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Purification and Crystallization of Flammulin, a Basic Protein with Anti-tumor Activities from *Flammulina Velutipes*

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Abstract: Flammulin, an anti-tumor protein, was purified from the aqueous extract of basidiomes of *Flammulina Velutipes* to electrophoretic homogeneity and crystallized by microdialysis against a polyethylene glycol- sodium phosphate buffer. The purified product was found to have marked anti-tumor effects and be able to affect the tumor cells directly.

Keywords: Flammulin, purification, crystallization.

Golden needle mushroom (*Flammulina velutipes*) is a popular edible mushroom in Asia. It has long been reported to have medical value and are devoid of undesirable effects[1]. Flammulin, a basic simple protein isolated from *Flammulina velutipes* with anti-tumor activities was reported by N. Komatsu *et al.*[2]. But the active fractions purified by their method are usually mixed with other impurities. In this study, we describe a new method of isolation. Flammulin was purified to electrophoretic homogeneity and crystallized by microdialysis against a polyethylene glycol sodium phosphate buffer.

Experimental

Isolation and Crystallization of Flammulin

The air-dried fruit bodies of *Flammulina velutipes* (1000 g)were extracted with 2 L water at about 10°C over night, the mixture was filtered and the insoluble material was re-extracted with water. Such extractions were repeated 2 times. The filtrates were combined and made to 66% saturation with ammonium sulfate. The resulting precipitates were dialyzed in cellophane bag against cooled water. The dialysate was added ethanol to a concentration of 50% in the cold. The precipitate was discarded and the supernatant was added ethanol to a final concentration of 75%. The precipitate was collected and then dialyzed against 0.02 mol/L Na_2HPO_4-NaH_2PO_4 pH 6.6 at 4°C. The dialysate was applied to a DEAE-Sephadex column (2.2×10 cm) which was previously equilibrated with 0.02 mol/L Na_2HPO_4-NaH_2PO_4 pH 6.6. The column was washed with

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the equilibration buffer. The active fractions were further purified by application to a CM-Sephadex-C-50 column (2.2×10 cm) pre-equilibrated with 0.02 mol/L Na_2HPO_4-NaH_2PO_4 pH 7.8, the column was first washed with 15 mL equilibration buffer and then eluted with 40 mL 0-1 mol/L NaCl in 0.02 mol/L Na_2HPO_4-NaH_2PO_4 pH 7.8. The active fractions (peak IV) were collected with about 35 mg purified flammulin normally being obtained.

Crystallization was performed according to the method of Dattagupta at room temperature in the dark[3]. The concentrated elutes from CM-Sephadex column were centrifuged and 50 μL of supernatant was pipetted into microdialysis tubes, and set up for crystallization against 5 mL of reservoir solution of PEG in sodium phosphate buffer.

Physicochemical and anti-tumor activities analysis

Active fractions insolated from each purification step were subjected to SDS/PAGE analysis for detection of the purity. SDS/PAGE (8.0%, w/v) was performed on a Bio-rad mini protein gel apparatus according to the method of Laemmli[4]. The gel was visualized by staining with Coomassie Brilliant Blue R-250. CZE for flammulin was performed on a Beckman P/ACETMMDQ capillary electrophoretic apparatus by the method of Wheat[5].

The amino acid composition was analyzed with an automatic Beckman 6300 system amino acid analyzer. Samples were hydrolyzed with 6 mol/L HCl for 24 h, 48 h, and 72 h at 105°C. After removal of HCl, the hydrolyzate was dissolved in pH 2.2 buffer for amino acid analysis. Tryptophan content was measured from spectroscopic absorbency at 280 nm and 288 nm in 6 mol/L HCl by method of Edelhoch[6]. Protein concentration was estimated by the method of Smith *et al.*, using bovine serum albumin as a standard[7].

In vitro effect on tumor cells: Cell-containing ascitic fluid was mixed flammulin and incubated at 37°C for 3 h, and then inoculated subcutaneously in ddD mice. After 10 d, the animals were sacrificed and the weight of solid tumor removed was compared.

In vivo anti-tumor effect: The intraperitoneal treatment with various amounts of flammulin on mice was started 2 h after the tumor transplantation and continued once daily for 4-5 d, the observation period being 50 d. The survival rate of the treated mice and effect on tumor growth were compared.

Results and Discussion

The crude extracts were fractionated on a DEAE-Sephadex column (2.2×10 cm) and the unbound protein peak was found to have anti-tumor activity. The active fractions were further purified with CM-Sephadex-C-50 column. Five peaks were obtained, and the highest specific activity was detected in peak IV (**Figure1**). The purified product gave a single band with an apparent molecular mass of 24 kDa by SDS/PAGE. The homogeneity of flammulin was further analyzed by CZE and the final product gave a single peak.

For crystallization, maximum dimension is 4 mm. Changes from the crystallization conditions that caused crystals to crack and melt (**Figure 2**).

The amino acid composition of flammulin is presented in **Table 1**. The results of amino acid analysis reveal that flammulin comprises large amounts of aspartic acid. In addition, flammulin has a higher number of arginine residues than of lysine residues, and no methionine was detected, which is one of the properties of flammulin. The aqueous solution of flammulin shows a typical protein ultraviolet absorption, with the maximum at 276-278 nm and the minimum at 250 nm (data not shown).

Figure 1 Ion-exchange column chromatography purification of flammulin

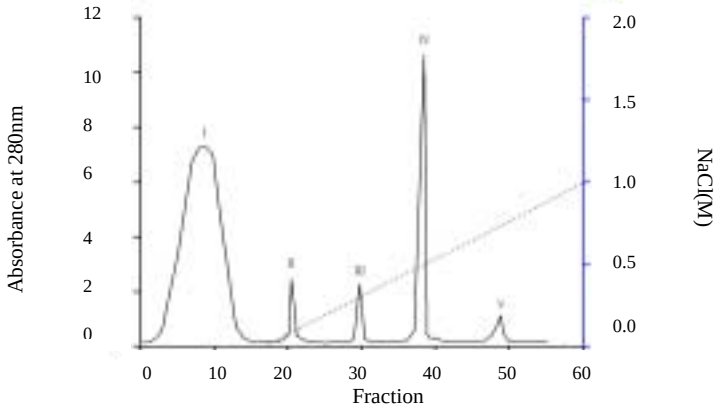
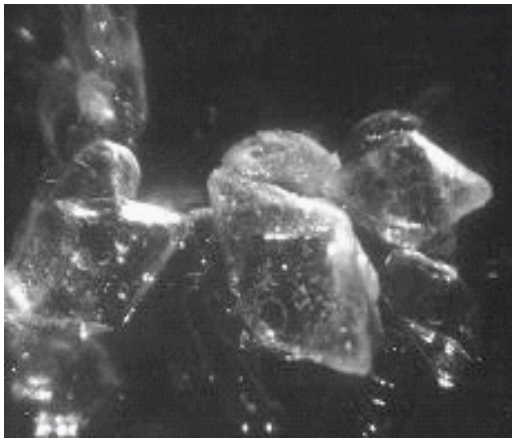


Figure 2 Crystals of flammulin grown in polyethylene glycol



The physicochemical and immunochemical analysis of this protein have been studied in details. Flammulin was proved to be able to affect the tumor cells directly. Cytopathogenic changes in the ascitic fluid of the treated animals were observed. Inhibition of mitosis, swelling of tumor cells, formation of vacuoles in both nucleus and cytoplasm, bubbling of the cells and finally cytolysis could be recognized. Similar marked tumor-inhibiting effects were obtained on Sarcoma 180.

Table 1 The amino acid composition of flammulin

Amino acid	Residues in Flammulin molecule
Ala	35(34.79)
Arg	15(14.53)
NH_3	25(25.02)
Asp	33(33.25)
Cys	7(7.06)
Val	6(5.77)
Glu	17(16.69)
Gly	24(24.18)
His	4(3.96)
Ile	5(4.88)
Leu	14(14.23)
Lys	3(3.03)
Met	0(0.00)
Phe	8(7.86)
Pro	19(18.79)
Ser	14(14.00)
Thr	19(19.12)
Trp	1(1.02)
Tyr	11(10.69)
Total residues	235

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