Analysis of Free Amino Acid Pools in Fungal Mycelia

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Free amino acid pools derived from three different types of fungal mycelia have been analyzed by the method of Heathcote and Haworth by using thin-layer chromatography. The preliminary extraction was carried out with boiling water and interfering proteins; peptides and salts were first removed by means of an ion-retardation resin. As far as determined, the results obtained represent the first quantitative analysis of fungal amino acid pools.

Although an extensive range of microorganisms, algae, and higher plants have been examined for their free amino acid pools, little is known at the present time concerning the composition of the pools within fungal mycelia.

Thornton and McEvoy (4) prepared free amino acid pools of several fungal mycelia by different methods, the most successful of which consisted of extraction into boiling water. They were unable, however, to obtain good chromatographic separation of the amino acids in these extracts because of the interference of proteins, peptides, and salts. Desalting with acid butanone improved the chromatographic picture, but the interference from peptides remained. Consequently, these workers were unable to determine the amino acid composition quantitatively.

Recently, we developed a method for the preparation of peptide- and salt-free extracts of amino acids from biological materials by using an ion-retardation resin (2). The extracts may then be examined and the amino acids quantitatively determined by the thin-layer chromatographic technique of Heathcote and Haworth (3). In the present paper, the method has been applied to the mycelia of Heliscus submersus, Tetracladium setigerum, and Aspergillus flavus.

**Chromatographic solvents.** The solvent for development in the first dimension consisted of propan-2-ol, butanone, and 1 N hydrochloric acid [60:15:25 (v/v/v)]; that for development in the second dimension was 2-methyl-2-butanol-butane-propa-none-methanol-water-ammonia (0.88) [50:20:10:5:15:5 (v/v/v)].

**Chromogenic reagents.** Ninhydrin-cadmium acetate reagent was used to detect the a-amino acids and isatin-cadmium acetate reagent, the imino acids. The reagents were prepared as previously described by Heathcote and Haworth (3).

**Chromatographic columns.** The columns for desalting were prepared from glass tubing (1.5 by 30 cm). Indentations were made about 1 cm from one end to support a glass wool plug which in turn supported the resin. The flow rate was controlled by means of a Hoffmann screw clip attached to a length of rubber tubing at the base of the column.

**Organisms used.** H. submersus, T. setigerum, and A. flavus were maintained on 2% malt-agar slopes until use.

**Growth media.** The growth medium used for the cultivation of mycelia from H. submersus and T. setigerum consisted of glucose (6.0 g), ferric chloride (0.02 g), potassium dihydrogen phosphate (1.0 g), magnesium sulfate (0.2 g), yeast extract (0.5 g), and distilled water (1 liter).

The growth medium used for the cultivation of mycelia from A. flavus consisted of yeast extract (30 g), glucose (100 g), and distilled water (1 liter).

**Cellulose.** MN300 cellulose was obtained from Macherey Nagel and Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge.

**Densitometer.** The instrument used was a “Chromoscan” double-beam densitometer (Joyce Loebl & Co. Ltd., Gateshead-on-Tyne, Great Britain) with thin-layer attachment, and the reflectance mode of operation was used throughout the work.
Inoculation and incubation of the organisms. The organisms *H. submersus* and *T. setigerum* were grown on slopes of malt-agar and *A. flavus* on slopes of Czapek-Dox at 23 C for 14 days. After this time, a quantity of mycelium was removed from each culture, homogenized in sterile water (5 ml) with a Waring Blender (10-ml attachment bottle), and then used as the inoculum. Two drops of each individual inoculum were then introduced aseptically into 24 Roux bottles containing sterile medium (50 ml), and surface growth was carried out at 23 C for 13 days in a stationary horizontal position. It had been previously established that after 13 days all the organisms were in the logarithmic phase of growth. Nevertheless, tests were carried out to confirm the presence of excess glucose (Benedict’s test) and unused peptone (biuret test) in the media to ensure that growth was active and not nitrogen-limited. After this time, the separate mycelia were harvested, combined, and then washed three times with 100 ml of sterile water. They were dried rapidly by suction and weighed. The total amounts of dried mycelia were 1.35 g/liter for *H. submersus*, 1.14 g/liter for *T. setigerum*, and 2.38 g/liter for *A. flavus*.

Extraction of the free amino acid pools from mycelia. The dried mycelia was divided into nine portions. Each portion was weighed and made to 5 ml with distilled water, homogenized by means of a Waring Blender for 5 min, and then made to 10 ml with distilled water. Three of these portions were extracted three times by boiling under reflux for 15 min, after which they were cooled and centrifuged and the mycelia residues were set aside. Each supernatant fluid was concentrated to a volume of 2 ml under reduced pressure at 40 C, and this procedure was repeated for the remaining six portions, three being refluxed for 30 min and three for 45 min.

The water extracts were then passed individually down a column of Bio-Rad AG11A8 as described below to remove interfering protein, peptide, and salt before examination of their amino acid content on thin layers of cellulose.

The mycelia residues remaining after water extraction were taken up in 5 ml of butan-2-one containing 6 N HCl [5% (v/v)], homogenized for 15 min, and then centrifuged. In each case, the supernatant fluid was preserved, and the extraction was repeated twice. The three supernatant fluids were pooled and evaporated to dryness in a current of air. The solid remaining was dissolved in distilled water (2 ml), and a fractional amount (5 ml) was put directly on thin layers of cellulose.

Desalting procedure. A column (1.5 by 12.5 cm) of Bio-Rad AG11A8 (30 to 100 mesh) ion-retardation resin was prepared from an aqueous suspension and washed with water (100 ml) to remove impurities. The mycelial extract (2 ml) was applied to the column followed by a solution of sodium chloride [1 ml of 1% (w/v)], and the amino acids were eluted from the resin with distilled water (19 ml) at a flow rate of 2 ml/min. The first 13 ml of eluate was discarded, and the next 6 ml which contained the amino acids was collected and analyzed by thin-layer chromatography. The column was regenerated by washing with distilled water (100 ml) at a fast flow rate (10 ml/min).

Thin-layer chromatographic procedure. The chromatograms were developed in the first dimension until the solvent front had reached 13 cm from the origin (2.5 hr), after which time they were dried in a current of cold air for 15 min and heated at 60 C for a further 15 min. The chromatograms were then cooled and developed in the second dimension until the solvent front had reached 13 cm from the origin (2.5 hr). The layers were next heated to dryness at 60 C for 15 min, allowed to cool, and sprayed with chromogenic reagent until they appeared translucent. The colored amino acid complexes were fully developed by heating at 60 C for 15 min and al-

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**Fig. 1. Separation of untreated free amino acid pool from *Heliscus submersus.***

**Fig. 2. Separation of untreated free amino acid pool from *Tetracladium setigerum.***
lowing the chromatograms to stand in the dark at room temperature for 4 hr. The imino acid complexes were developed by heating at 90°C for 10 min and allowing to stand for 1 hr.

The spots were scanned by reflectance densitometry at 490 nm for the amino acids and 620 nm for the imino acids. The area under the densitometric curve was measured by using the following relation-

Second Dimension

**FIG. 3.** Separation of untreated free amino acid pool from Aspergillus flavus.

**FIG. 4.** Separation of free amino acid pool in Heliscus submersus after Bio-Rad treatment. The amino acids are numbered as follows: 1 = alanine; 2 = arginine; 3 = aspartic acid; 4 = glutamic acid; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 9 = isoleucine; 10 = leucine; 11 = histidine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 16 = proline; 18 = cysteine; 20 = cysteic acid; 25 = glutamine; 29 = methionine.

**FIG. 5.** Separation of free amino acid pool in Tetradium setigerum after Bio-Rad treatment. The amino acids are numbered as in the legend to Fig. 4.

**FIG. 6.** Separation of free amino acid pool in Aspergillus flavus after Bio-Rad treatment. The amino acids are numbered as follows: 1 = alanine; 2 = arginine; 3 = aspartic acid; 4 = glutamic acid; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 9 = isoleucine; 10 = leucine; 11 = histidine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 15 = tryptophan; 16 = proline; 18 = cysteine; 20 = cysteic acid; 25 = glutamine; 29 = methionine; 34 = arginino-succinic acid.
ship: area = peak height \times width at half height. The value obtained was then related to the amount of amino acid present by reading from prepared standard graphs (3).

RESULTS AND DISCUSSION

It has been known for some time that the extraction of fungal mycelia with boiling water is the most efficient of the available procedures. The chromatographic analysis of this type of extract has proved very difficult for the determination of free amino acid pools because of interference by salts and peptides. The thin-layer chromatographic patterns obtained with boiling water extracts of *H. submersus*, *T. setigerum*, and *A. flavus* are shown in Fig. 1–3, and it is clear that the picture is greatly distorted. Desalting of the extracts by no means clarifies the chromatographic pattern since peptide still interferes with the resolution of several amino acids and they cannot all be estimated quantitatively. However, complete separation of all of the naturally occurring amino acids and some other ninhydrin-positive compounds is possible when the boiling water extracts are treated with Bio-Rad AG11A8 before thin-layer chromatography. The improved resolution is shown clearly in Fig. 4–6, and no difficulty was found in determining the individual amino acids quantitatively after this treatment.

The results of extraction with boiling water for various periods of time are given in Table 1. For most amino acids, the aqueous extraction appears to be complete after 15 min, but further extraction of the mycelial residues with acid butanone results in small additional amounts of tryptophan. The full results for the amino acid pools of the three organisms are presented in Table 2.

The pool in *H. submersus* (656.9 &mu;g/250 mg) was found to be about half that of *T. setigerum* (1,187 &mu;g/250 mg) and considerably less than that of *A. flavus* (1,562.2 &mu;g/250 mg).

An interesting feature of the metabolism of *H. submersus* is the unusually large percentage (53.4%) of basic amino acid which is present in the pool. Arginine alone accounts for 34%. In the case of *T. setigerum*, alanine, glycine, and histidine account for 46% of the total amino acids. A more balanced distribution of amino acids was found in the pool from *A. flavus*, and a larger content of tryptophan (4.5%) was present than occurred in the other two organisms. These two facts, together with the unexpected finding of arginino-succinic acid, may be related to a more advanced metabolism in this organism.

### Table 1. Free amino acid pool extracted from Heliscus submersus with water

| Amino acid   | Conc (&mu;g/250 mg of dry mycelium) after refluxing period of |
|--------------|---------------------------------------------------------------|
|              | 15 min 30 min 45 min                                          |
|              | 1 2 3 1 2 3 1 2 3                                           |
| Alanine      | 64.0 0 0 65.1 0 0                                           |
| Arginine     | 222.0 0.3 0.2 217.4 0.7 0.2 224.0 1.0 0.1                   |
| Aspartic acid| 13.0 0 0 12.8 0 0                                           |
| Cysteic acid | 25.2 0 0 25.9 0 0                                           |
| Cysteine     | 14.0 0 0 14.1 0 0                                           |
| Glutamic acid| 50.0 0 0.1 49.2 0.2 0                                        |
| Glutamine    | 8.7 0 0 8.7 0 0                                            |
| Glycine      | 25.0 0 0 25.4 0 0                                           |
| Histidine    | 58.1 0 0 60.0 0.2 0.1                                        |
| Isoleucine   | 2.5 0 0 2.5 0 0                                             |
| Leucine      | 5.0 0.1 0 4.9 0 0                                           |
| Lysine       | 74.0 0.6 0 72.1 0.8 0.4                                       |
| Methionine   | 5.1 0 0 5.1 0.1 0.2                                         |
| Phenylalanine| 17.2 0.1 0 17.3 0 0.2                                        |
| Proline      | 12.9 0.1 0 12.9 0 0                                          |
| Serine       | 14.0 0 0.1 14.5 0 0                                          |
| Threonine    | 26.2 0.2 0 27.0 0 0                                          |
| Tryptophan   | 0 0 0 0 0.2 0.1 0                                           |
| Tyrosine     | 13.0 0 0 13.2 0 0                                           |
| Valine       | 7.0 0 0 7.0 0 0                                             |
| Total        | 656.9 1.4 0.6 655.1 2.0 1.2 664.8 2.2 0.4                   |

* Indicates number of extraction.
### Table 2. Free amino acid pools extracted from fungal mycelia successively with water and with acidified butanone

| Amino acid        | Heliscus submersus | Tetracadium setigerum | Aspergillus flavus |
|-------------------|--------------------|-----------------------|--------------------|
|                   | Boiling water      | Acidified butanone    | Boiling water      | Acidified butanone |
| Alanine           | 64.0               | 2.1                   | 230.0              | 4.8                 |
| Arginine          | 222.0              | 4.8                   | 36.7               | 0.2                 |
| Arginino-succinic acid | 0                  | 0                     | 60.2               | 0                   |
| Aspartic acid     | 13.0               | 0                     | 20.2               | 0                   |
| Cysteic acid      | 25.2               | 0.7                   | 15.8               | 0                   |
| Glutamic acid     | 50.0               | 0                     | 40.9               | 0                   |
| Glutamine         | 8.7                | 0                     | 4.8                | 0                   |
| Glycine           | 25.0               | 1.2                   | 141.0              | 3.3                 |
| Histidine         | 58.1               | 3.2                   | 173.0              | 4.2                 |
| Isoleucine        | 2.5                | 0                     | 38.9               | 0                   |
| Leucine           | 5.0                | 0                     | 71.0               | 0                   |
| Lysine            | 74.0               | 8.1                   | 76.5               | 7.9                 |
| Methionine        | 5.1                | 0                     | 35.2               | 6.1                 |
| Phenylalanine     | 17.2               | 0                     | 23.8               | 0                   |
| Proline           | 12.9               | 1.0                   | 62.7               | 1.9                 |
| Serine            | 14.0               | 0                     | 32.7               | 0.5                 |
| Threonine         | 26.2               | 0                     | 31.0               | 0                   |
| Tryptophan        | 0                  | 3.7                   | 0                  | 10.1                |
| Tyrosine          | 13.0               | 0                     | 3.4                | 0                   |
| Valine            | 7.0                | 0                     | 89.2               | 0                   |
| Total             | 656.9              | 24.8                  | 1,187.0            | 39.0                |

**LITERATURE CITED**

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