Constituents of a Cationic Peptide-rich Fraction of *Lentinus edodes*

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A new γ -glutamylpeptide, γ -glutamylnicotianine was isolated from a cationic peptide-rich fraction of *Lentinus edodes*, and the structure was confirmed by enzymatic synthesis. The presence of lactam of saccharopine and γ -glutamylglutamic acid was demonstrated in the fraction.

In the course of our studies^{1~4)} on nitrogenous compounds of dried Shiitake mushroom, *Lentinus edodes* (Berk) Sing., we have observed that about 20% of total nitrogen of a 70% ethanol extract of the mushroom was attributable to oligopeptides. The cationic peptide-rich fraction prepared from the extract by fractionations using ion-exchange resins and a Sephadex gel, was found to contain a large number of unidentified ninhydrin-positive components. Up to present, some of them were isolated and identified as nicotianine, cystathionine,¹⁾ saccharopine,²⁾ lentinic acid, *N,N'*-bis- γ -glutamylcystine,³⁾ γ -glutamylcystine, and *N,N'*-bis- γ -glutamylcystinylglycine.⁴⁾ However, many of the other components are still not yet identified.

This paper deals with the isolation and identification of lactam of saccharopine, γ -glutamylnicotianine and γ -glutamylglutamic acid from the fraction.

MATERIALS AND METHODS

Subfractions of AI-2. The subfractions of dried *L. edodes* extract used in this paper, AI-2-6, AI-2-12, AI-2-15 and AI-2-17 were the same as prepared in the previous paper.³⁾

General methods. All evaporations were carried out with a rotary evaporator under reduced pressure at a bath temperature not exceeding 40°C. Paper chromatographies were performed on Toyo Roshi, No. 51 for the analytical

runs and No. 514 for the preparative runs. The solvent systems used are as follows: (A) *n*-butanol-acetic acid-water (4:1:2), (B) *n*-propanol-ammonia water (7:3), (C) methanol-pyridine-water (4:1:2). Ion-exchange chromatographies were carried out on a column (2.7 $\times$ 100 cm) of Dowex 50W $\times$ 2 resin (pyridinium form) and a column (2.7 $\times$ 100 cm) of Dowex 1 $\times$ 2 (acetate form). Ten ml fractions were collected and assayed using the ninhydrin reaction. Amino acid analyses were performed with an automatic amino acid analyzer; Yanagimoto L-7, using the methods developed by Spackman *et al.*⁵⁾ and the N-terminals of the isolated peptides were determined by the DNP method.⁶⁾ Specific rotations were measured with a Union automatic polarimeter PM-201. IR spectra were obtained from the KBr disks using a Hitachi EPI-S2 spectrophotometer. MS spectra were measured on a JEOL JMSP-100 spectrometer. ¹H-NMR spectra were taken with a Hitachi R-40 spectrometer on solutions prepared in D₂O containing internal standard 3-trimethylsilylpropionate-*d*₄.

Synthesis of L-nicotianine. Nicotianine was synthesized from L-homoserine and ethylnicotinate according to Noguchi *et al.*⁷⁾ A mixture of methyl-L- α -amino- γ -iodobutyrate hydrochloride (100 mmol) prepared from L-homoserine, and ethylnicotinate (100 mmol) was kept at 85°C for 12 hr. The viscous precipitate obtained from the reaction mixture was hydrolyzed with 50 ml of 3N HCl at 100°C for 30 min. After removal of HCl by evaporation, the hydrolyzate was treated with a Dowex 50W $\times$ 2 resin column and fractionated by a Dowex 1 $\times$ 2 resin column successively, to produce L-nicotianine (900 mg, recrystallized twice from aqueous ethanol), mp 244~246°C, $[\alpha]_D^{28} = +24.5$ (*c* = 1.55, H₂O). Anal. Found: C, 49.16; H, 5.81; N, 12.19%. Calcd. for C₁₀H₁₂N₂O₄·H₂O: C, 49.58; H, 5.83; N, 11.57%.

Synthesis of γ -L-glutamyl-L-nicotianine. A mixture of L-nicotianine (1 mmol), reduced glutathione (2 mmol) and γ -glutamyltranspeptidase (porcine kidney, Sigma Co., Ltd., 50 units) dissolved in 100 ml of a 0.1 M Tris-HCl buffer (pH 7.6) was incubated at 37°C with gentle and continuous shaking for 22 hr. Subsequently, the reaction mixture was passed through a Dowex 50W $\times$ 2 resin column. After washing with distilled water, the adsorbed substances were eluted with 1 N pyridine. The eluted solution was evaporated to dryness and redissolved in about 10 ml of water. The solution was chromatographed on a column of Dowex 1 $\times$ 2 resin using a gradient of acetic acid, and the eluate was collected in 10 ml fractions. γ -L-Glutamyl-L-nicotianine was obtained from the fractions and purified by rechromatography on the same column. The active fractions were concentrated and freeze-dried, yielding 200 mg (54%) of colorless powder, $[\alpha]_D^{28} = +9.4$ ($c = 1.04$, H₂O). *Anal.* Found: C, 46.22; H, 5.33; N, 10.58%. Calcd. for C₁₅H₁₉N₃O₇ $\cdot$ 2H₂O: C, 46.27; H, 5.95; N, 10.79%.

RESULTS AND DISCUSSION

Isolations and identifications of saccharopine and lactam of saccharopine

The subfraction, AI-2-6, exhibited one strongly colored (*R_f* 0.18) and two faintly colored spots on the paper chromatography in solvent (A) as a result of the ninhydrin reagent. After purification by rechromatography on a Dowex 1 $\times$ 2 resin column, the *R_f* 0.18 compound was crystallized from a concentrated aqueous solution by adding droplets of ethanol. The compound was recrystallized twice from aqueous ethanol to yield 58 mg of colorless needles, mp 237 ~ 239.5°C (decomp.), $[\alpha]_D^{20} = +12.9$ ($c = 0.25$, H₂O). *Anal.* Found: C, 47.51; H, 7.44; N, 10.22%. Calcd. for C₁₁H₂₀N₂O₆: C, 47.84; H, 7.30; N, 10.14%. The data and chromatographic properties, *R_f*-values on the paper chromatographies and elution time on amino acid analyzer, strongly suggested that the compound is saccharopine which was previously isolated from the fraction, AI-5.²⁾ Furthermore, this suggestion was confirmed by the direct comparison of IR and MS spectra with that of authentic saccharopine.

The main constituent of the subfraction, AI-2-15, showing *R_f* 0.26 on the paper chromatography in solvent (A), was purified by chromatography on a column of Dowex 1 $\times$ 2 resin.

FIG. 1. Saccharopine and Lactam of Saccharopine.

The compound was then crystallized from the aqueous ethanol solution in a cold state, and recrystallized twice in the same manner. The colorless needles (75 mg) possessed mp 225 ~ 227°C (decomp.), $[\alpha]_D^{20} = +9.3$ ($c = 0.5$, H₂O). *Anal.* Found: C, 51.40; H, 6.98; N, 11.05%. Calcd. for C₁₁H₁₈N₂O₅: C, 51.15; H, 7.02; N, 10.85%. The chromatographic properties and behavior after treatment with 6 N HCl at 110°C for 11 hr, closely resembled with those of lactam of saccharopine.²⁾ Moreover, the MS spectrum *m/z* 258 (*M*⁺), 240, 213, 167, 153, 140, 112, 84, and the IR spectrum $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3550–3350, 3040–2600, 2050 (–NH₃⁺), 1720 (–COOH), 1655 (5-membered lactam), 1605 (–COO[–]), 1560, 1472; 1410 (–COO[–]), 1367, 1330, 1275, 1226, 1190, 1145, 1103, 1080, 980, 910, proved this assumption.

Previously, we found saccharopine in the fraction, AI-5, but now also found it in the subfraction, AI-2-6. However, these conflicting facts may be accounted for by the presence of lactam in the subfraction, AI-2-15, that is, the saccharopine in AI-2-6 was formed in the course of the fractionations from the lactam (Fig. 1).

Isolation and identification of γ -glutamyl-nicotianine

The subfraction, AI-2-12, showed two ninhydrin positive spots on the paper chromatography using solvent (A). The lower spot (AI-2-12-a) absorbing UV light was isolated. The subfraction dissolved in 2 ml of water was chromatographed on 4 sheets of Toyo Roshi No. 514 (40 $\times$ 40 cm) for 12 hr in solvent (A). Areas of the dried chromatograms containing AI-2-12-a were located by UV light. The compound was eluted with water from the area, concentrated to a few ml, and rechromatographed on a column of Dowex 1 $\times$ 2 resin.

(acetate form). Elution was made with 2N acetic acid and the fractions containing AI-2-12-a were collected. After removal of acetic acid by evaporation, the solution redissolved in about 5 ml of water was lyophilized. The resulting powder (15 mg) showed a single spot on the paper chromatographies in solvent (A), (B) and (C), and emerged between the serine and glutamic acids on the chromatogram of the amino acid analyzer, indicating that it is chromatographically pure.

AI-2-12-a, when hydrolyzed in 6N HCl at 110°C for 16 hr, produced glutamic acid and an unidentified UV-absorbing spot on the paper chromatography in solvent (A). This compound was reacted with the ninhydrin reagent and subsequent chromatographic investigations showed it to be nicotianine which was previously isolated from the fraction, AI-6.¹⁾ The amino acid analysis of the hydrolyzate revealed that the glutamic acid and nicotianine were present in a 1:1.04 molar ratio. The N-terminal of the peptide was determined by hydrolysis of the DNP derivative. AI-2-12-a contained single N-terminal consisting of glutamic acid. Results of the pyridoxal test⁸⁾ and

Cu^{2+} treatment⁹⁾ on the peptide indicated that there were only a free amino group adjacent to a free carboxyl in the molecule. Consequently, it was suggested that the peptide is γ -glutamyl-nicotianine. The dipeptide was then synthesized in order to make critical comparisons.

In the first experiment to prepare γ -glutamyl-nicotianine, the coupling of carbobenzyloxylglutamic anhydride to nicotianine in water was tried according to the procedure applied to synthesis of γ -glutamyl peptides by Kasai *et*

TABLE I. CHROMATOGRAPHIC BEHAVIORS OF AI-2-12-a AND SYNTHETIC γ -GLUTAMYLNICOTIANINE

	AI-2-12-a γ -GluNic	
	Solvent*	
<i>R_f</i> on PC	A	0.15
	B	0.12
	C	0.46
Elution time on amino acid analyzer** (min)		47.3

* A, *n*-BuOH-AcOH-H₂O (4:1:2); B, *n*-PrOH-ammonia water (7:3); C, MeOH-pyridine-H₂O (4:1:2).

** Yanaco L-7, Na-citrate buffer.

FIG. 2. ¹H-NMR Spectrum of Synthetic γ -Glutamyl-nicotianine in D₂O Using 3-Trimethylsilylpropionate-*d*₄ as the Internal Standard.

*al.*¹⁰⁾ This attempt, however, was unsuccessful to yield a mixture of α and γ -glutamyl-nicotianine. The problem could be solved by the enzymatic method using porcine kidney γ -glutamyltranspeptidase. The synthetic γ -glutamyl-nicotianine could not yield as crystal, but the structure was confirmed by several examinations. The $^1\text{H-NMR}$ spectrum of the peptide is presented in Fig. 2.

Chromatographic comparisons of AI-2-12-a with synthetic γ -glutamyl-nicotianine (Table I) strongly support that these were identical.

Isolation and identification of γ -glutamylglutamic acid

One main ninhydrin positive component was detected on a paper chromatography of the subfraction, AI-2-17, in solvent (A). The compound showing *R_f* 0.28 was purified by rechromatography on a Dowex 1 $\times$ 2 resin column and crystallized from the concentrated aqueous solution by adding droplets of ethanol. The colorless crystals were then recrystallized twice from the aqueous ethanol solution (Yield: 120 mg).

This compound was eluted just before aspartic acid from the column of the amino acid analyzer and possessed mp 172.5~173°C. *Anal.* Found: C, 43.46; H, 5.86; N, 10.15%. Calcd. for $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_7$: C, 43.48; H, 5.84; N, 10.14%. Acid hydrolysis of the compound in 6N HCl at 110°C for 16 hr followed by

determination of the constituent amino acids using automatic amino acid analyzer, indicated that it produced about 2 mol of glutamic acid per mol and no other detectable amino acids. The N-terminal analysis of the peptide showed only DNP-glutamic acid. These data as well as positive results of the Cu^{2+} musking and the pyridoxal test for α -amino group, strongly suggest that it is γ -glutamylglutamic acid. The final confirmation was achieved by direct comparison of the IR spectra of the compound with that of the authentic compound (Fig. 3).

In the present paper, the occurrence of one non-protein amino acid, lactam of saccharopine, and two γ -glutamylpeptides, γ -glutamyl-nicotianine and γ -glutamylglutamic acid, in *L. edodes* was shown. This is the first report on the isolation of lactam of saccharopine from fruit bodies of fungi. But it is difficult to substantiate whether saccharopine, previously isolated from the fraction, AI-5, or the lactam is the predominant form in the native state, because they are easily convertible to each other in the isolation process.¹¹⁾

Though a large number of γ -glutamylpeptides have been isolated from natural products,^{12~14)} this is the first report on the isolation of γ -glutamyl-nicotianine. Nicotianine has been isolated from the leaves of *Nicotina tabacum*¹⁵⁾ and fruit bodies of *L. edodes*.¹⁾ The isolation of the γ -glutamyl derivative of this

FIG. 3. IR Spectra of AI-2-17 and Authentic γ -Glutamylglutamic Acid in KBr Disks.

pyridinium amino acid may increase interest in its physiological role.

γ -Glutamylglutamic acid is widely found in plants.¹⁴⁾ This peptide possess an astringent taste,¹⁶⁾ so may be attributable to complex taste of the dried Shiitake mushroom.

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