Phytochemistry, 1974, Vol. 13, pp. 1703 to 1706. Pergamon Press. Printed in England.

FERREDOXIN ACTIVATION BY RHODANESE

UMBERTO TOMATI, ROSAMARINA MATARESE and GIORGIO FEDERICI

Laboratory of Radiobiochemistry and plant Ecophysiology, National Research Council, and the Institute of Biological Chemistry, University of Rome, Rome, Italy

(*Received* 7 *December* 1973)

Key Word Index—*Brassica oleracea*: Cruciferae: cabbage; rhodanese; apoferredoxin; ferredoxin activation.

Abstract—Rhodanese was extracted from *Brassica oleracea* leaves and purified 150-fold. The enzyme was shown to have optimum activity at pH 8–8·5 and a temperature range of 50–55°; a K_m of 0·4 mM at 30° for thiosulphate and cyanide, and mol. wt around 32000. The electrophoretically pure enzyme is able to produce the biological and spectral properties of ferredoxin when added to apoferredoxin in the presence of thiosulphate.

INTRODUCTION

RHODANESE (thiosulphate:cyanide sulphur transferase E.C. 2.8.1.1) is a well known enzyme which occurs widely, especially in mammals.[1,2] *In vivo*, its physiological role is uncertain; *in vitro* it catalyses the transfer of sulphur from thiosulphate to a nucleophilic acceptor such as cyanide and reduced lipoate.[3,4]

It has been previously demonstrated that, in the presence of thiosulphate, mammalian rhodanese is able to reactivate ferredoxin from apoferredoxin.[5] The resulting active ferredoxin has the same biological and spectral properties and is indistinguishable from the native oxidase.[6] This fact seems to suggest that rhodanese might be an activator for non heme iron proteins by providing labile sulphide from thiosulphate. We have also established the presence of rhodanese in chloroplasts and suggest a correlation between rhodanese activity and labile sulphide in chloroplasts.[7]

We have, therefore, purified rhodanese from cabbage in order to investigate the role of this enzyme in plants, and to ascertain if the reactivation of the biological and spectral properties of ferredoxin from apoferredoxin, previously reported for mammalian rhodanese, can be observed with the plant enzyme.

RESULTS AND DISCUSSION

We used cabbage leaves as the source of rhodanese because we found that this plant[7] contained a much larger amount of the enzyme than do parsley leaves. The properties of parsley rhodanese are similar to those of cabbage with regard to electrophoretic behaviour, K_m for cyanide and thiosulphate and optimal activity conditions.[8]

[1] SORBO, H. B. (1951) *Acta Chem. Scand.* **5**, 724.
[2] WESLEY, J. (1959) *J. Biol. Chem.* **234**, 1857.
[3] WILLAREJO, M. and WESTLEY, J. (1963) *J. Biol. Chem.* **238**, 1185.
[4] WILLAREJO, M. and WESTLEY, J. (1963) *J. Biol. Chem.* **238**, 4016.
[5] FINAZZI-AGRÒ, A., CANNELLA, C., GRAZIANI, M. T. and CAVALLINI, D. (1971) *FEBS Lett.* **3**, 172.
[6] FEE, J. A. and PALMER, G. (1971) *Biochim. Biophys. Acta* **245**, 175.
TOMATI, U., FEDERICI, G. and CANNELLA, C. (1972) *Physiol. Chem. Phys.* **4**, 193.
[8] TOMATI, U., CANNELLA, C., PECCI, L., ARDUINI, E. and PENSA, B. (1972) 8th FEBS Meeting, Amsterdam.

Rhodanese activity in cabbage is about 10-fold less than in mammalian liver and kidney. Its solubilization is rather difficult even after treatment with surface active agents and with buffers at varying pH (5–8) and ionic strength (10^{-3} to 10^{-1} M). The difficulty might be due to the binding of the enzyme to the supporting structures as happens for many other enzymes.

Rhodanese was purified 150-fold by $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G75, using Na-phosphate buffer 0·01 M pH 7·6 containing 10^{-3} M $Na_2S_2O_3$ (this reagent has been reported as capable of stabilising mammalian rhodanese[9,10]). The results are summarized in Table 1. The resulting enzyme is electrophoretically pure and behaves as a single protein during determination of its molecular weight by gel filtration according to the method of Cavallini[11]. The electrophoretically pure enzyme has an optimal pH between 8 and 8·5 in Tris or phosphate butter (10 mM), but the enzyme is also active at higher pH. This result is in contrast to the report of Chew and Boey[12] that the pH optimum of rhodanese from tapioca is between 10·2 and 11·0. Optimum temp. is between 50 to 55° and the enzyme is completely inactivated at 65°. Rhodanese is still active after 15 days at −20°, but the activity is partially lost after dialysis against water.

TABLE 1. SUMMARY OF THE PURIFICATION OF RHODANESE FROM CABBAGE

Step	Vol (ml)	Total protein (mg)	Rhodanese Total units†	Sp. Act. (μ/mg)	Yield (%)
(1) Crude solution*	3600	25200	12600	0·5	100
(2) Ammonium sulphate after acidification with 0·1 M HCl	43	77	2470	32	20
(3) Gel filtration on Sephadex G75	30	30	1800	60	14
(4) Solubilitation with 1 M ammonium sulphate at pH 7·9	0·16	4	320	80	2·4

* Homogenate of 2 kg of cabbage leaves in 0·01 M Na-phosphate buffer pH 7·6. Data are the average of 10 preparations.

† μmol KCNS/mg protein/min.

The preparations obtained showed no transulphurase activity with mercaptoethanol and 3-mercaptopyruvate as substrate, CNS^- being produced only when thiosulphate is present. The K_m for thiosulphate and for cyanide is 0·4 mM, and the approximate mol. wt (*ca* 32000) is comparable with that of mammalian rhodanese used as a marker in the determination.

Our results do not indicate the presence in cabbage rhodanese of aggregates or subunits, as found by Bowen, *et al.*[13] for rhodanese of *Thiobacillus denitrificans*. They purified a "monomeric form" with mol. wt of *ca* 38000, similar to that of mammalian rhodanese.

[9] SORBO, B. H. (1953) *Acta Chem. Scand.* **7**, 1137.
[10] SORBO, B. H. (1953) *Acta Chem. Scand.* **7**, 1129.
[11] CAVALLINI, D., CANNELLA, C., FEDERICI, G., DUPRÈ, S., FIORI, A. and DEL GROSSO, E. (1970) *European J. Biochem.* **16**, 537.
[12] CHEW, M. Y. and BOEY, C. G. (1972) *Phytochemistry* **11**, 167.
[13] BOWEN, T. J., BUTLER, P. J. and HAPPOLD, F. C. (1965) *Biochem. J.* **97**, 651.

and detected a subunit with mol. wt 9000 and a tetramer (mol. wt 150000) by gel filtration. This tetramer, when treated with mercaptoethanol, produced enzymatically active fragments with mol. wt of 2000 and 7000 but no subunits with a mol. wt of 19000, corresponding to the monomeric form of mammalian rhodanese.[13]

We did not obtain aggregates or subunits of cabbage rhodanese by gel filtration on Sephadex G200, 100, 75, 50, 25 using columns equilibrated with phosphate buffer (pH 7·8 10 mM) containing $Na_2S_2O_3$ (1 mM). The assays were repeated after treatment with 3% mercaptoethanol.

RECONSTITUTION OF FERREDOXIN

Fee and Palmer have demonstrated the reconstitution of ferredoxin from apoferredoxin in the presence of Na_2S and a ferric salt.[6] Finazzi-Agrò, *et al.* subsequently found that rhodanese from mammals and thiosulphate produce the same effect in the presence of ferric nitrate.[5] Both authors followed ferredoxin reconstitution by measuring increase in absorptivity (A) at 422 nm. The same method has been used in this work to establish a similar behaviour of cabbage rhodanese.

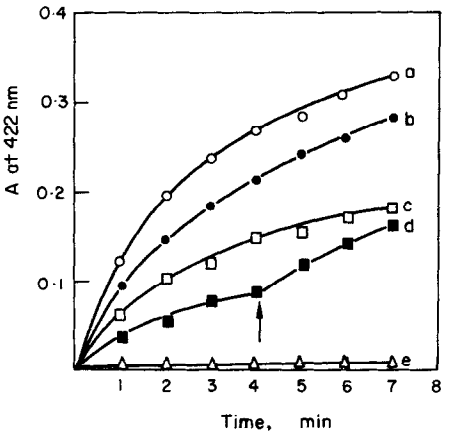

FIG. 1. RATE OF RECONSTITUTION OF FERREDOXIN.
The reconstitution of ferredoxin was followed by measuring the increase of A at 422 nm. The standard incubation mixture contained: 0·17 M Tris-HCl pH 7·6; 1·25 10^{-4} M apoferredoxin; 3 10^{-4} M $Fe(NO_3)_3$; 2 10^{-3} M dithiothreitol. Curve a: standard incubation mixture; 4 10^{-4} M Na_2S; Curve b: standard incubation mixture; 1·25 10^{-3} M $Na_2S_2O_3$; 1·2 10^{-5} M rhodanese; Curve c: standard incubation mixture; 1·25 10^{-3} M $Na_2S_2O_3$; 6·0 10^{-6} M rhodanese; Curve d: standard incubation mixture; 1·25 10^{-3} M $Na_2S_2O_3$; at the arrow 6·0 10^{-6} M of rhodanese was added; Curve e: without dithiothreitol.

We added cabbage rhodanese and thiosulphate to the apoferredoxin and got an increase of A at 422 nm proportional to the amount of enzyme added (Fig. 1). The reconstituted ferredoxin is indistinguishable from the native protein. It is known that *in vitro* recombination of iron with apoferredoxin takes place only in presence of sulphide and a reducing agent.[6] The fact that rhodanese, in the presence of thiosulphate (probably acting *in vivo* as a reducing agent[14]), is able to restore ferredoxin activity, supports the hypothesis that rhodanese produces labile sulphide, useful for the preservation of ferredoxin activity.[5]

[14] KOJ, A., FRIEDNO, J. and JANIK, Z. (1967) *Biochem. J.* **103**, 791.

EXPERIMENTAL

Rhodanese purification. Cabbages were purchased from a local market and treated on the same day. All operations were carried out at 4°. 2 Kg of leaves were homogenized, for 10 min, in 2 l. of 0·01 M Na-phosphate containing 1 mM thiosulphate, filtered and centrifuged at 5000 *g* for 10 min. The supernatant was quickly acidified to pH 3·8 with 0·1 M HCl and centrifuged at 8000 *g* for 10 min. The clear supernatant was precipitated with $(NH_4)_2SO_4$ at 35–80% saturation and the ppt dissolved in 0·01 M Na-phosphate buffer containing 1 mM Na-thiosulphate. The solution, concentrated by ultrafiltration, was filtered on Sephadex G75 (column 90 × 4 cm equilibrated with the same buffer; flux 20 ml/hr). The fraction containing rhodanese was concentrated by ultrafiltration. The pH was adjusted to 7·9 with 0·1 M ammonia, precipitated with $(NH_4)_2SO_4$ at 90% saturation and the precipitate dissolved in 1 M $(NH_4)_2SO_4$ previously adjusted to pH 7·9. The enzyme so obtained is pure electrophoretically. Electrophoresis was carried out according to the method reported by Ornstein & Davis.[15]

Enzyme assay. A mixture containing 1·5 ml of 0·01 M Na-phosphate buffer pH 8·2, 0·5 ml of 0·1 M Na-thiosulphate and 0·5 ml of enzyme soln was incubated at 30°. After 10 min, 0·5 ml of 0·1 M KCN was added. After further 10 min the reaction was stopped with 0·25 ml of formalin at 38%. Then 2·75 ml of Sorbo reagent[9] were added and A read at 460 nm. Under these conditions, A = 0·758 corresponds to 1 μmol of KCNS.

Other methods. Protein was determined according to Lowry,[16] chloroplasts were prepared using the method of Whatley and Arnon,[17] chlorophyll was determined by Arnon's method,[18] mol. wt was determined according to Cavallini.[11] Ferredoxin concn was determined by absorption measurement at 280 and 422 nm, using $\epsilon = 9200$ and mol. wt of 10700.[6] Apoferredoxin was made and ferredoxin reconstituted according to Fee and Palmer.[6] Ferredoxin activity was tested as described by San Pietro.[19]

Acknowledgements—The authors wish to thank Prfs. G. Giovannozzi-Sermanni, D. Cavallini and E. N. Ugochukwu for useful discussions, Prof. A. Finazzi-Agrò for supplying parsley ferredoxin and Professor C. Cannella for assistance with disk electrophoresis and determination of molecular weight.

[15] ORNSTEIN, L. and DAVIS, B. J. (1962) *Disk Electrophoresis*, Rochester N.Y. Distillation Products Industries.
[16] LOWRY, O. M., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[17] WHATLEY, F. R. and ARNON, D. I. (1963) In: *Methods in Enzymology* (COLOWICK, S. and KAPLAN, K. O., eds.), Vol. VI, p. 308, Academic Press, New York.
[18] ARNON, D. I. (1949) *Plant Physiol.* **24,** 1.
[19] SANPIETRO, A. (1963) In: *Methods in Enzymology* (COLOWICK, S. and KAPLAN, K. O., eds.), Vol. VI, p. 439, Academic Press, New York.