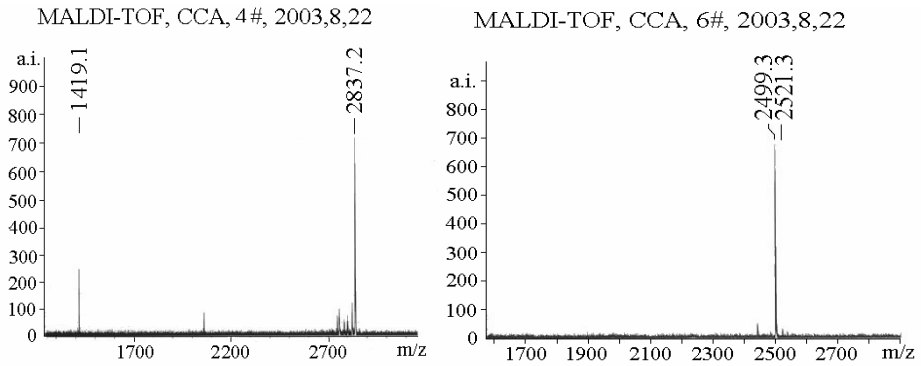



**Figure 4** MS spectra of fragment 4 , 6



4967 Da and the RAM-viscotoxin B2 was 5319 Da. The results showed viscotoxin B2 contained six cys residues. The monoisotopic mass of fragment 4 from enzyme digestion (peak 4 in **Figure 3**) and fragment 6 (peak 6 in **Figure 3**) were determined to be 2836.2 Da and 2498.3 Da (**Figure 4**), respectively. The calculated monoisotopic mass of the fragment KSCCKNTTGRNIYNTCRFAGGSRE (Cys was alkylated with iodo- acetamide) was 2836.29 Da, which was consistent with the result of 2836.2 Da of fragment 4 from mass spectrum. The sum of the monoisotopic mass of the fragment 4 and 6 was 5334.5 Da. Subtracted the MW of $H_2O$, the value was 5316.5 Da, which was the same as the monoisotopic mass of RAM-viscotoxin B2. These results indicated that fragment 6 was the unknown one that should be sequenced again. The amino acid sequence of fragment 6 was determined to be RCAKLSGCKIISASTCPSDYPK by Edman degradation. The calculated monoisotopic mass of it (Cys was alkylated with iodoacetamide) was 2498.21 Da, which was confirmed by the result of 2498.3 Da from mass spectrum. So the complete sequence of viscotoxin B2 was determined to be KSCCKNTTGRNIYNT CRFAGGSRERCAKLSGCKIISASTCPSDYPK.

## Discussion

Viscotoxin B2 shared high sequence homology with viscotoxins isolated from *Viscum album* L. Following showed the primary structures of viscotoxin B2 and viscotoxin A3, which was the main component of viscotoxins.

Viscotoxin B2 KSCCKNTTGRNIYNTCRFAGGSRERCAKLSGCKIISASTCPSDYPK
Viscotoxin A3 KSCCPNTTGRNIYNACRLTGAPRPTCAKLSGCKIISGSTCPSDYPK

The underlined letter indicated the different amino acids between viscotoxin B2 and viscotoxin A3. Compared with other five viscotoxins that had been studied, the positions of Cys residues in viscotoxin B2 were found to be the same as those in viscotoxins. But the 5$^{th}$ residue in all the viscotoxins from *Viscum album* L. was Pro, which was substituted by Lys in viscotoxin B2, and most residues´ substitutions were not conservative. Viscotoxin B2 had the most abundant basic residues among viscotoxins from mistletoe. Pharmacological experiments showed that viscotoxin B2 had distinct antitumor activity. Viscotoxin B2 has a potential value to be used as medicine for the treatment of cancer. Experiments about its structure and activity relations are currently under investigation.

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