Purification of Thymidine 2′-Hydroxylase from *Neurospora crassa* *

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Thymidine 2′-hydroxylase (EC 1.14.11.3) catalyzes the oxygenation of the deoxyribose moiety of thymidine and deoxyuridine. The enzyme is a 2-oxoglutarate-dependent oxygenase and high enzyme activities have been found in *Neurospora crassa*[1,2] and *Rhodutorula glutinis*.[3] Since low and variable concentrations of deoxypyrimidine nucleosidase have been found in Neurospora, thymidine 2′-hydroxylase seems to be the key enzyme for reutilization of thymidine in this organism.[4-6] After hydrolysis of ribosylthymine, thymine is oxidatively demethylated in another 2-oxoglutarate-dependent oxygenase reaction catalyzed by thymine 7-hydroxylase (EC 1.14.11.6).

These enzymes have been investigated by us in an attempt to improve the understanding of 2-oxoglutarate-dependent oxygenase reactions.[2,7] We have previously used partially purified enzyme preparations, but have now isolated thymidine 2′-hydroxylase from Neurospora in a 5-step procedure which gives a 8 000-fold purification from an intial 140 000 *g* supernatant (Table 1).

The molecular mass of the native enzyme is 47 kdalton as determined by gel filtration.[2] The enzyme consists of one polypeptide chain as judged from SDS polyacrylamide gel electrophoresis, where one major band is found with a molecular mass of 46 kdalton (Figs. 1 and 2).

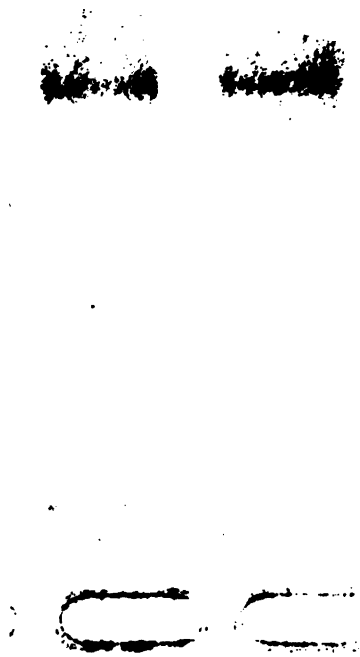

Fig. 1. SDS polyacrylamide gel electrophoresis of thymidine 2′-hydroxylase.

The specific activity of 765 μkat g^{-1} is the highest value reported for any 2-oxoglutarate-dependent oxygenase. The most active 2-oxoglutarate-dependent oxygenase reported hitherto is the bacterial γ-butyrobetaine hydroxylase (EC. 1.14.11.1) with a specific activity of 360 μkat g^{-1}.[9] If, however, the difference in molecular mass between these two enzymes is taken into account and molecular activity is calculated, the figures obtained are very similar *i.e.* 36 s^{-1} for thymidine

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Table 1. Purification of thymidine 2′-hydroxylase from *Neurospora crassa*.

Purification step	Protein g[a]	Total Activity μkat	Spec. Activity μkat g^{-1}
140 000 *g* supernatant	2.8	0.26	0.093
DEAE-cellulose chromatography	0.15	0.32	2.1
Hydroxylapatite chromatography	$8.6 \cdot 10^{-3}$	0.15	17
Sephadex G-100 filtration	$2.5 \cdot 10^{-3}$	0.082	33
Chromatofocusing on Mono P™	$0.35 \cdot 10^{-3}$	0.043	123
Anion exchange on Mono Q™	$28 \cdot 10^{-6}$	0.021	765

[a] Protein was determined according to Lowry *et al.*[11] except for the last step where $A_1^{1\%}{}_{cm} = 10$ at 280 nm was used.

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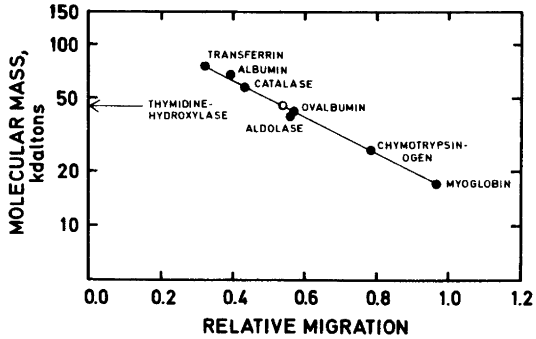

Fig. 2. Molecular mass determination of thymidine 2′-hydroxylase in SDS polyacrylamine gel electrophoresis.

2′-hydroxylase and 34 s^{-1} for γ-butyrobetaine hydroxylase. On high resolution chromatography on a Mono Q™ column (Fig. 3) coincidence of enzyme activity and a protein peak, which gives one dominating band on PAGE is found. Together with the high specific activity this indicates that the enzyme is essentially pure.

Experimental. Soluble extract. A soluble extract of *Neurospora crassa* strain STA 4 (FGSC 262 A) was prepared as described previously.[9]

DEAE Cellulose chromatography. After desalting on a Sephadex G-25 column the soluble extract (365 ml) was put onto a column (5×26 cm) of DE-cellulose (Whatman DE 52) equilibrated in 10 mM potassium phosphate buffer, pH 6.5, containing 25 mM KCl, 0.1 M glycine, and

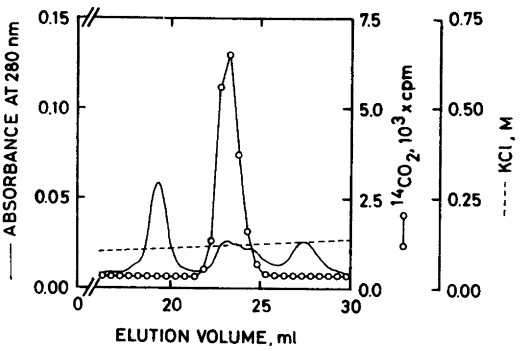

Fig. 3. Chromatography of thymidine 2′-hydroxylase in the final purification step on Mono Q™. The eluate was monitored for absorbance at 280 nm in a cell with 1 cm light path and collected in 0.5 ml fractions. The enzyme assay was performed as described in *Experimental;* 0.45 μl of eluate was used in the assay.

0.1 mM EDTA. The chromatogram was developed with a 7-1 linear gradient from 25 mM to 175 mM KCl. The enzyme activity was eluted around 150 mM KCl well separated from the preceding activity of thymine 7-hydroxylase.

Hydroxylapatite chromatography. The enzyme preparation from the DE cellulose column was concentrated by ultrafiltration on PM-10 Diaflo ultrafiltration membrane and the buffer was changed by passage through a Sephadex G-25 column. The enzyme preparation was then put onto a column (5×16 cm) of hydroxylapatite (Bio-Gel^R HTP from Bio-Rad Laboratories) equilibrated with 10 mM potassium phosphate buffer, pH 6.5, containing 0.1 M glycine. The chromatogram was developed with a linear gradient from 10 mM to 80 mM potassium phosphate buffer, pH 6.5, in a volume of 2.4 l. The enzyme activity was eluted around 60 mM.

Sephadex G-100 filtration. The hydroxylapatite eluate (350 ml) was concentrated by ultrafiltration on PM-10 Diaflo ultrafiltration membrane to 10 ml and further concentrated to 2 ml by vacuum dialysis in a Sartorius Membrane filter 13200 collodion bag. The enzyme preparation was then applied onto a Sephadex G-100 superfine column (1.5×90 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM NaCl amd 0.03 % NaN_3.

Chromatofocusing. The buffer was changed to 25 mM BIS-TRIS, pH 6.0, by passing the previous eluate through a Sephadex G-25 column. The preparation was then chromatographed in a fast protein liquid chromatograph, FPLC, on a 3 ml Mono P™ column with an ampholyte, 7.5 % polybuffer in water (all components from Pharmacia fine chemicals, Uppsala, Sweden). The enzyme was eluted at pH 4.2.

QAE cellulose chromatography. The combined fractions (1 ml) from the preceding step were put through a Sephadex G-25 in order to change the buffer to 10 mM potassium phosphate pH 6.8. This solution was chromatographed in the FPLC-system on a 1 ml Mono Q™ column. The chromatogram was developed with a gradient that was 2 mM to 100 mM KCl in 10 ml and then 100 mM to 200 mM KCl in 40 ml (Fig. 3). The eluate was collected in 0.5 ml fractions and the enzyme activity was mainly found in three fractions which were combined and concentrated to 75 μl by vacuum dialysis in a collodion bag.

SDS polyacrylamine gel electrophoresis. Slab gels (12.5×26×0.3 cm) with a 7.5 % polyacrylamide gel were run with the continuous buffer system desbribed by Weber and Osborn.[10] Samples of protein were denaturated in 0.5 % SDS and 0.5 % mercaptoethanol for three minutes at 100 °C. Aproximately 3 μg of the purified thymi-

dine 2'-hydroxylase was applied to the gel. After staining in Coomassie brilliant blue the mobility was calculated relative to that of bromophenol blue.

Enzyme assay. The incubation system (0.2 ml) contained thymidine 2'-hydroxylase (about 10 pkat) in 50 mM potassium phosphate buffer, pH 7.5. The concentration of substrates and cofactors were: thymidine 0.5 mM, 2-oxo[1-^{14}C] glutarate 0.25 mM, 50 nCi, ascorbate 5 mM, dithiothreitol 5mM, Fe_2SO_4 1 mM, and catalase 2 g/l. The incubations were carried out for 10 min at 37 °C and stopped by the addition of 0.2 ml 0.3 M trichloroacetic acid. The incubations were carried out in 10-ml plastic test tubes. $^{14}CO_2$ was trapped on a piece of filter paper attached to a wire in the rubber stopper of the test tube. The filter papers had been soaked in 20 µl of a 1 M solution of hydroxide of Hyamine 10-X [*p*-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide from Packard Instrument Company Inc.]. The diffusion was allowed to proceed for 1 h at 37 °C before the radioactivity was measured.

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