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PURIFICATION OF TAPIOCA LEAF RHODANESE

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Abstract—Tapioca (*Manihot esculenta*, kekabu variety) leaf rhodanese was purified 7.8 fold by column chromatography on Sephadex G-10, acetone fractionation and gel electrophoresis. The purified enzyme was homogeneous in sedimentation velocity studies and analytical gel electrophoresis, and hetero-dimer was demonstrated by SDS gel electrophoresis. The enzyme kinetics were also studied.

INTRODUCTION

Rhodanese (thiosulphate: cyanide sulphur transferase, E.C. 2.8.1.1) activity was first detected in liver tissue by Lang [1] who obtained a 20 fold purification. Cosby and Summer [2] purified the enzyme further to 100 fold from bovine liver, and later Sörbo [3] purified the same enzyme 150 fold. Sörbo eventually isolated the enzyme in crystalline form [3]. Subsequently rhodanese from other sources was also investigated, for example, Westley and Green [4] crystallised rhodanese from bovine kidney while Tabita, *et al.* [5] purified the enzyme 40 fold from *Ferrobacillus ferrooxidans*. The techniques used in the purification of rhodanese by these authors included ammonium sulphate fractionation, acetone fractionation, lead acetate treatment and DEAE cellulose fractionation. Chew and Boey [6] were the first to report rhodanese from tapioca leaf. An excellent review on rhodanese was recently presented by Westley [7].

RESULTS AND DISCUSSION

Tapioca leaf rhodanese obtained was purified 7.8 fold. Table 1 shows the various stages of preparation and its sp act at each stage. Sephadex G-10 separated the rhodanese fraction from the smaller molecules including a diffuse band of yellow pigment. Rhodanese was eluted together with other macromolecules in the void volume on Rhodanese was precipitated maximally with 70 to 90% acetone. The enzyme was highly mobile in preparative polyacrylamide gel electrophoresis and could be collected at the early stages of fractionation.

Attempts to use ammonium sulphate and DEAE cellulose fractionation to purify the enzyme incurred a great loss of activity.

The enzyme was demonstrated to be homogeneous by analytical polyacrylamide gel electrophoresis and sedimentation studies. The purified enzyme was detected as a single protein band on electrophoresis in 3 different polyacrylamide gel concentrations. However, when the enzyme was subjected to SDS polyacrylamide gel electrophoresis, it dissociated into 2 very closely related (in terms of *Rm*) fast moving subunits, suggesting that it was a hetero-dimer. The subunits had MWs of 16000 and 17000 ± 2000, respectively. The $S_{20,w}$ value obtained from sedimentation velocity was 2.65 sec. Assuming that $D_{20,w}$ is 7.50×10^{-7} cm^2/sec and the partial specific volume is 0.742 g/ml^3, the MW was *ca* 33000; close to that of the liver rhodanese which was 37600 [3].

The kinetics of tapioca leaf rhodanese were studied. The apparent K_m for thiosulphate and cyanide were

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Table 1. Purification of tapioca leaf rhodanese

Purification Steps	Total activity (μmol SCN formed per 15 min)	Total protein (mg)	Specific activity (μmol SCN formed per mg protein per 15 min)	Purification factor
1. Crude homogenate	1180	979	1.20	1.0
2. Supernatant centrifuged at 30000 *g* for 60 min	1010	603	1.67	1.4
3. Dialysis against 0.1 M borate buffer at pH 7.2	992	545	1.82	1.5
4. Sephadex G-10 gel filtration of lyophilised extract	348	139	2.50	2.1
5. Acetone fractionation	210	41	5.12	4.3
6. Preparative polyacrylamide gel electrophoresis	150	16	9.40	7.8

1.5×10^{-2} M and 6.6×10^{-2} M, respectively. The activation energy was 6.0 kcal per mol. Methanol and sodium formate affected the K_m but not V_{max}. Tapioca leaf rhodanese was inactivated by 2-mercaptoethanol but activity was fully restored by addition of thiosulphate. The enzyme was also competitively inhibited by *p*-toluene sulphonate implicating tryptophan to be part of the active site of the enzyme. These studies thus showed similar molecular properties between tapioca leaf rhodanese and rhodanese obtained from other sources [7].

EXPERIMENTAL

Freshly harvested young tapioca leaves were washed and dried between blotters. Pooled leaf blades were ground in liquid N_2 and extracted with 0.1 M borate in 0.1 M mannitol at pH 7.2 (1 g fr. wt leaf/2 ml buffer). The homogenate was filtered through cheese-cloth and the filtrate centrifuged at 30000 *g* for 60 min. Supernatant was dialysed against 0.1 M borate buffer at pH 7.2 in the cold (4°) and then lyophilised. *Sephadex G-10 column chromatography.* Lyophilised extract was dissolved in H_2O (6 g fr. wt leaf equivalent/ml) and 5 ml was applied to the column (2.5 × 25 cm) and eluted with 0.1 M borate (pH 7.2) and 10 ml fractions were pooled. Me_2CO *fractionation* was performed from 0 to 90% Me_2CO at −5°. The ppt. was centrifuged at 30000 *g* for 10 min and washed twice with cold Me_2CO. The active fractions were pooled. Further purification was effected with *preparative polyacrylamide gel electrophoresis.* In a 7.5% gel at pH 10.5. The upper and lower reservoir buffers consisted of 0.1 M glycine–NaOH at pH 10.5. A sample size of 3 ml containing *ca* 40 mg protein was introduced into the column and 40 mA was applied. Fractions of 5 ml were collected and assayed for enzyme activity.

Analytical polyacrylamide gel electrophoresis was carried out on the purified enzyme sample. The enzyme was electrophoresed in 0.1 M Tris–glycine buffer at pH 8.3 in 5, 7.5 and 10% polyacrylamide gel concentrations (4mA per running tube). The gel was stained in 1% amido black in 7% HOAc.

SDS polyacrylamide gel electrophoresis was carried out in 0.025 M Tris–0.192 M glycine buffer at pH 8.3 and in 0.1% SDS and 5 M urea following the method of ref. [8]. Standard proteins used for calculation of the curve for *RM* against MW were cytochrome-*c*, chymotrypsinogen, bovine serum albumin and α and β subunits of rat brain tubulins which were kindly supplied by our colleaque Dr. L. P. Tan. The gel was stained in 0.25% Coomassie Blue in 50.1 MeOH and 10% HOAc.

Detection of rhodanese. The presence of rhodanese in the polyacrylamide gel was detected using the method of ref. [9] with slight modifications. The gel was incubated in 0.125 M $Na_2S_2O_3$ and 0.125 M KCN in arginate buffer at pH 10.5 at 55° for 15 min. The enzyme zone appeared as a white band. Rhodanese activity was assayed using the method of ref. [3] with some modifications. The reaction mixture consisted of 0.125 M $Na_2S_2O_3$ and 0.125 M KCN in borate buffer at pH 11.5. Incubation was carried out for 15 min at 55°. *Protein* was assayed using the method of ref. [10].

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