Effects of Ethionine and Parafluorophenylalanine on the Formation of Peptidase A in *Penicillium janthinelium*

An extracellular proteolytic enzyme, produced by a *Penicillium* mold and capable of activating trypsinogen in the pH range of 3–4, was discovered by KUNITZ in 1938[1]. Similar enzymes were subsequently found in the growth media of *Aspergillus saitoi*[2] and *Aspergillus oryzae*[3]. One such enzyme, peptidase A, was purified from *Penicillium janthinellum* by HOFMANN and SHAW[4] who studied some of its properties. Since the enzyme was seen to be produced after the post-logarithmic phase of growth was reached and when extensive sporulation had set in, it was suggested that the enzyme formation might be related to sporulation[1]. It was, however, shown by THANGAMANI and HOFMANN[5], using the inhibitors acridine Orange and 6-ethyl thiopurine, that sporulation and formation of peptidase A were not directly related, in *P. janthinellum*. During the course of the above work, it was observed that washed suspensions of the mycelium could produce considerable amounts of peptidase A, even in the absence of an external source of nitrogen. This suggested the possibility that the enzyme protein might exist in the mycelium as an inactive zymogen. Zymogens of extracellular proteases have been demonstrated for *Streptococcus haemolyticus*[6] and *Pseudomonas myxogenes*[7]. Evidence for the existence of protein precursor of α-amylase in *Bacillus subtilis* was presented by NOMURA et al.[8,9], who found that washed suspensions of the cells released α-amylase in a nitrogen-free medium and the release was not affected by inhibitors of protein synthesis like ethionine and *p*-fluorophenylalanine, even though protein synthesis as a whole was inhibited. The present communication describes the effects of ethionine and *p*-fluorophenylalanine on the formation of peptidase A and on total protein synthesis in washed suspensions of *P. janthinellum*, in a nitrogen-free medium.

The mold was grown for 2 days, with shaking, in Erlenmeyer flasks containing the complete synthetic medium described earlier[4]. The mycelium was washed and suspended in nitrogen-free medium (complete medium without nitrate). C^{14}-amino acid mixture ('reconstituted protein hydrolysate') was added to the flasks at a level of 1 μC per 40 ml of the medium. DL-ethionine and *p*-fluoro-DL-phenylalanine were added to some flasks as required. The flasks were shaken for another 24 h. Peptidase A in the culture supernatants was assayed as described earlier[4]. Prior to assay, the amino acid analogues in the samples were removed by dialysis against 2 m*M* citrate buffer, pH 3.8. Total protein in the mycelium was obtained as a trichloroacetic acid precipitate by the method of SEIKEVITZ[10]. The washed residue was suspended in hot 2*N* sodium hydroxide and centrifuged. This was repeated and the supernatants pooled. This fraction represented total mycelial protein. Assay of protein was carried out by the method of LOWRY et al.[11]. Measurement of radioactivity in the total protein fraction was carried out in a liquid scintillation counter.

The Figures 1 and 2 show the effects of ethionine and *p*-fluorophenylalanine on the formation of peptidase A, weight of mycelium, total protein and specific radioactivity of total mycelial protein. It is seen that both the amino acid analogues inhibited the formation of peptidase A to a great extent, whereas they had little effect on uptake of C^{14}-amino acids into total cellular protein and on mycelial weight. If the analogues had inhibited total cellular protein synthesis but not peptidase A, it could indicate the presence of a zymogen of the enzyme. However, it is not possible to rule out the existence of a zymogen from the present data since the analogues could have inhibited the activating mechanism. The lack of effect on total cellular protein synthesis by the analogues could be due to their incorporation into cellular proteins. But analyses of mycelial protein hydrolysate (after performic

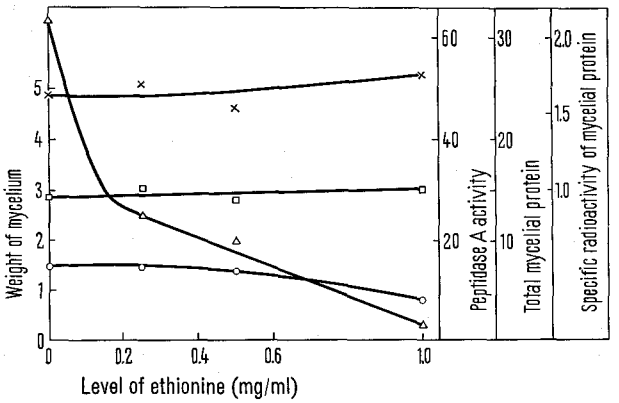

Fig. 1. Effect of ethionine on peptidase A formation and total protein synthesis. ○—○, weight of mycelium (g); △—△, peptidase A activity ($\times 10^4$ U/ml of culture supernatant); ×—×, specific radioactivity of total protein ($\times 10^{-4}$ dpm/mg); □—□, total mycelial protein (mg).

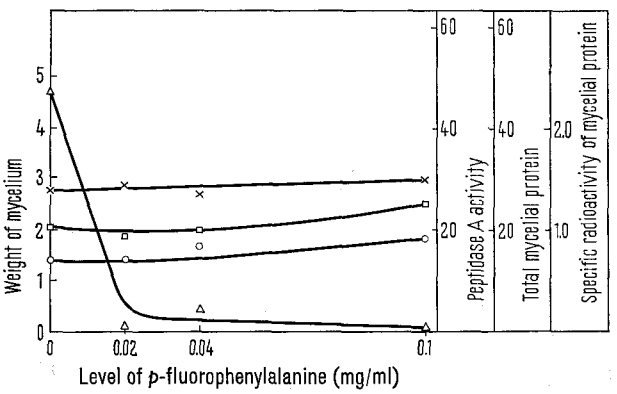

Fig. 2. Effect of *p*-fluorophenylalanine on peptidase A formation and total protein synthesis. ○—○, weight of mycelium (g); △—△, peptidase A activity ($\times 10^4$ U/ml of culture supernatant); ×—×, specific radioactivity of total mycelial protein ($\times 10^{-4}$ dpm/mg); □—□, total mycelial protein (mg).

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acid oxidation in the case of the ethionine experiment, to form ethionine sulphone) on the amino acid analyser showed no trace of the analogues. It is possible that the analogues were metabolysed in other ways[12]. Incorporation of ethionine into peptidase A, making it biologically inactive, is ruled out since the enzyme does not contain sulphur amino acids[4].

Résumé. La DL-éthionine et la *p*-fluorophénylalanine inhibent la formation de la peptidase A extracellulaire chez le *Penicillium janthinellum* sans affecter la synthèse de la protéine cellulaire.

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Identification of Flavin in the Purified Beef Brain Mitochondrial Monoamine Oxidase

Several reports have appeared concerning the physicochemical properties of mitochondrial monoamine oxidase [EC 1.4.3.4. monoamine : oxygen oxidoreductase (deaminating)]. NARA, GOMES and YASUNOBU[1] first reported that beef liver mitochondrial monoamine oxidase is flavo-enzyme. IGAUE, GOMES and YASUNOBU[2] further reported that the flavin in beef liver mitochondrial monoamine oxidase is covalently attached to the enzyme and catalytically involved in the enzyme reaction. ERWIN and HELLERMAN[3] also reported that beef kidney mitochondrial monoamine oxidase contains flavin which is tightly associated with the protein. However, there has been little knowledge about the prosthetic group of brain mitochondrial monoamine oxidase because of the difficulty in its solubilization and purification. TIPTON[4] has reported the extraction of the enzyme from pig brain mitochondria by the combined effects of sonication and freezing-thawing and the extensive purification. TIPTON[5] has also shown that the prosthetic group of pig brain mitochondrial monoamine oxidase is FAD which is dissociable from the protein.

One of the authors showed that the enzyme can be extracted from beef brain mitochondria by sonication in the presence of a detergent[6]. We also found that the enzyme can be extracted by the use of a detergent after the mild heat treatment of beef brain mitochondria[7]. Present communication describes purification of monoamine oxidase from beef brain mitochondria and the identification of the prosthetic group as FAD.

Monoamine oxidase activity was measured by the disappearance of kynuramine[8]. Protein was determined by the method of LOWRY et al.[9]. Beef brain mitochondria were prepared according to the method of BRODY and BAIN[10]. The mitochondrial suspension in 0.01 *M* potassium phosphate buffer, pH 7.4 (10 mg protein/ml) was incubated at 40 °C with continued stirring for 10 min. After cooling the suspension in an ice bath, a 5% (w/v) aqueous solution of Nonion NS-210 (polyoxyethylene-nonyl phenol ether-containing detergent, Nippon Oils & Fats Co. Ltd., Tokyo) was added dropwise to 1% final concentration with gentle stirring in an ice bath. 30 min of equilibration with stirring was allowed. The suspension was centrifuged at 106,000 × g for 1 h and the clear supernatant layer was pipetted out carefully. To the supernatant was added saturated ammonium sulphate solution (pH 7) to 30% saturation. The suspension was then centrifuged at 9,500 × g for 15 min. The precipitate rose to the surface. The solution beneath precipitate was aspirated and discarded. The precipitate was redissolved in 0.01 *M* phosphate buffer (pH 7.4) and extensively dialyzed against the same buffer. The buffer was changed 3 times during the 15 h dialysis. The dialyzed solution was centrifuged, and the supernatant was applied to a DEAE-cellulose column equilibrated with 0.01 *M* phosphate buffer (pH 7.4). Stepwise elution was carried out with 0.01 *M* and 0.1 *M* phosphate buffers and then 0.1 *M* phosphate buffer containing 0.4% Nonion NS-210. The monoamine oxidase activity was eluted with 0.1 *M* phosphate buffer containing the detergent. The active fraction was concentrated by the addition of saturated ammonium sulphate solution (pH 7) to 30% saturation. The precipitate obtained by centrifugation was dissolved in 0.01 *M* phosphate buffer (pH 7.4) and then dialyzed against the same buffer. The dialyzed enzyme solution was subjected to calcium phosphate gel[11]-cellulose powder (gel/powder, 1:5, w/w) column chromatography. The column was equilibrated with 0.01 *M* phosphate buffer (pH 7.4). The elution procedure was the same as in the DEAE-cellulose chromatography. The enzyme was also eluted with 0.1 *M* phosphate buffer containing 0.4% Nonion NS-210 (pH 7.4). The enzyme solution was faintly yellow.

A typical example of the purification is summarized in the Table. Analysis of the purified enzyme preparation by disc electrophoresis showed that it was nearly homogeneous. However, the purification was only 28-fold as shown in the Table. This low value of the increase in the specific activity may be due to inactivation of the enzyme during the purification procedure, especially at the stage of ammonium sulphate fractionation. Freezing of the enzyme preparations after the extraction from beef brain mitochondria caused rapid loss of the enzyme activity.

The absorption spectrum of the purified enzyme exhibited a maximum at 410 nm. The spectrum was nearly identical with that of purified rat liver mitochondrial enzyme reported by YOUDIM and SOURKES[12]. Fluorescence activation spectrum of the native enzyme measured at 530 nm with Aminco-Bowman spectrophotofluorometer

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