A Comparison of Methods for the Extraction of Amino Acids from Fungal Mycelium

The free amino acid pools of an extensive range of microorganisms, algae and higher plants have been analysed but the reports show that a very wide range of solvents have been used for their extraction. For example, 90% ethanol (CHILD and FOTHERGILL[1]), 70% ethanol (THORNTON and FOX[2], TOUZE-SOULET[3]), 50% ethanol (MURRAY and ZSCHEILE[4]), 1.5 *N* perchloric acid (KANAZAWA[5]), 50% glycerol (SHELLARD and JOLLIFFE[6]) and an aqueous solution of trichloracetic acid (BALDY, PIQUEMAL and LACHÉ[7]).

Apart from work by BENT and MORTON[8] using *Penicillium griseofulvum* and HANCOCK[9] using *Staphylococcus aureus*, little attention has been paid to assessment of efficiencies of amino acid pool extraction techniques. This work was stimulated when the ninhydrin positive substances extracted from the mycelium of 3 aquatic Hyphomycetes using 5 different methods were compared and found to be different (see Table I). Subsequent thin-layer chromatography and comparison using a Joyce-Loebl 'Chromoscan' densitometer showed that different solvents extracted different amino acids and quantities of amino acids from the mycelium of *Heliscus submersus*.

Materials and methods. The organisms used were *Heliscus submersus*, *Varicosporium elodeae* and *Clavariopsis aquatica*, maintained on 2% malt agar slopes. The medium used for cultivation of mycelium consisted of 6.0 g glucose, 0.02 g $FeCl_3$(tech), 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract (oxoid), 1 l distilled water. Inoculation and incubation was as described by THORNTON and FOX[2].

After 6 days incubation the mycelium was filtered off, washed in distilled water, suction dried and subjected to the extraction techniques described. 0.5 g samples of mycelium were homogenized for 1 min in 10 ml solvent then poured into 20 ml hot or boiling solvent in 100 ml flasks on a boiling water bath for 15 min. Where necessary reflux condensation was used. Amino acid extraction solvents used were (1) boiling water, (2) boiling 70% ethanol, (3) boiling methanol, chloroform, water (MCW), 12:5:3, (4) 5% w/v trichloroacetic acid (TCA) at 100 °C, (5) boiling water on oven dried mycelium (see THORNTON and FOX[2]).

The extracts were cooled, centrifuged and the supernatant evaporated to dryness at 60 °C using a rotary evaporator, then resuspended in 2 ml distilled water and used for chromatography.

Chromatography was carried out on cellulose thin layer as described by JONES and HEATHCOATE[10] with 50 µl loadings per plate. The relative concentrations of the amino acids so detected were compared quantitatively by the 'Chromoscan' densitometer using a 620 filter and a 1.0 dioptre density filter at maximum sensitivity. The comparative values given are those evaluated automatically by the 'Chromoscan' automatic integrator and are directly proportional to the concentration of the amino acids detected on the chromatograms.

Results and discussion. In preliminary experiments total ninhydrin positive substances were assessed by loading 10 µl samples of the 5 extracts on to cellulose thin layer and sprayed and developed as described by JONES and HEATHCOATE[10] and their comparative densities measured (Table I). The results show that method 4 (TCA) extracted considerably more ninhydrin-positive material than all other methods and method 1 (boiling water) extracted consistently less than other methods. Method 4 extracts were found to give poor and confused chromatograms and was later discarded as inferior to other methods described.

The comparative amino acid concentrations on the various chromatograms as assessed by the densitometer are given on Table II. Alanine, arginine, aspartic acid, and glutamic acid are seen to be extracted in greatly varying quantities by different solvents from standard weights of

Table I. Comparative assessment of total ninhydrin positive substances extracted from fungal mycelium by 5 different methods

Organism	Chromoscan reading				
	Technique number				
	1	2	3	4[a]	5
H. submersus	122[b]	134	131	819	160
V. elodeae	45	100	60	920	82
C. aquatica	23	97	240	1104	120

[a] High readings probably due to peptide formation. [b] Figures given are the readings of the automatic integrator of the densitometer and are directly proportional to the concentrations of the ninhydrin positive substances extracted.

Table II. Relative concentrations of various amino acids extracted by different methods from *H. submersus*

Amino acid	Chromoscan reading			
	Technique number			
	1	2	3	5
Alanine	1050	1000	110	560
Arginine HCl	50	+	200	+
Aspartic acid	480	180	130	590
Glutamic acid	250	200	60	550
Glutamine	+	—	—	—
Histidine HCl	—	—	280	—
Leucine	+	+	+	+
Lysine	150	—	+	+
Methionine	+	—	+	+
Ornithene	+	—	+	—
Phenyalanine	+	—	+	+
Isoleucine	+	+	+	+
Serine	+	250	+	+
Threonine	+	100	80	+
Tyrosine	+	—	—	—
Valine	+	—	—	+
Taurine	+	200	250	+
Cystine	+	100	450	500
Total	17	10	15	13

—, not detected; +, detected in quantities below accurate measurement by the densitometer. Glycine, cysteine HCl, cysteic acid, proline, hydroxyproline and tryptophan were not detected in any extracts.

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mycelium of *Heliscus submersus*. Boiling water appears to be the most efficient in extracting 17 amino acids whilst only 10 amino acids could be detected in the 70% ethanol extract.

Subsequent to this work, THORP[11] showed that methyl ethyl ketone/6 *N* HCl (MEK/HCl, 10:1) not only proved to be an efficient desalting medium in which to resuspend impure amino acid residues, but proved to be a very satisfactory free amino acid extractant. This was done by homogenizing freeze-dried mycelium in cold MEK/HCl for 15 min. This gave a salt-free solution of 16 amino acids from mycelium of *Tetracladium setigerum* which gave very clear chromatograms by the method described.

MEK/HCl was found unreliable for the extraction of tyrosine and leucine, which are readily extracted by boiling water, complementary to this, boiling water was found poor for the extraction of histidine, arginine and taurine, all of which are extracted readily under the conditions described by MEK/HCl.

The results indicate that no single method is absolutely satisfactory for the extraction of free amino acid pools from fungal mycelium but a combination of extracts obtained by boiling water, and MEK/HCl should extract the widest possible range of free amino acids contained in the mycelium used.

Résumé. En comparant plusieurs techniques pour extraire l'amino-acide libre de la mycose fongique on a observé de grandes différences entre elles. L'eau bouillante et la méthyléthylcétone HCl se sont montrés les solvents les plus efficaces et donnant chromatogrammes les plus clairs.

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The Effect of Glucose, Glucose Monophosphates and Inorganic Phosphate on the Tryptic Digestion of Phosphorylase b

Our earlier work[1] showed that both allosteric activator AMP and inhibitor ATP protect phosphorylase b (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) against tryptic digestion. As is known, phosphorylase b is also inhibited allosterically by glucose and Glc-6-P. In the present study we have examined the effect of these inhibitors on the tryptic digestibility of phosphorylase b. In addition, the effect of Glc-1-P and inorganic phosphate (P_i), the substrates of the enzyme, were also studied.

Materials and methods. 4-times recrystallized rabbit-muscle phosphorylase b was prepared according to FISCHER and KREBS[2]. It was further purified by passing it through a column of Sephadex G-100 immediately before use. The gel column was equilibrated with 0.05 *M* *Tris*-HCl buffer at pH 7.2. Tryptic digestion was carried out at 37 °C, buffered in 0.05 *M* *Tris*-HCl at pH 7.2. The final concentration of the constituents in the incubation mixture (total volume 3.5 ml) were: mercaptoethanol 0.01 *M*; trypsin 3.6×10^{-7} *M*; and phosphorylase b 3.1×10^{-6} *M* or casein 1.43 mg/ml. Various concentrations of glucose, Glc-6-P, Glc-1-P and P_i are indicated in the figures. The reaction was stopped after 20 min incubation by adding 1.5 ml of 10% trichloroacetic acid. After centrifugation the acid soluble protein residues were determined spectrophotometrically at 280 nm. In the case of the fluorimetric investigations, the digestion was carried out in the constant temperature compartment of an Opton spectrofluorometer ZFM 4C. At various incubation times the emission and excitation spectra of the samples were estimated.

Results and Discussion. Various concentrations of glucose and Glc-6-P yielded a significant inhibition of tryptic digestion of phosphorylase b (Figure 1). As Figure 1 shows, Glc-6-P had a greater inhibitory effect than glucose. Glc-1-P and P_i were found, however, to have no influence on the tryptic digestion of the enzyme. That the above-mentioned compounds had an effect on phosphorylase b and not on trypsin was verified by the fact that the rate of tryptic hydrolysis of casein remained unaffected by these compounds at the concentrations used.

The tryptic digestion of the enzyme led to a decrease in the fluorescence intensity and to a slight shift of the fluorescence maximum from 340–350 nm (Figure 2A). The excitation spectrum of the protein did not alter during the digestion. The protective effect of Glc-6-P against the tryptic digestion of phosphorylase b can also be detected fluorimetrically (Figure 2B). Similar results were obtained also with glucose.

It is known that after treatment of proteins with 8 *M* urea, i.e. after all the tryptophan residues of the protein

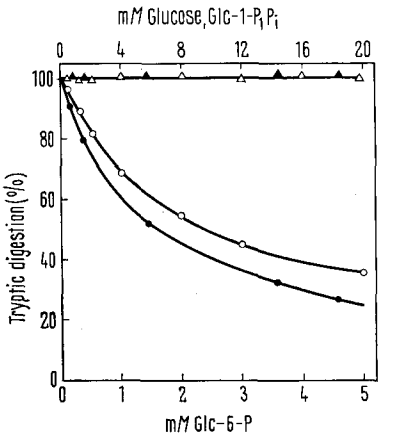

Fig. 1. Effect of glucose (○—○), Glc-6-P (●—●), Glc-1-P (△—△) and inorganic phosphate (P_i) (▲—▲) on the tryptic digestion of phosphorylase b.

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