Quantitative Determination of the Free Amino Acids and Their Derivatives in the Common Edible Mushroom, *Agaricus bisporus*

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Summary Nitrogen in the ethanol extract of the fruiting bodies of the common edible mushroom, *Agaricus bisporus*, accounted for about 60% of the total nitrogen of the fruiting bodies, and about 70% of the ethanol-extractable nitrogen was attributable to the free amino acids and their derivatives. Identification and quantitative determination of the free amino acids and their derivatives contained in this fraction were performed after group separation.

Of the 44 ninhydrin-positive compounds detected, alanine, glutamic acid, proline and arginine were the most predominant protein amino acids occurring in the free form, and ornithine, *N*-(γ -L-glutamyl)-4-hydroxyaniline and γ -aminobutyric acid were the most plentiful non-protein amino acids. An analytical method of the aromatic compounds characteristic of the mushroom is also described.

Key Words *Agaricus bisporus*, mushroom, free amino acids, aromatic compounds, γ -glutamyl conjugates

Several commercially cultivated mushrooms have become popular food items owing to a continuous supply in all seasons. *Agaricus bisporus* is one of the most widely consumed mushrooms and contains the unusual aromatic compounds, *N*-(γ -L-glutamyl)-4-hydroxyaniline (GHA), *N*-(γ -L-glutamyl)-3,4-dihydroxyaniline (GDA) and β -*N*-(γ -L-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine) (see Ref. 1). During the course of the investigation on the biosynthesis of these unusual aromatic compounds (1, 2), the extract of the fruiting bodies was found to contain many unidentified ninhydrin-positive compounds. Among them, γ -L-glutamyl-glycine, *N*-(γ -L-glutamyl)ethanolamine and L-saccharopine were identified as the

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new constituents of the mushroom (3, 4).

In the present investigation, the free amino acids and their derivatives¹ occurring in the mushroom were fully determined. A convenient method is described for the quantitative determination of the above-mentioned unusual aromatic compounds.

MATERIALS AND METHODS

Materials. Freshly harvested fruiting bodies of *Agaricus bisporus* (stage 2 (6), namely, the common edible stage) were obtained from a commercial grower in this district.

γ -L-Glutamylglycine, *N*-(γ -L-glutamyl)ethanolamine, GHA and GDA used as the analytical standards were synthesized as described previously (1-4). Agaritine was prepared from the fruiting bodies of the mushroom according to Kelly *et al.* (7). The crystalline compound gave mp 205-208°C (decompn). *Anal.* Found: C, 53.71; H, 6.50; N, 15.65. Calcd for $C_{12}H_{17}N_3O_4$: C, 53.92; H, 6.41; N, 15.72%. The other chemical properties agreed with those reported by Levenberg (8). L-Saccharopine was kindly supplied by Dr. Larsen, Royal Veterinary and Agricultural University, Denmark. L-Cystathionine was prepared from the 1-aminoproline-treated rats as described previously (9). The other reagents used were commercial products of analytical grade.

Preparation of the amino acid fraction. The fruiting bodies (300 g) were homogenized and extracted with 3 vols. of 95% ethanol. The homogenate was centrifuged (5,000 rpm, 30 min) and the precipitate was further extracted with 75% ethanol three times. The combined extract was applied to a 4 × 35 cm column of Amberlite IR-120B (20-50 mesh, H^+ form). The compounds adsorbed on the column were eluted with 2N NH_4OH (1.5 liters) and the eluate (amino acid fraction) was evaporated to dryness under reduced pressure at 30°C. The above procedure was repeated four times and the amino acid fractions obtained (corresponding to 1.2 kg of the fruiting bodies) were combined.

For the analysis of the unusual aromatic compounds, the fruiting bodies were homogenized and extracted with 3 vols. of 75% ethanol containing 1% ascorbic acid and 0.02N HCl at 4°C. The extraction was repeated three times and the combined extract was evaporated to dryness under reduced pressure below 30°C. The residue was dissolved in distilled water and the sample solution was used for the analyses described below.

Fractionation of the amino acid fraction. The group separation of the amino acid fraction was carried out as follows. One-half portion of the amino acid fraction (corresponding to 600 g of the fruiting bodies) was applied to a 5 × 20 cm column of Dowex 1 × 4 (100-200 mesh, acetate form). The column was washed with water (F-

¹ In this paper, the term "protein amino acids" is used to express the amino acids found in normal proteins and the word "non-protein amino acids" means those other than the protein amino acids including their derivatives (see Ref. 5).

I) and the compounds adsorbed were then eluted with 2 liters of 3 N acetic acid (F-II). The F-I and F-II fractions were evaporated to dryness under reduced pressure at 30°C. To isolate individual compounds for identification, the F-I fraction was further chromatographed on a 1.4 × 50 cm column of Dowex 50W × 4 (100–200 mesh, H⁺ form). The compounds adsorbed on the column were eluted successively with 500 ml of HCl solutions of the following concentrations, 0.1 N (F-III), 0.11 N (F-IV), 0.12 N (F-V), 0.13 N (F-VI), 0.14 N (F-VII), 0.15 N (F-VIII), 0.16 N (F-IX) and 0.17 N (F-X), and finally with 500 ml of 2 N NH₄OH (F-XI).

Paper chromatography and electrophoresis. Paper chromatography was performed on Toyo No. 51A filter paper with the following solvent systems: I, *n*-butanol/acetic acid/water (4:1:1, by vol.); II, phenol/water (4:1, w/w); III, 2,4-lutidine/water (3:1, v/v); IV, pyridine/*n*-butanol/water (1:1:1, by vol.); V, pyridine/*n*-butanol/acetic acid/water (10:1:4:2, by vol.). Two-dimensional paper chromatography was carried out by the combination of the solvent systems I and II or I and III.

Paper electrophoresis was carried out with a voltage gradient of 15V/cm for 3 hr in a pyridine/acetic acid buffer of pH 3.1.

Nitrogen content. Nitrogen content was determined by Kjeldahl method according to AOAC (10).

Amino acid analyses. Quantitative determination of the free amino acids and their derivatives was carried out with an automatic amino acid analyzer, Yanaco LC-5S. Two analytical systems were used: System I, SCX1001 resin/lithium citrate buffer which is commonly used for analyses of physiological fluids (11); System II, Aminex A-4 resin/sodium citrate buffer which is routinely used for analyses of protein hydrolysates (12). The aromatic compounds were analyzed by the combination of the following analytical systems: System III, Aminex A-4 resin (0.9 × 60 cm)/0.5 N sodium citrate buffer of pH 3.8 containing 2.5% *n*-propanol; System IV, Aminex A-4 resin (0.9 × 60 cm)/0.5 N sodium citrate buffer of pH 4.45, and the analyses were carried out at the flow rate of 80 ml/hr at 52°C.

Tryptophan was determined fluorometrically (13) after separation by gel-filtration on a Sephadex G-10 column (1) and its identity was confirmed by two-dimensional paper chromatography and the reaction with Ehrlich reagent (14).

RESULTS AND DISCUSSIONS

About 60% of the total nitrogen of the fruiting bodies of the mushroom was extracted with 75% ethanol. A chromatogram of the ethanol extract by the automatic amino acid analyzer is shown in Fig. 1, in which over 35 ninhydrin-positive compounds were detectable. Two-dimensional paper chromatograms showed the occurrence of more than 40 ninhydrin-positive compounds in the amino acid fraction prepared from the ethanol extract. This indicates that the complexity of the amino acid fraction resulted in an incomplete resolution in Fig. 1. Therefore, the amino acid fraction was further fractionated as described under

Fig. 1. Chromatogram of the extract of the fruiting bodies of *A. bisporus*. The 75% ethanol extract was analyzed by the amino acid analyzer equipped with System I. The sample used corresponded to 0.075 g of the fruiting bodies. The arrows in this figure indicate the elution positions of the protein amino acids.

MATERIALS AND METHODS and the compounds in the fractions were identified by the use of the amino acid analyzer, two-dimensional paper chromatography, electrophoresis, gel-filtration and specific color reactions (14, 15). Co-chromatography with the authentic compounds was carried out in every identification. The identity of some compounds was further confirmed by chemical degradation or by spectroscopic analyses such as infra-red absorption, nuclear magnetic resonance and gas-liquid chromatography-mass spectrometry. As to the unusual aromatic compounds, systematic analytical methods have not been reported, and they could be conveniently determined by the amino acid analyzer using the newly developed systems described under MATERIALS AND METHODS, as shown in Fig. 2.

Forty-four ninhydrin-positive compounds including NH_3 and urea determined by the above procedures are listed in Table 1.

Alanine, glutamic acid, proline and arginine were the most predominant protein amino acids. The occurrence of the first two amino acids at high concentrations has also been described in other species of mushrooms (16, 17). As shown in Table 2, the composition of the protein amino acids in the amino acid fraction resembled that of the amino acids in the ethanol-insoluble fraction (corresponding to about 30% of the total nitrogen of the fruiting bodies). This result shows that about one-half of the total protein amino acids in the fruiting bodies

Fig. 2. Chromatogram of the aromatic compounds in the fruiting bodies of *A. bisporus*. The extract with 75% ethanol containing 1% ascorbic acid and 0.02 N HCl was chromatographed by the amino acid analyzer equipped with Systems III and IV. The sample applied corresponded to 0.04 g of the fruiting bodies. In the analyses, the ninhydrin color yields of the aromatic compounds were as follows: agaritine, 85.0; 3,4-dihydroxyphenylalanine, 93.5; tyrosine, 94.3; GDA, 86.2; phenylalanine, 98.7; and GHA, 87.2 (norleucine, 100). As shown in Fig. 2-A, the elution position of GDA coincided with that of tyrosine, while phenylalanine was able to be separated from the other compounds. And, in Fig. 2-B, GDA overlapped with phenylalanine. Therefore, GDA was calculated by subtracting the value for phenylalanine obtained in Fig. 2-A from the value for phenylalanine-GDA obtained in Fig. 2-B. A, chromatogram with System III; B, chromatogram with System IV. 1, agaritine; 2, 3,4-dihydroxyphenylalanine; 3, tyrosine; 4, GDA; 5, phenylalanine; 6, GHA; IS, internal standard (norleucine).

existed in the free form.

Among the non-protein amino acids detected, ornithine, GHA and γ -amino-butyric acid were predominant and agaritine, cystathionine, ethanolamine, *N*-(γ -L-glutamyl) ethanolamine, kynurenine, saccharopine, α -amino adipic acid, methionine sulfoxide, α -amino-*n*-butyric acid and β -alanine occurred in the range of 0.3 to 5 μ mol per g of the fruiting bodies.

GHA was isolated from the gill tissue of the mushroom by Weaver *et al.* (18) and this compound seems to be the main browning principle in that tissue. Vogel

Table 1. Free amino acids and their derivatives in the fruiting bodies of *Agaricus bisporus*.

The compounds in group I were determined by the amino acid analyzer equipped with System I. The F-I and F-II fractions were used for the analyses. Those in group II except for tryptophan were determined by the analyzer equipped with Systems III and IV as described in the text and Fig. 2. The extracts with 75% ethanol containing 1% ascorbic acid and 0.02 N HCl described in the text were used for the analyses. The compounds of both the groups were listed in the table according to their elution positions on the chromatograms.

Compound	Amount ($\mu\text{mol/g}$ fruit- ing bodies) ^a	Compound	Amount ($\mu\text{mol/g}$ fruit- ing bodies) ^a
Group I		Cystathionine	0.63
Phosphoserine ^b	0.08	Methionine	0.40
Phosphoethanolamine ^b	0.10	Isoleucine	2.79
Urea	3.30	Leucine	3.59
γ -L-Glutamylglycine ^c	0.05	β -Alanine	0.30
Aspartic acid	2.10	γ -Aminobutyric acid	3.52
Methionine sulfoxide	0.32	Kynurenine	0.45
4-Hydroxyproline	0.18	Ornithine	7.33
Threonine	3.64	Lysine	2.27
Serine	2.73	Ethanolamine	0.57
<i>N</i> -(γ -L-Glutamyl)- ethanolamine ^d	0.50	Ammonia	16.89
Asparagine	5.83	Histidine	3.49
Glutamic acid	1.67	3-Methylhistidine ^e	0.05
Glutamine	4.25	Arginine	8.68
Sarcosine ^e	0.05	Group II	
α -Amino adipic acid	0.52	Agaritine ^g	1.04
Proline	10.06	3,4-Dihydroxyphenyl- alanine	0.06
Glycine	2.24	Tyrosine	0.31
Alanine	12.72	GDA	0.18
Citrulline	0.12	Phenylalanine	0.29
α -Amino- <i>n</i> -butyric acid	0.32	GHA	4.49
Valine	3.34	Tryptophan ^h	0.06
Saccharopine ^f	0.37		
Cystine	0.24		

^a Stage 2(6): average fresh weight of a fruiting body, 6.1 g; average cap diameter, 2.8 cm.

^b These compounds were identified by co-chromatography with the authentic compounds on the amino acid analyzer (System I) and by their disappearance after acid hydrolysis. Further identification could not be carried out, owing to the limited quantities available.

^c This compound appeared together with aspartic acid in the F-II fraction after group separation, and they could not be separated by the amino acid analyzer (Systems I and II). Therefore, both the compounds in the F-II fraction were separated by preparative paper chromatography before determination. Preparative paper chromatography (sol-

Table 2. Protein amino acids in the fruiting bodies of *A. bisporus*.

Amino acids in the ethanol-insoluble fraction were determined as follows. The residue of the ethanol-insoluble fraction was hydrolyzed with 6N HCl at 105°C for 24 hr and the hydrolysate was assayed by the amino acid analyzer equipped with System II. The compounds were listed according to their elution positions. Glucosamine (7.79 $\mu\text{mol/g}$ fruiting bodies) was the only ninhydrin-positive compound other than the protein amino acids and NH_3 , and occurred at trace level in the amino acid fraction.

Amino acid	A	B	Ratio
	Amino acid ^a fraction Amount ($\mu\text{mol/g}$ fruiting bodies)	Ethanol-insoluble fraction	$\frac{A}{A+B} \times 100$
Aspartic acid ^b	7.93	8.98	47
Threonine	3.64	5.68	39
Serine	2.73	5.41	34
Glutamic acid ^b	16.92	9.87	63
Proline	10.06	6.27	62
Glycine	2.24	7.29	24
Alanine	12.72	7.22	64
Valine	3.34	4.64	42
Cystine	0.24	0.31	44
Methionine	0.40	0.69	37
Isoleucine	2.79	4.03	41
Leucine	3.59	6.60	35
Tyrosine	0.31	2.07	13
Phenylalanine	0.29	3.49	8
Lysine	2.27	7.10	24
Histidine	3.50	1.88	65
Arginine	8.68	3.84	69

^a The values derived from Table 1. ^b The dicarboxylic acids and their amides.

(Continued from the footnote of Table 1)

vent system I) could be carried out quantitatively (recovery, 97%).

^d This compound appeared together with serine in the F-V fraction by group separation, and they could not be separated by the amino acid analyzer (System I). Therefore, both the compounds in the F-V fraction were determined after separation by preparative paper chromatography, as described in c).

^e These compounds were identified only by co-chromatography with the authentic compounds on the amino acid analyzer (System I), owing to the limited quantities available.

^f When the amino acid fraction was analyzed by the amino acid analyzer (System I), this compound overlapped with cystine. However, by group separation, they were able to be separated (cystine, in the F-I fraction; saccharopine, in the F-II fraction).

^g The decomposition of agaritine during the extraction was estimated to be $10.7 \pm 2.6\%$ (mean $\pm$ S.D., $n=3$). This value was used for the correction.

^h Tryptophan was determined as described in the text.

Table 3. Distribution of GHA and tyrosine in the fruiting bodies of *A. bisporus*. GHA and tyrosine were determined by the amino acid analyzer as described in the text and Fig. 2. The extracts with 75% ethanol containing 1% ascorbic acid and 0.02 N HCl were used for the analyses.

Part	Average weight	GHA	Tyrosine
		Amount ($\mu\text{mol/g}$ fresh weight)	
Whole fruiting body ^a	5.65 ± 0.81^b	4.24 ± 1.15	0.27 ± 0.07
Cap ^c	2.41 ± 0.29	1.44 ± 0.57	0.32 ± 0.09
Stipe	1.96 ± 0.47	4.17 ± 0.92	0.20 ± 0.09
Gill	1.28 ± 0.15	9.61 ± 1.75	0.28 ± 0.01

^a Stage 2 (6). ^b The fruiting bodies were analyzed individually and the values were shown as the means $\pm$ S.D. ($n=3$). ^c The gill tissue was completely eliminated.

and Weaver (19) have speculated on the role of an enzymatically oxidized product of GHA (20) in the induction of dormancy of the spore, this speculation subsequently being questioned by Rast *et al.* (21). Table 3 shows the distribution of GHA and tyrosine in the fruiting bodies of the mushroom. The occurrence of GHA in the stipe and the cap was observed by Biston *et al.* (22) by paper chromatography, and Rast *et al.* (21) also demonstrated its distribution in the fruiting bodies by spectrophotometric analyses after separation on a Sephadex column. The present authors proved active incorporation of radioactive shikimic acid into this monophenol in the stipe and the cap (1). But this is the first systematic analysis by the amino acid analyzer in which tyrosine has been simultaneously determined. As shown in Table 3, the occurrence of GHA was not restricted to the gill tissue, but was found to be distributed in every part of the fruiting bodies at higher concentrations than tyrosine which had been assumed to be a major browning principle in the mushroom (23). The possible participation of this monophenol in the enzymatic browning reaction of the whole fruiting bodies should be considered.

Another interesting finding is the occurrence of various γ -glutamyl conjugates. The unusual aromatic compounds exist in this conjugate form and the present authors found the occurrence of γ -L-glutamylglycine (3) and *N*-(γ -L-glutamyl)-ethanolamine (4).

Furthermore, the predominant occurrence of the compounds metabolically related to glutamic acid, proline, arginine, ornithine, glutamine and γ -aminobutyric acid, was noteworthy. In addition, the existence of saccharopine and α -amino adipic acid, the lysine metabolites, in considerable amounts was also interesting.

There are a few published reports (16, 17, 24) on the free amino acids in *A. bisporus*, but their attention was mainly focused on the protein amino acids in the free form. It should be kept in mind that the amino acid fraction of the mushroom is

complex and that this complexity sometimes leads to misinterpretation, especially when an amino acid analyzer equipped with System II is used. Even with System I which is routinely used for the analyses of physiological fluids, the separation of the free amino acids and their derivatives was incomplete as shown above, so that group separation as described under MATERIALS AND METHODS was essential for precise identification and determination. Even by such group separation, γ -L-glutamylglycine and *N*-(γ -L-glutamyl)ethanolamine could not be separated from aspartic acid and serine, respectively. Their identification and determination were carried out after separation by preparative paper chromatography, as described in the footnote to Table 1.

Altamura *et al.* (25) have reported the ninhydrin-positive compounds in *A. campestris*, a closely related species of *A. bisporus*. However, the unusual aromatic compounds and the compounds identified by the present authors (3,4) were left undetermined, and a more precise identification of several compounds described in their paper seems to be necessary.

The results presented in this report give the first critical information on the free amino acids and their derivatives in the mushroom and provide useful knowledge for the fields of food chemistry and biochemistry of mushrooms.

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