Rapid purification and crystallization of *Neurospora crassa* tyrosinase

A. M. Fuentes, M. A. J. Taylor, J. Jenkins and I. Connerton - AFRC, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 2EF, UK

A rapid method for the purification of *Neurospora crassa* tyrosinase (EC 1.14.18.1) has been developed using an FPLC system that yields sufficient pure protein for protein crystals to be grown.

Neurospora crassa wild type (74A) was grown at 30 C in shake culture in full-strength Vogel's medium, 2% sucrose, from a large conidial inoculum according to the procedure by Lerch (1987 Meth. Enzymol. 142:165-169). Tyrosinase was induced by transferring the mycelia into fresh half-strength Vogel's medium, 0.5% sucrose, containing cycloheximide to a final concentration of 1.5 uM (Horowitz et al. 1970 J. Biol. Chem. 245: 2784-2788). The mycelia were harvested by filtration under low vacuum, dried with paper towels, and ground in sand with a pestle and mortar in the presence of 0.1 M Tris-HCl buffer, pH 8.0 and 2 mM sodium benzoate (buffer A). After stirring for one hour, the crude extract was centrifuged and the supernatant collected. The supernatant was then applied to a Q-Sepharose column (2.6 x 28 cm) pre-equilibrated with buffer A. After washing the column with two column volumes of buffer A, tyrosinase activity was eluted with a linear gradient (0-0.75 M) of NaCl over 600 ml in buffer A. A wide peak of protein showing tyrosinase activity was eluted between 0.18 and 0.54 M NaCl. The pooled activities from the tyrosinase peak were desalted on a Sephadex G-15 column (5.0 x 16 cm), pre-equilibrated in 50 mM sodium citrate buffer pH 5,0 containing 2 mM sodium benzoate (buffer B). Active fractions were loaded onto a Mono-S HR5/5 column pre-equilibrated in buffer B. Activity was eluted with a linear gradient (0-0.3 M) of NaCl over 12 ml in buffer B. Protein containing tyrosinase activity eluted as a single peak at 0.2 M NaCl. The peak fractions appear as a single band on SDS-PAGE. Samples of each of the steps during the purification are presented in Figure 1.

The purified protein was concentrated to 15 mg/ml using a Centricon 10 spun at 5,000 x g for 1 h. Protein was crystallized by the hanging drop method (Ducroix and Giege, Methods of Crystallization, pp. 73-98 in Crystallization of Nucleic Acids and Proteins: A Practical Approach. A. Ducroix and R. Giege (eds.), IRL Press, Oxford, UK). The reservoir contained 4-6% w/v polyethylene glycol MW 12,000 in 0.3 M MOPS/ethanolamine buffer, pH 7.2, at 18 C, in the presence of 50 mM thiourea as an inhibitor of tyrosinase activity. After three days tetragonal needles 0.03 x 0.03 x 1.5 mm were formed, as shown in Figure 2. Improving the size of the crystals to enable X-ray structure analysis of tyrosinase is the subject of current work.

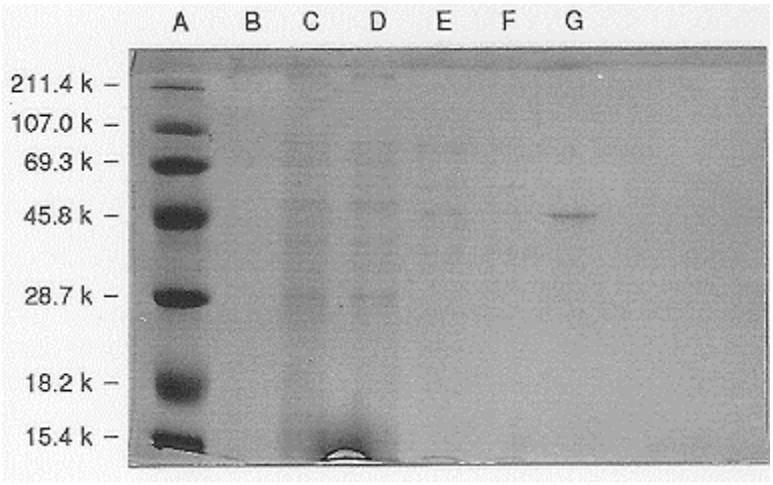

Figure 1. SDS-PAGE gel stained with Coomasie brilliant blue R250 showing the purification of tyrosinase. (A) Prestained protein molecular weight standards, (B) Cell culture supernatant, (C) Crude extract of Neurospora, (D) Post-cell debris supernatant, (E) Tyrosinase from Q-Sepharose, (F) Tyrosinase from G-15, (G) Tyrosinase from Mono S HR5/5.

Figure 2. Long tetragonal needles of Neurospora tyrosinase. The square cross-section of the needle can be clearly seen.