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A Rapid Method to Quantify Free Tryptophan in Mycelia of Ectomycorrhizal Fungi*

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Abstract

A rapid procedure for the extraction and high performance liquid chromatography quantitative estimation of free tryptophan in crude extracts of the ectomycorrhizal fungus *Hebeloma cylindrosporum* is described.

Keywords: free tryptophan, ectomycorrhizal fungus, high performance liquid chromatography, *Hebeloma cylindrosporum*.

Introduction

Tryptophan (Trp) is an amino acid which plays an important role in plant-microorganism relationships. It is released in root exudates of higher plants and stimulates the growth of numerous bacteria and fungi. This amino acid can also be used by symbiotic microorganisms as a precursor of indole-3-acetic acid (IAA) which is involved in different associations and particularly in ectomycorrhizal symbiosis. Therefore free Trp quantification in root exudates or plant material is of particular interest. Different methods for the analysis of Trp in plant extracts or root exudates have been described. They generally involve a complex purification of the extracts by many steps of ion exchange chromatography (Atsumi, 1980; Rybicka, 1980; Haissig, 1982; El Bahr et al., 1984) or precolumn derivatization followed by different quantification methods. These are either colourimetric (Spies and Chambers, 1948; Messineo and Musarra, 1972) or fluorometric (Denckla and Dewey, 1967 modified by El Bahr et al., 1984). Trp can also be quantified by the tryptophanase method (Delmer and Mills, 1968) or with automatic amino acid

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analyser (Rybicka, 1980). These methods are time consuming and are suitable only for a limited number of samples. A very sensitive and rapid method for Trp separation and quantification by high performance liquid chromatography (HPLC) has been developed by Tarr and Arditti (1981). However, analytical conditions were optimized to separate Trp and its metabolites by using standard solutions and not biological extracts. Hence, the aim of the present work was to develop a method avoiding the use of derivatives to extract and quantify free Trp in mycelia of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. This material has been used because the free Trp level in mycelia is thought to be the main limiting factor for fungal IAA production by ectomycorrhizal fungi (Gay et al., 1989). Therefore, free Trp quantification in fungal hyphae is a prerequisite for the study of the metabolism of endogenously and/or exogenously supplied Trp as IAA precursor and subsequently for the study of the role of fungal IAA in the ectomycorrhizal symbiosis.

Materials and Methods

Fungal pure culture

H. cylindrosporum is an ectomycorrhizal fungus, the characteristics of which have been described by Debaud et al. (1986). The strain used in this work is a monokaryotic mycelium (h1) isolated after laboratory fruiting of the HC1 wild dikaryon (Debaud and Gay, 1987). It was cultivated according to Gay and Debaud (1987).

Extraction procedure

Two extraction procedures have been compared: (1) the conventional procedure for extracting amino acids: 50 mg of fresh weight (FW) of tissue were extracted once with 1 ml 80% ethanol and twice with 1 ml 70% ethanol at ambient temperature. The extracts were centrifuged (15 000 g for 20 min at 4°C), pooled and concentrated to dryness under vacuo at 20 ± 1°C. (2) the method proposed by Bieleski and Turner (1966) as modified by Haissig (1982) to extract free amino acids from higher plant tissues: fungal tissues (50 mg FW) were homogeneized at 4°C and extracted twice with 1 ml methanol/chloroform/water 12/5/3 by volume (MCW) and extracts were pooled for each sample. A methanol water fraction (sugar + amino acids) and a residue were obtained for each sample by adding 1.8 ml water which caused separation of the chloroform phase. A centrifugation (15 000 g for 20 min at 4°C) allowed removing chloroform droplets from the methanol water. The methanol fraction was then evaporated to dryness under vacuo at 20 ± 1°C.

Other different methods frequently used to extract total amino acids involve boiling

water or methanol (Bent and Morton, 1964; Vanables, 1988) and cannot be used to extract Trp because of its instability toward heat.

Thin layer chromatography

A preliminary Trp detection in extracts was performed by thin layer chromatography (TLC) using aluminium plates coated with silica gel G 60 (Merck). The solvent system was either butanol/acetic acid/water (6/1.5/2.5 by volume) or methanol/chloroform/water (3/2/0.5 by volume). Trp was detected on the plates by reference to standard Trp by spraying them either with ninhydrine or paradimethylcinnamaldehyde.

High performance liquid chromatography

Reverse phase HPLC was performed using a Waters Associates instrument. The column (4 mm × 30 cm) was Micro Bondapak C 18 (10 μm). Different solvent systems were used and analytical conditions were adapted to each analysis. Trp was detected and quantified by its absorbance at 280 nm using standard Trp as reference.

Results

Extraction

No Trp could be detected by TLC in ethanol extracts. In contrast, MCW extracts contained Trp which was easily detectable by TLC. This amino acid was quantified by HPLC in MCW extracts before and after pigment removal in the chloroform layer (Table 1). This comparison allowed demonstrating that removal of the chloroform fraction was not necessary in the case of fungal extracts which contain a limited amount of pigments. Hence, the selected extraction procedure was as follows: free Trp was extracted from mycelia (1 g FW) by homogeneizing twice with 20 ml methanol/chloroform/water (12/5/3 by volume) at 4°C followed each time by centrifugation (15 000 g for 20 min at 4°C). The extract was subsequently evaporated to dryness under vacuo at 20 ± 1°C and resuspended with 500 μl of distilled water just before HPLC.

Table 1. Comparative study of different procedures to extract tryptophan from *Hebeloma cylindrosporum* mycelia. MCW: methanol/chloroform/water (12/5/3 by volume), TLC: thin layer chromatography, HPLC: high performance liquid chromatography.

		Ethanol extract	MCW extract
TLC detection of tryptophan		Not detected	+
HPLC tryptophan quantification (n mol/g fresh weight)	Crude extract	—	0.24
	After removal of the chloroform fraction	—	0.21

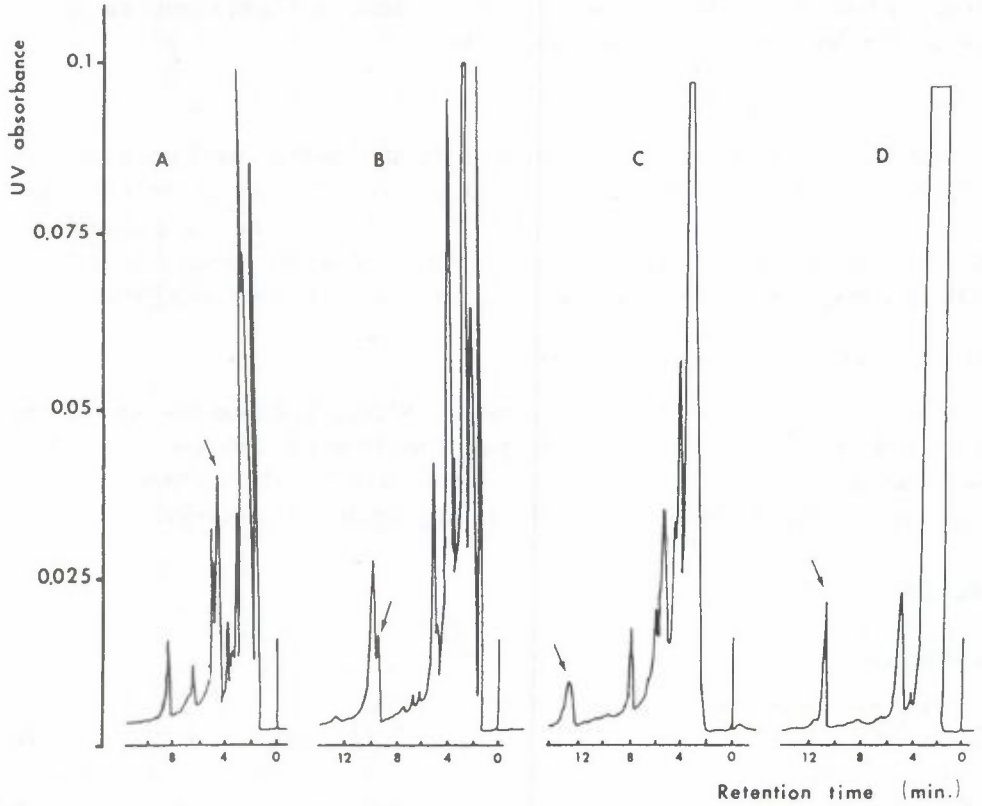

Figure 1. Elution pattern from the HPLC column during analysis of free tryptophan extracted from *Hebeloma cylindrosporum* mycelia. Column: Micro Bondapak C 18, sample injection: 10 μl, flow rate: 1 ml min^{-1}, UV detection at 280 nm, detector sensitivity: 0·1 absorbance unit full scale, solvents: A: acetonitrile/water (15/85 by volume), B: acetonitrile/water (5/95 by volume) C: acetonitrile/acetic acid/water (15/1/84 by volume) D: acetonitrile/25% ammonium hydroxide/water (2/0.02/97.98 by volume) Arrows indicate tryptophan peak.

Tryptophan quantification by HPLC

The solvent system used by Tarr and Arditti (1981) to separate Trp from its metabolites (10 mM sodium acetate, pH 4.8) did not allow the separation and subsequently the quantification of Trp extracted from *H. cylindrosporum* mycelia. Therefore, several solvent systems were tested. Acetonitrile/water at different ratios was used as a basis for these solvents. It was or was not supplemented with either acetic acid or ammonium hydroxide to obtain different pH conditions. Acetonitrile/water solvents did not allow a good separation of Trp (Fig. 1A and B). The addition of acetic acid allowed a better separation (Fig. 1C). The best separation of the Trp peak under acidic conditions was obtained with acetonitrile/acetic acid/water (15/1/84 by volume).

Solvent systems in which acetic acid was replaced by ammonium hydroxide were also tested (Fig. 1D). The eluant acetonitrile/25% ammonium hydroxide/water (2/0.02/97.98 by volume) allowed the best separation and quantification of Trp.

Discussion

As can be seen from the results reported, the procedure of Trp analysis described in this paper is simple and much more rapid than most of the methods previously described. Each of the steps is based on existing methods, but the combination of steps chosen have resulted in a procedure with many advantages. As previously demonstrated by Bieleski and Turner (1966), methanol/chloroform/water is a much more efficient amino acid extractant than ethanol; it has already been used by Botton and Es Sagouri (1985) to extract free amino acids of the Ascomycete *Sphaerostilbe repens*. The two-phase extraction medium which was necessary to remove pigments and lipids in higher plant tissues was not necessary in the case of fungal material. HPLC allowed cutting out extract purification by ion exchange chromatography. Moreover, this method is sensitive since it allowed the detection of 250 pmole of Trp in fungal extracts whereas colourimetric methods generally detected with difficulty Trp amounts lower than 5 nmole. The only report of a more sensitive method avoiding the use of derivatives was by Tarr and Arditti (1981) which could detect by HPLC up to 5 pmole of standard Trp. Procedures involving derivatives (e.g. orthophthaldialdehyde, phenylthiohydantoin or phenylthiocarbamyl) associated with fluorescence detection also allowed the detection of ca. 5 pmole of Trp. The use of fluoroenylmethyloxycarbonylchloride with a fluorometer offers an approximately tenfold increase in detection sensitivity over OPA fluorescence procedures (0.5 pmole). Thus, although procedures involving derivatization are more sensitive, the main advantage of the method proposed in this work is to be one of the most sensitive procedures avoiding the use of derivatives. It will be used to study the Trp metabolism and the physiology of IAA biosynthesis by ectomycorrhizal fungi.

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