The Isolation of Rhodanese from *Pseudomonas aeruginosa* by Affinity Chromatography

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INTRODUCTION

Rhodanese (thiosulphate: cyanide sulphur-transferase; EC 2.8.1.1) was originally described by Lang (1933). The enzyme catalyses the formation of thiocyanate from cyanide and thiosulphate according to the reaction: $S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$. Rhodanese activity has been demonstrated in most mammalian tissues, with the greatest activity present in liver and kidney. The enzyme has been purified from liver (Horowitz & DeToma, 1970) and kidney (Westley & Green, 1959) as well as from several micro-organisms (Bowen, Butler & Happold, 1965; McChesney, 1958; Smith & Lascelles, 1966; Tabita, Silver & Lundgren, 1969).

In this communication, the use of affinity chromatography in the partial purification of rhodanese from *Pseudomonas aeruginosa* is reported.

METHODS

Organism and growth conditions. The strain of *Pseudomonas aeruginosa* used in this study was a water isolate supplied by Dr C. W. Houston, Department of Bacteriology, University of Rhode Island. The organism was maintained on trypticase soy agar (BBL) slants at 4 °C. A Fernbach flask containing 1 l trypticase soy broth (BBL) was inoculated with an overnight culture of *P. aeruginosa* to a density of 0.1 mg dry wt ml⁻¹, and shaken at 200 rev. min⁻¹ in a New Brunswick Psychrotherm Incubator at 35 °C. After 6 h growth, the bacteria were harvested by centrifugation at 8 °C in a Sorvall RC-2B centrifuge at 10000 g for 15 min.

Disruption of bacteria. The harvested bacteria were washed once with 6.7 mM-potassium phosphate buffer, pH 8.0, and resuspended in 10.0 ml of the same buffer containing 1 mM-sodium thiosulphate. The bacteria were sonicated at 5 °C for 30 s periods for a total of 3 min at maximum frequency, with a Bronwill Biosonik ultrasonicator (Bronwill Scientific, Rochester, New York, U.S.A.). The remaining whole bacteria and debris were removed by centrifuging at 10000 g for 30 min. The suspension was then centrifuged at 30000 g for 45 min to remove the membrane fraction. The 30000 g supernatant was the starting material for the purification of rhodanese from *P. aeruginosa*.

Gel filtration. The initial step in the purification was fractionation on a Sephadex G-100 column. The Sephadex G-100 was swollen in eluant buffer (6.7 mM-potassium phosphate buffer, pH 8.0) for 48 h and then degassed under vacuum. The crude cell-free extract was fractionated on a 2.5 × 30 cm column at 5 °C. The fraction volume was 5.0 ml. The three peak fractions containing rhodanese activity were selected for further purification using affinity chromatography.

Preparation of affinity column. The affinity gel was prepared by a procedure similar to that used by Mosbach *et al.* (1972). Settled Sepharose 4B (60 ml) was activated by the cyanogen bromide method (Cuatrecasas, 1970). 1,6-Diaminohexane (10 g), dissolved in 100 ml 0.1 M-NaHCO₃ buffer, was added to the activated Sepharose and the final pH was adjusted to 8.5 with 4 M-NaOH. The suspension was stirred for 15 h at room temperature. The gel was then filtered through a sintered glass funnel and washed extensively with, in turn, 0.1 M-NaHCO₃, 0.1 M-HCl, 0.5 M-NaCl, and distilled deionized water. It was then washed with a large volume of aqueous 80% (v/v) pyridine. After filtration, the gel was transferred to a 250 ml Erlenmeyer flask and 0.5 g lipoic acid, dissolved in 12 ml pyridine was added, followed by 20 g dicyclohexylcarbodi-imide dissolved in 45 ml pyridine. The flask was stoppered and gently agitated on a rotary shaker at room temperature. After shaking for 14 days, the gel was filtered and washed successively with 400 ml of each of the

Fig. 1. Elution of rhodanese from a Sepharose 4B-lipoic acid affinity column. Eluant buffer: 6.7 mM-phosphate buffer with various concentrations of sodium chloride, pH 8.0. Fraction volume: 2.5 ml. ▲, Rhodanese activity; ●, protein concentration.

following: distilled deionized water; ethanol; butanol; ethanol; distilled deionized water; 0.001 M-HCl for 20 min; 0.1 M-NaHCO₃; 0.5 M-NaCl; and distilled deionized water.

Affinity chromatography. The gel was equilibrated with 6.7 mM-phosphate/1 mM-sodium thiosulphate buffer, pH 8.4, and the partially purified enzyme preparation was applied. The lipoic acid affinity column was then washed successively with 6.7 mM-potassium phosphate/1 mM-sodium thiosulphate buffer, pH 8.0, the same buffer containing 0.02 M-NaCl, and finally buffer containing 0.06 M-NaCl. The affinity column was regenerated prior to reuse by washing with 0.5 M-NaCl and re-equilibrating with 6.7 mM-phosphate/1 mM-sodium thiosulphate buffer, pH 8.4.

Enzyme assay. A modification of the procedure of Sorbo (1953) which measures the formation of thiocyanate from cyanide and thiosulphate, was used to measure rhodanese activity. The assay system consisted of 1.0 ml 0.1 M-sodium thiosulphate, 1.0 ml 0.1 M-KCN in 0.01 M-Tris/HCl buffer, pH 9.3, and enzyme, in a final volume of 3.0 ml. The reaction mixture was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.2 ml of 37% (w/w) formaldehyde. Two ml of 16% (w/v) ferric nitrate in 1 M-HNO₃ was added for colour development. The absorbance of the reaction mixture was measured in a Beckman DK-2A ratio recording spectrophotometer at 470 nm, and compared with a standard curve constructed using known amounts of sodium thiocyanate. Protein was measured according to the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Hall & Berk (1968) first reported the presence of rhodanese in *Pseudomonas aeruginosa*. In this organism, rhodanese is a constitutive enzyme and its level cannot be elevated by the incorporation of either thiosulphate or cyanide into the growth medium. This contrasts with the finding of Bowen *et al.* (1965), who demonstrated a several-fold increase in rhodanese activity by the incorporation of cyanide into the growth medium of *Thiobacillus denitrificans*.

Villarejo & Westley (1963) reported that rhodanese isolated from beef liver catalysed the reduction of thiosulphate by reduced lipoic acid. It became apparent that if rhodanese from *P. aeruginosa* could interact with lipoic acid in the same way as the mammalian enzyme, it might be possible to further purify rhodanese by the use of a lipoic acid-Sepharose affinity column.

Mosbach and co-workers (1972) proposed the use of a hexacarbon 'spacer' molecule introduced between the Sepharose-gel matrix and the desired ligand. They reasoned that by displacing the ligand from the gel matrix, the steric availability for the desired enzyme(s) would be improved. An affinity gel was prepared by linking 1,6-diaminohexane to Sepharose 4B using the cyanogen bromide method and subsequently coupling the lipoic acid, by means of a peptide bond, to the free amino group of the spacer molecule using the condensing

agent dicyclohexylcarbodi-imide. When a partially purified rhodanese preparation was applied to this lipoic acid-Sepharose gel, containing a spacer molecule of approximately 0.7 nm in length, essentially all of the enzyme remained bound to the gel (Fig. 1). Any 'non-specifically' bound protein was washed from the gel with buffer containing 0.02 M-sodium chloride. All of the rhodanese was eluted in a sharp peak by increasing the sodium chloride concentration in the buffer to 0.06 M.

The lipoic acid-Sepharose affinity gel might also be valuable in the isolation of rhodanese from other bacterial and mammalian sources. At present, the methods available for purifying rhodanese from mammalian sources in large quantities involve several ammonium sulphate fractionations, pH manipulation, and in some cases batch fractionation with DEAE-cellulose (Sorbo, 1953; Westley & Green, 1959; Davidson & Westley, 1965; Horowitz & DeToma, 1970). These methods are quite time-consuming. A one-step purification system using lipoic acid bound to Sepharose should equal or surpass the level of purification achieved by either procedure.

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