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Studies on the Constituents of the Higher Fungi of Korea(XIX)

Sterols and Amino Acids of *Lampteromyces japonicus* (Kawam.)Singer

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韓國產 高等 菌類의 成分 研究(XIX)

Lampteromyces japonicus (Kawam.)Singer의 스테롤 및 아미노산

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Abstract: To investigate constituents of *Lampteromyces japonicus* (Kawam.) Singer which grows wildly in Korea, the carpophores of the fungus were collected in Gyeong Gi Province and analyzed for sterols and amino acids by gas chromatography and an amino acid autoanalyzer. The results showed that it contained stigmasterol and ergosterol and that it contained eleven free amino acids. These free amino acids were also determined quantitatively. Furthermore, sixteen amino acids were identified in the total amino acid fraction of the fruiting body.

Recently numerous investigations have been carried out on Korean higher fungi. In Korea more than 600 species of them have been identified (Kim, 1978).

In the field of studies of constituents of these fungi, Kim reported the identification of amino acids in 15 edible mushrooms (1958). Yoon reported that various extracts of 33 species among 81 species of wild mushrooms had antibiotic activities(1959). Huh identified amino acids of 27 species of edible mushrooms(1960). Kim *et al.* reported on the detection of alkaloids in mushrooms (1970). Kim and Park reported that three species of Korean mushrooms had antiumor activities (Kim *et al.*, 1979; Park *et al.*, 1979).

To investigate constituents of *Lampteromyces japonicus* (Kawam.)Singer (*Pleurotus japonicus* Kawam.), a species of the family *Tricholomataceae*, that grows wildly in Korea, its carpophores were collected and examined for sterols and amino acids by gas chromatography and an amino acid autoanalyzer.

Materials and Methods

1. Material

The carpophores of *Lampteromyces japonicus* (Kawam.) Singer were collected in the Kwang-Neung area, Gyeong Gi Province in October of 1978 and dried in the dark.

2. Methods

1) Extraction and Purification of Sterol

Thirty grams of the dried mushroom were mixed with 600 ml of chloroform and methanol(2:1) and homogenized in a blender. This sludge was divided into three 500-ml flasks and shaken on a shaker for 40 hours and filtered. The filtrate was stored and the residue was shaken with 300 ml of chloroform and methanol mixture (2:1) for 24 hours and filtered.

The entire filtrate was concentrated in vacuum with a rotary evaporator. Then the concentrated solution was mixed with 200 ml of 10% alcoholic KOH and left on a water bath with reflux condensor at 80~85°C for four hours. The saponified solution was transfered to a 2-liter separatory funnel and 200 ml of ether and 250 ml of distilled water were poured into it. The ether layer was separated after it was shaken severely and left for 30 minutes.

The ether layer (the unsaponified fraction) was washed with distilled water several times until the final washed water was neutral. The separated ether layer was dehydrated by leaving it with anhydrous sodium sulfate for 15 hours. It was filtered and the filtrate was concentrated in vacuum in a rotary evaporator. The yellowish brown material was obtained. Liebermann-Bürchard test was carried out with this material. Since the chloroform and acetic anhydride layer was greenish blue and the sulfuric acid layer was red, this material was found to contain sterols. Chloroform solutions of the brown material and standard sterols were spotted on T.L.C. plates and developed with benzene and acetone mixture(4:1). It was sprayed with concentrated sulfuric acid and three spots were detected.

The chloroform solution of this material was spotted on three preparative T.L.C. plates and developed with benzene and acetone mixture (4:1). Methanol was sprayed on them and three bands appeared. The three bands were scraped and extracted with ether and concentrated on a water bath respectively. Each band was assigned A, B and C in sequence of increasing R*f* values.

Liebermann-Bürchard tests were carried out on samples A, B and C to find out wheather any sample had a sterol.

Gas liquid chromatography was carried out with standard sterols and B fraction under the conditions shown in Table I.

2) Free Amino Acids Analysis (G.C.)

A) Reagents

n-Butanol(HCl): 100ml of *n*-butanol was saturated with dry HCl gas. Methanol(HCl): 100ml of methanol was saturated with dry HCl gas. Dichloromethane (anhyrous): 100 ml of dichloromethane was mixed with 25g of calcium chloride (anydrous) and distilled.

Table I. Operation condition of G.L.C.

Packing material	3% OV-17
Carrier gas	Nitrogen 60ml/min.
Column temperature	280°C
Detecor	Flame ionization detector
Chart speed	5 mm/min

Table II. Operating condition (G.C.)

Column	3% OV-17 (80~100 mesh. Shimalite.)
	3 mm×2 m boronsilicate glass column.
Temperature	Injection port: 200°C
	Column: 100-210°C (5°C/min.)
	Detector: 270°C
Flow Rate	N_2 40 ml/min.
	H_2 60 ml/min.
	Air 88 ml/min.
Attenuation	4×10^2 a.f.s.

B) Instruments and conditions of chromatography.

The instrument used was Gas Chromatography (Shimatzu G.C. —4 B.M.) and the conditions were as in Table II.

C) Synthesis of derivatives for analysis

Standard amino acid mixture containing 1.5mg of 16 standard amino acids respectively was mixed with 3ml of methanol (HCl) and reacted at room temperature for 30 minutes on a shaker. Methyl ester was obtained and concentrated in an evaporator at 60°C and dried completely.

This methyl ester was mixed with 10ml of butanol(HCl) and reacted at 150°C for five minutes and at 100°C for 60 minutes. This was concentrated in an evaporator and dried completely.

This butyl ester was mixed with three ml of dichlormethane and one ml of trifluoroacetic anhydride and reated at 90°C for an hour. This was concentrated in an evaporator and dried completely. This material obtained was dissolved in one ml of chloroform. One and a half mg of each standard amino acid were reacted in the same manner.

D) Extraction

Ten grams of the material were mixed with 250ml of ethanol and blended. This sludge was shaken for two days and filtered. The filtrate was recovered and the residue was extracted with ethanol again. The entire filtrate was concentrated in an evaporator and 0.746 g of solid material was obtained. Lipid material was removed from this material obtained with ethyl ether. The water layer was concentrated in an evaporator and 0.55 g of solid material was obtained.

The lipid-free extract was mixed with dichloromethane and concentrated in an evaporator and carried out in the same manner as standard amino acids.

3) Total Amino Acid Analysis

A) Treatment of sample.

One-hundred mg of the mushroom (dry wight) were mixed with 20ml of 6N-HCl and poured into amples, and they were saturated with nitrogen gas and sealed. They were hydrolyzed at 110° ± 5°C for 48 hours and the hydrolyzed solution was filtered. The filtrate was concentrated in a vacuum evaporator and dried completely. This material was dissolved in 10 ml of 0.1N-HCl and the concentration of the solution was adjusted.

Total amino acids were analyzed by an amino acid autoanalyzer in the conditions stated in Table III.

B) Preparation of standard amino acid solution.

Two-tenth μM/ml of each of 14 standard amino acids with exceptions of glutamic acid (0.1584μM/ml.) proline (0.4μM/ml.) cysteine (0.1906μM/ml.) were prepared and 0.5ml of solution was injected.

C) Instrument and operating conditions.

The instrument used was Hitachi amino acid autoanalyzer (Model KLA-5). The operations are stated in Table III.

D) Quantitative analysis of total amino acids

The chromatogram of sample was compared with chromatogram of standard amino acid mixture and each amino acid was identified and quantitative analysis was carried out by H.W. method because the amount is proportional to the area of the peak.

Table III. Operating conditions of amino acid autoanalysis

Column size	9 mm ID×550 mm
	9 mm ID×100 mm
Ion exchange resin	Hitachi-custom ion exchange 2613
	Hitachi-custom ion exchange 2617
Flow rate	Buffer solution: 60 ml/hr.
	Ninhydrin: 30 ml/hr.
Wave length	15mm tubular flow cell: 570 nm(red)
Buffer solution	pH 3.25 pH 4.25 pH 5.28.
	Na. citrate buffer solution.

Results and Discussion

1. Sterols

Three spots were identified from T.L.C. plates of the samples. The R*f* values of them were 0.39, 0.45 and 0.59. Since the R*f* value of standard sterol was 0.45, the fraction whose R*f* value was 0.45 was found to be a sterol. Each band of preparative T.L.C. was scraped and extracted with ether, and then Libermann-Bürchard test was carried out with each fraction. As a result the fraction whose R*f* value was 0.45 was sterol. Each standard sterol that dissolved in chloroform was analyzed by G.L.C. and retention times were obtained(Table IV).

The chromotogram of sample was shown in Figure 1 and it was found that the sample contained ergosterol and stigmasterol.

To confirm it, the sample solution was mixed with ergosterol and stigmasterol solution and analyzed by G.L.C. As a result of this chromatogram it was confirmed that these bands were ergosterol's and stigmasterol's.

2. Free Amino Acid

One μl of N- T.F.A. butyl ester of 16 stundard amino acids mixture was injected and a chromatogram of standard amino acid was obtained. One μl of sample solution was injected and a chromatogram of sample (Figure 2) was obtained and each amino acid was analyzed quantitatively (Table V).

Since isoleucine and leucine, lysine and glutamic acid have the same peaks as two pairs, they were

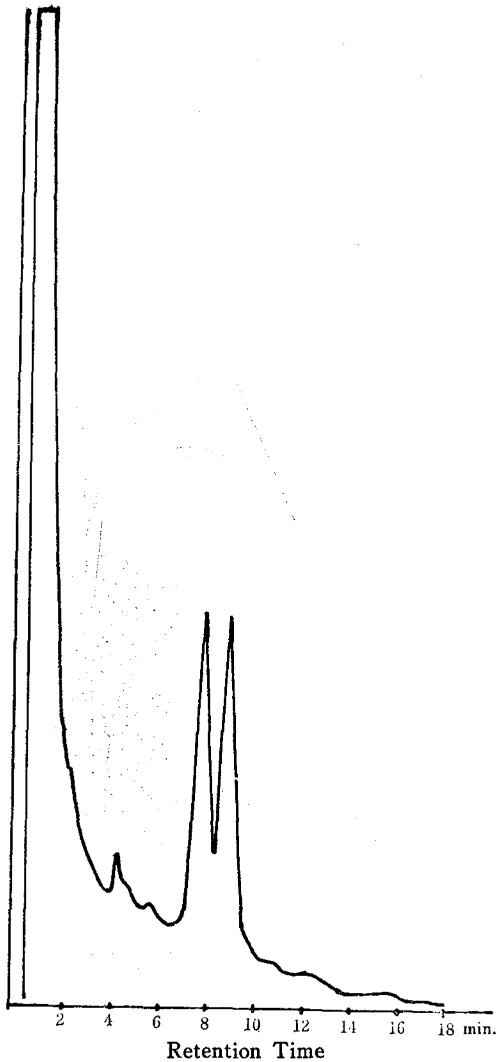

Fig. 1. Chromatogram of sterols of the carpophore.

Table IV. Retention times of standard sterols.

Standard	Retention time(min.)
Cholesterol	6.1
Ergosterol	7.8
Stigmasterol	8.4
β-Sitosterol	9.2

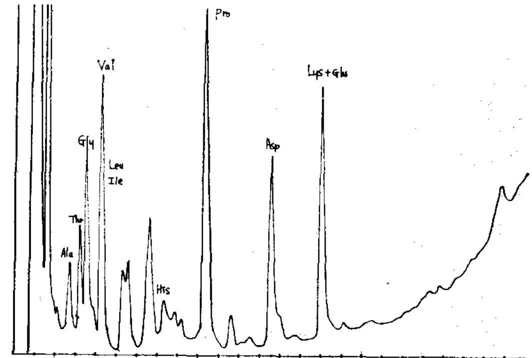

Fig. 2. Chromatogram of free amino acid of the carpophore

Table V. Contents of free amino acids in the carpophore

Amino acid	Content (mg/g)	Amino acid	Content (mg/g)
Alanine	1.73	Aspartic acid	1.62
Threonine	0.25	Lysine Glutamic acid	0.99
Glycine	2.93	Histidine	1.29
Valine	2.30	Proline	2.43
Leucine Isoleucine	0.96		

analyzed as total contents of amino acids.

3. Total Amino Acid

Five-tenth ml of standard amino acids mixture solution was injected and a chromatogram (Figure 3) was obtained. Five-tenth ml of sample solution was injected and a chromatogram (Figure 4) and content of each amino acid(Table VI) was obtained

In the total amino acid analysis, the mushroom was found to contain large amounts of aspartic acid, glutamic acid and proline. Tryptophan was destroyed during the hydrohysis and was not detected.

In addition, Moore *et al.* (1960) reported that about five % of threonine, cysteine, and tyrosine, 10% of serine were destroyed during the hydrolysis for 22 hours. It was reported by Rees (1946) that 5.3% of threonine and 10.5% of serine were destroyed. When they were hydrolyzed in 6N-HCl at 100°C. Fukuda (1975) reported that the polysaccharide component of this mushroom had an antitumor activity.

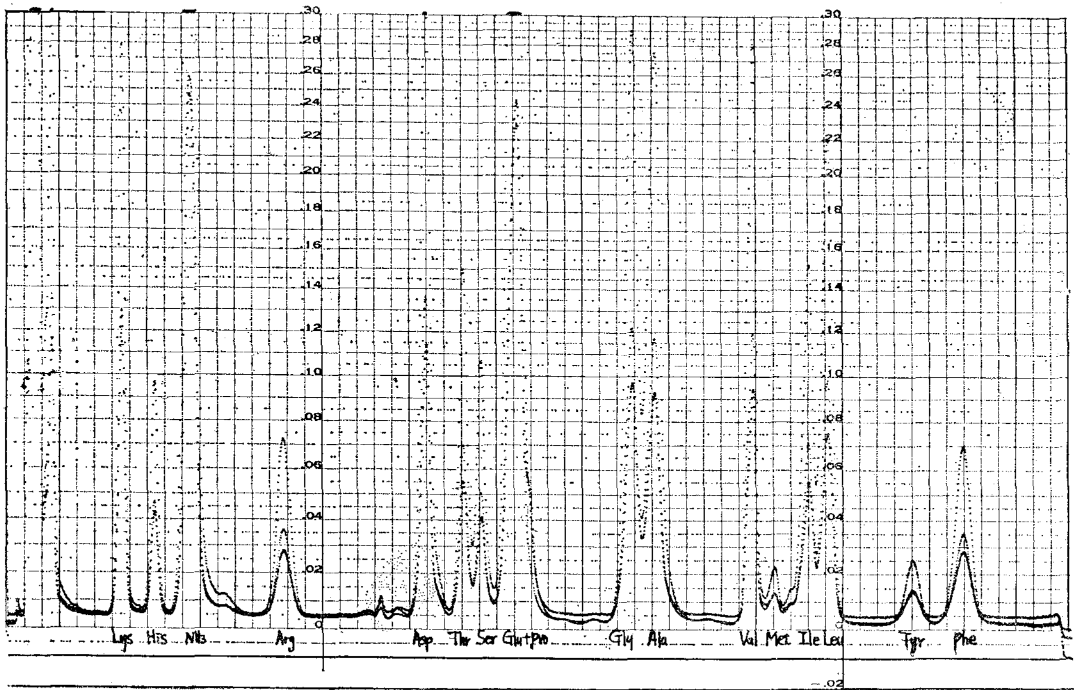

Fig. 3. Chromatogram of standard amino acids(A.A.A.)

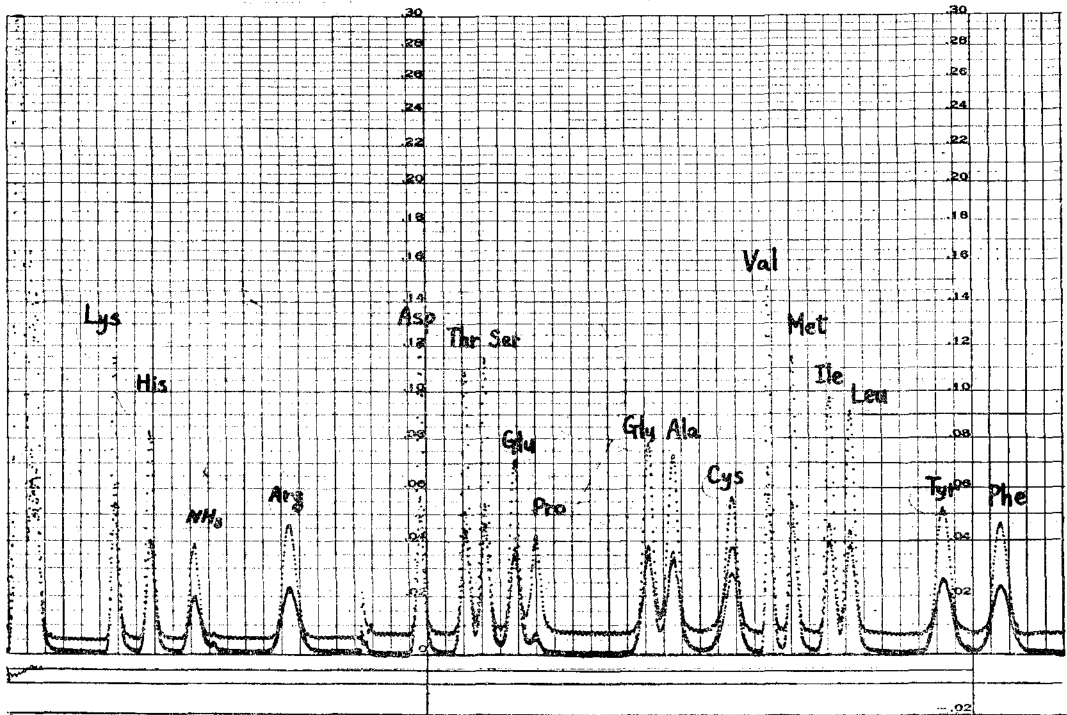

Fig. 4. Chromatogram of amino acids of the carpophore,

Table VI. Contents of amino acids.

Amino acid	Content (mg/g)	Amino acid	Content (mg/g)
Lysine	17.38	Alanine	16.66
Histidine	8.97	Valine	16.04
Arginine	12.24	Methionine	0.83
Aspartic acid	much	Isoleucine	4.81
Threonine	8.44	Leusine	9.35
Serine	3.10	Tyrosine	3.98
Glutamic acid Proline	much	Phenylalanine	9.70
Glycine	12.89		

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