# *Flammulin, an Antitumor Substance**

By Yumi WATANABE, Koji NAKANISHI, Nobuhiko KOMATSU,
Tomohei SAKABE and Hirofumi TERAKAWA

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From the aqueous extract of fruit bodies of *Flammulina velutipes*, an edible mushroom, a high molecular weight substance with antitumor qualities against the Ehrlich ascites tumor was isolated by Komatsu et al.[1] and designated "flammulin."

The present paper deals with a procedure for flammulin's purification, some of its physical and chemical properties, and its amino acid composition.

Although the separation of the active component from the material is rendered very difficult by its unstable properties, considerable purification was achieved by the combination of ammonium sulfate fractionation and ethanol fractionation in the cold. Preliminary experiments showed that the precipitates obtained at 66% ammonium sulfate saturation and 75% ethanol were active, while the precipitates, obtained at 50% ethanol were inactive. Further purification was carried out by ion exchange chromatography using DEAE-Sephadex and CM-Sephadex columns. The active component was not bound to DEAE-Sephadex equilibrated with 0.02 M phosphate buffer at pH 7.0. The active fraction, purified on a DEAE-Sephadex column, seemed to be still contaminated with a small amount of colored impurities, and zone electrophoresis on starch showed that it consisted of two or more components, as Fig. 1 shows. Therefore, it was chromatographed on a CM-Sephadex column equilibrated with a 0.005 M acetate buffer at pH 5.6 eluted step by step with 0.01 M, 0.05 M, 0.1 M, and 0.2 M acetate buffers, and separated into two fractions, active and inactive, as is shown in Fig. 2. The active fraction was subjected to rechromatography on a CM-Sephadex column under gradient elution from a 0.005 M to a 0.1 M acetate buffer and then eluted with about a 0.08 M concentration, as Fig. 3 shows. The purified flammulin by chromatography was dialysed against cold tap water and lyophilized. The lyophilization often caused a slight denaturation, but lyophilized flammulin was obtained in an almost colorless state in an average yield of about 120 mg. from 2 kg. of dried fruit bodies. The final fractionation scheme for 2 kg. of the material is given in Scheme 1.

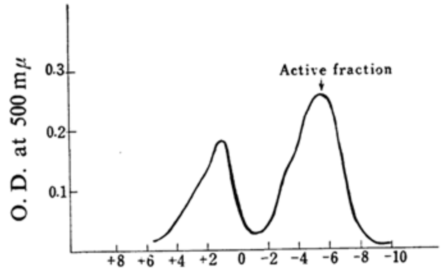

Fig. 1. Zone electrophoresis of partially purified flammulin.
(pH 8.6, $\mu = 0.05$, Veronal buffer)

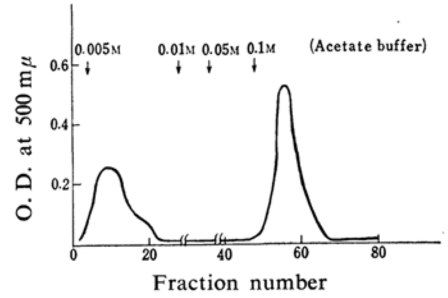

Fig. 2. Chromatogram of partially purified flammulin on CM-Sephadex.

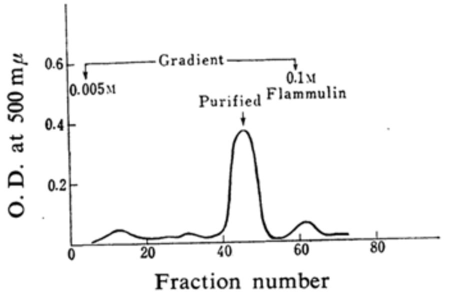

Fig. 3. Rechromatogram of flammulin on CM-Sephadex.

Flammulin purified as above works against the Ehrlichascites tumor when administered at the daily dose of 2.5 γ per mouse.

The purity of lyophilized flammulin was determined by moving boundary electrophoresis, paper electrophoresis, and sedimentation, it was found to be homogeneous in all

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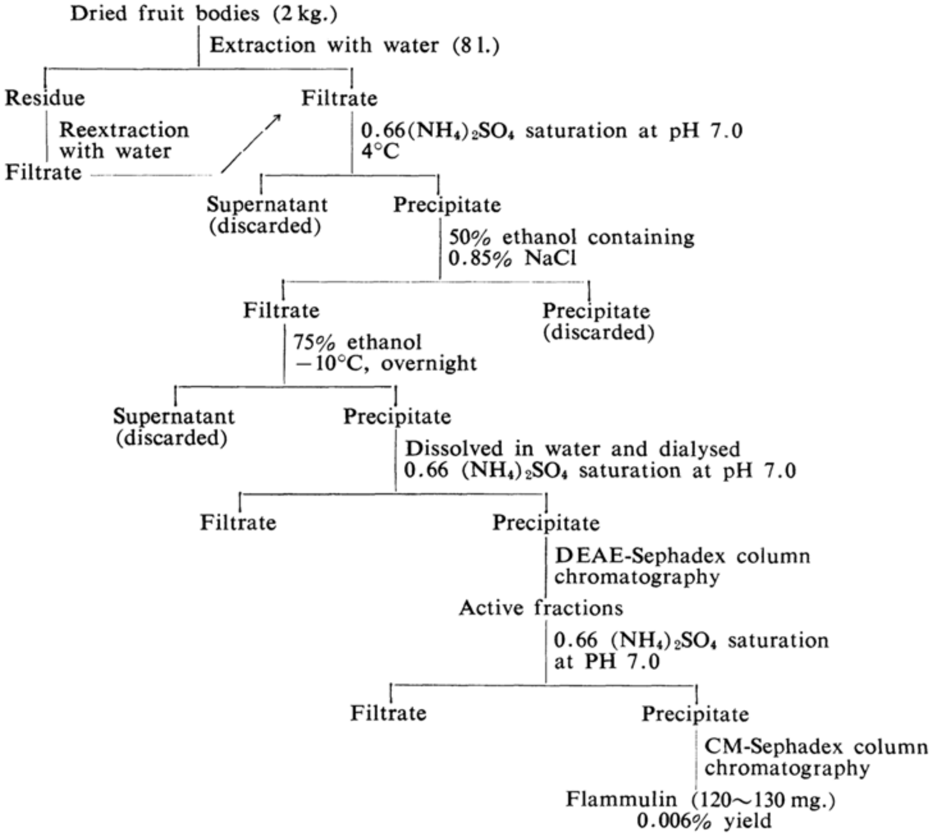
* Reported at the 16th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1963.

1) N. Komatsu, H. Terakawa, K. Nakanishi and Y. Watanabe, *J. Antibiotics, Ser. A*, 16 (3), 139 (1963).



Scheme 1. Purification procedure of flammulin.

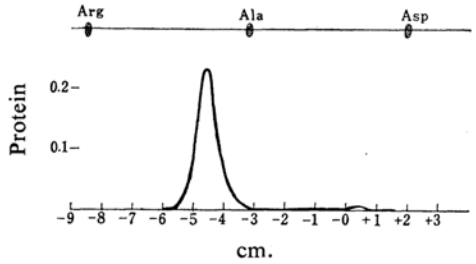


Fig. 4. Paper electrophoresis of flammulin.

these analyses. On the moving boundary electrophoresis, flammulin yielded a single homogeneous peak with a descending mobility of $0.87\times10^{-5}$ cm$^2$/sec. V. Upon paper electrophoresis at pH 7.0, flammulin moved toward the cathode and showed a single symmetrical peak, as is shown in Fig. 4. The same single bands were obtained on paper electrophoresis over the range from pH 4.6 to 12, and the relative position to the standard amino acids seemed to be unchanged. The sedimentation pattern of flammulin showed only a single symmetrical boundary, indicating that it was homogeneous. The calculated values of the sedimentation and diffusion coefficients were found to be $s_{20w}=2.5$s and $D_{20w}=9.93\times10^{-7}$ cm$^2$/sec. respectively, figures of which indicate a molecular weight of 24000. The aqueous solution of flammulin shows a typical protein ultraviolet absorption, with the maximum at 277 m$\mu$ and the minimum at 251∼252 m$\mu$. The nitrogen content of 16.2%, as determined by the micro-Dumas method, is a general value for proteins. Some physical properties are summarized in Table I.

Amino acid analyses of the acid hydrolysates of flammulin were performed on paper chromatography and with an automatic amino acid analyzer; the average results of three separate analyses for the relative stable amino acids are summarized in Table II. The analytical results show that an almost complete liberation of the constituent amino acids was obtained during the first 24-hr. hydrolysis. Since a partial decomposition with an increase in the time of hydrolysis occured with aspartic acid, threonine, serine and proline, the values for these amino acids were corrected for the losses by linear extrapolation to zero time. Similarly, ammonia was found to increase with an increase in the time of hydrolysis, so the value was corrected in the same way as above. As is clear in Table II, flammulin

TABLE I. SOME PROPERTIES OF FLAMMULIN

| | |
|---|---|
| Electrophoretic mobility ($\mu=0.1$, descending) | $0.87\times10^{-5}$ cm$^2$/sec. V. (pH 7.7) |
| Sedimentation coefficient | $S_{20w}=2.5$ s |
| Diffusion coefficient | $D_{20w}=9.93\times10^{-7}$ cm$^2$/sec. |
| Molecular weight | 24000 |
| Nitrogen content | 16.2% |
| UV-absorption | $\lambda_{max}=277$ m$\mu$<br>$\lambda_{min}=251\sim252$ m$\mu$ |

TABLE II. AMINO ACID COMPOSITION

| Amino acid | Amino acid residue per 100 g. protein g. | Amino acid per 100 g. protein g. | N as % of total N | Calcd. No. of residue for mol. wt. 24000 | No. of residue to nearest integer |
|---|---|---|---|---|---|
| Lys | 2.42 | 2.76 | 3.27 | 4.54 | 5 |
| His | 2.73 | 3.09 | 5.16 | 4.78 | 5 |
| Arg | 9.08 | 10.13 | 20.12 | 13.97 | 14 |
| Asp | 17.60 | 20.35 | 13.70 | 36.72 | 37 |
| Thr | 8.30 | 9.78 | 7.10 | 19.72 | 20 |
| Ser | 4.35 | 5.25 | 4.32 | 12.00 | 12 |
| Glu | 9.50 | 10.83 | 6.36 | 17.68 | 18 |
| Pro | 7.60 | 9.01 | 6.76 | 18.80 | 19 |
| Gly | 5.20 | 6.84 | 7.88 | 21.89 | 22 |
| Ala | 10.17 | 12.75 | 12.37 | 34.38 | 34 |
| Half-cys | 1.28 | 1.51 | 1.08 | 3.02 | 3 |
| Val | 2.12 | 2.51 | 1.85 | 5.14 | 5 |
| Met | 0 | 0 | 0 | 0 | 0 |
| isoLeu | 2.86 | 3.32 | 2.18 | 6.08 | 6 |
| Leu | 4.21 | 4.88 | 3.22 | 8.94 | 9 |
| alloisoLeu | 0.33 | 0.38 | 0.25 | 0.70 | 1 |
| Tyr | 2.50 | 2.78 | 1.32 | 3.69 | 4 |
| Phe | 5.14 | 5.77 | 3.02 | 8.39 | 8 |
| Try | 0.62 | 0.68 | 0.28 | 0.82 | 1 |
| Amide $NH_3$ | 1.80* | 1.80* | 9.16 | 25.41 | 25* |
| Total | 97.48 | 112.24 | 99.71 | — | 222 |

* Omitted from the total.

contained all of the common amino acids except methionine, and no new amino acid composition; this indicates that flammulin is a simple protein composed of only common amino acids. *N*-Terminal amino acid analysis was carried out according to the procedure of Sanger.[2] In chromatography only a single spot of DNP-amino acid was obtained at the position corresponding to DNP-valine. Flammulin is characterized by its high content of aspartic acid and alanine, but the basic properties of flammulin suggest that the carboxyl groups of aspartic acids are masked in the form of amide as well as peptide linkages.

## Experimental

**The Determination of Antitumor Activity and Protein.** — The activity of all the fractions in the purification procedure were assayed by in vivo experiments, using Ehrlich ascites tumor of mice, while the protein contents were determined by the Folin reaction,[3] using the absorption at 500 m$\mu$.

**The Extraction of the Active Substance.** — Fruit bodies of *Flammulina velutipes* were air-dried and extracted with water (8 l. for 2 kg.) three times in the cold, and aqueous extracts were fractionated.

**Fractionation with Ammonium Sulfate and Ethanol.** — All fractionations were carried out in the cold to avoid denaturation; ethanol fractionations, especially, were done below −5°C. Ammonium sulfate fractionation was performed by adding solid ammonium sulfate to the aqueous solution while stirring it in the cold.

**Chromatographic Purification.** — The preparation of DEAE-Sephadex and CM-Sephadex columns and the chromatographic procedure were carried out according to the general method of Sober et al.[4] About one gram of DEAE-Sephadex A-50 was used for the extract from 2 kg. of dried fruit bodies. To the dialysed flammulin solution, a one-tenth volume of 0.2 M phosphate buffer was added at pH 7.0. The effluent was collected in 2 ml. fractions with an automatic fraction collector, and the

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[2] F. Sanger, *Biochem. J.* (*London*), **39**, 507 (1945).

[3] O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[4] H. A. Sober, F. G. Gutter, M. M. Wyckoff and E. A. Perterson, *J. Am. Chem. Soc.*, **78**, 756 (1956).

activity and the protein were measured as has been described above. The active fractions were collected, dialysed, and adsorbed on a CM-Sephadex C-50 column equilibrated with 0.005 M acetate buffer at pH 5.6; the concentration of buffer was changed step by step. The effluent was treated in the same way as above. The crude flammulin preparation obtained in the above chromatography was subjected to rechromatography, using a gradient elution system—one chamber filled with 125 ml. of a 0.005 M, and the other, with 125 ml. of a 0.3 M acetate buffer. The other procedures were the same as have been described above. The purified flammulin was then dialysed and lyophilized.

**Electrophoresis.** — Moving-boundary electrophoresis was carried out at 8°C for two hours in a phosphate buffer ($\mu=0.1$, pH 7.7) using the Tiselius electrophoresis apparatus. Paper electrophoresis was carried out over the range from pH 4.6 to 12 under the following conditions:

Buffer system: 1/10 M-phosphate or 1/10 M-Veronal-Na-HCl, 1/10 M-Veronal-Na-NaOH.
Filter paper: Toyo-Roshi No. 51, 14.5×35 cm.
Voltage: 300 V./35 cm. (5 mamp.)
Temperature and time: 20°C, 3 hr.

After electrophoresis, the paper was colored with a B. P. B. solution. The excess dye was thoroughly washed out, and the paper was dried and cut off in 0.5 cm. widths. These were then eluted with 3 ml. of a 0.01 N sodium hydroxide aqueous solution, and the protein content was determined from the optical density at 590 m$\mu$. Some amino acids were run under the same conditions, and the positions were detected with ninhydrin.

**Sedimentation.** — The lyophilized sample of flammulin was dissolved in a phosphate buffer of pH 7.2, $\mu=0.1$, and this solution was subjected to sedimentation. Sedimentation experiments were conducted in a Spinco model E ultracentrifuge at 59780 r.p.m., and the observed sedimentation coefficient was corrected to the value in water at 20°C ($s_{20\mathrm{W}}$) in the conventional manner.

**Diffusion.** — The diffusion measurements were made with a 0.25% solution in a 1/10 M sodium bicarbonate-carbonate buffer solution (pH 9.75) at 20°C with a Spinco model H diffusion apparatus. The diffusion coefficient was also determined by applying the gel diffusion method of Allison.[5]

*Amino Acid Analyses. — The Acid Hydrolysis of Flammulin.* — Flammulin (10 mg.) was hydrolysed with 1 ml. of 6 N hydrochloric acid or 0.5 ml. of 12 N hydrochloric acid in a sealed tube at 110°C for 10 to 45 hr. At the end of the hydrolysis period, the hydrolysate was concentrated to dryness on a steam-bath, to remove the excess hydrochloric acid, and finally dried over sodium hydroxide in vacuo.

*Amino Acid Composition* — The amino acid analyses of the hydrolysated flammulin were performed by two-dimensional paper chromatography (*n*-butanol - acetic acid - water, phenol - water) and with a Beckman-Spinco model 120 automatic amino acid analyser.

*Analyses of Tryptophane.*[6,7] — To 3 to 5 mg. of flammulin in 1 ml. of water, 30 mg. of dimethylaminobenzaldehyde in 9 ml. of 21.4 N sulfuric acid were added. The solution was allowed to stand at 25°C for 12 hr. in the dark and treated with 0.1 ml. of a freshly-prepared 0.4% sodium nitrite aqueous solution; then, after it had been kept standing for another 30 min. in the dark, the optical density at 580 m$\mu$ was measured. The tryptophane content was determined from the standard curve after correction for the blank value.

*N-Terminal Amino Acid.* — Lyophilized flammulin (10 mg.) was dinitrophenylated in 66% ethanol (3 ml.) containing 8 mg. of 2,4-dinitrofluorobenzene and 10 mg. of sodium bicarbonate at room temperature for 10 hr. The acid hydrolysis of DNP-flammulin was carried out in 1 ml. of 6 N hydrochloric acid at 105°C for 12 hr. The ether-soluble DNP-amino acids were subjected to two-dimensional paper chromatography using *n*-butanol saturated with 2 N ammonium hydroxide as the first solvent and a 1.5 M phosphate buffer as the second solvent. DNP-alanine was used as the standard.

## Summary

(1) Flammulin, an antitumor substance of *Flammulina velutipes*, has been demonstrated to be a basic simple protein with a molecular weight of about 24000 and to be homogeneous in electrophoresis and sedimentation.

Analyses of amino acids indicated the following composition:

$\mathrm{Lys}_5$, $\mathrm{His}_5$, $\mathrm{Arg}_{14}$, $\mathrm{Asp}_{37}$, $\mathrm{Thr}_{20}$, $\mathrm{Ser}_{12}$, $\mathrm{Glu}_{18}$, $\mathrm{Pro}_{19}$, $\mathrm{Gly}_{22}$, $\mathrm{Ala}_{34}$, $\mathrm{Half\text{-}cys}_3$, $\mathrm{Val}_5$, $\mathrm{Met}_0$, $\mathrm{isoLeu}_6$, $\mathrm{Leu}_9$, $\mathrm{alloisoLeu}_1$, $\mathrm{Tyr}_4$, $\mathrm{phe}_8$, $\mathrm{Try}_1$, $(\mathrm{CONH}_2)_{25}$.

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*Department of Chemistry*
*Tokyo Kyoiku University*
*Otsuka, Tokyo (Y. W.)*

*Department of Chemistry*
*Tohoku University*
*Katahira-cho, Sendai (K. N)*

*The Institute for Infectious Diseases*
*The University of Tokyo*
*Minato-ku, Tokyo (N. K.)*

*Department of Chemistry*
*Nagoya University*
*Chikusa-ku, Nagoya (T. S.)*

*Biological Institute*
*Tokyo Medical and Dental University*
*Bunkyo-ku, Tokyo (H. T.)*

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[5] A. C. Allison and J. H. Humphrey, *Immunology*, **3**, 95 (1960).

[6] J. R. Spies and D. C. Chamber, *Anal. Chem.*, **20**, 30 (1948).

[7] J. R. Spies and D. C. Chamber, ibid., **21** 1249 (1949).