Phytochemistry, Vol. 23, No. 3, pp. 497–499, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

PARTIAL PURIFICATION AND SOME PROPERTIES OF SHIKIMATE DEHYDROGENASE FROM TOMATOES

EUCLIDES J. LOURENÇO* and VALDIR A. NEVES

Department of Food Science and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of S. Paulo, S. Paulo, Brasil

(*Revised received* 18 *April* 1983)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; shikimate dehydrogenase; enzyme purification.

Abstract—The partial purification of shikimate dehydrogenase (SDH) from tomato fruit was achieved by precipitation with ammonium sulphate, and chromatography on DEAE-cellulose and hydroxyapatite. The enzyme has a MW of 73 000, shows an optimum at pH 9.1 and K_m values of 3.8×10^{-5} M and 1.0×10^{-5} M with shikimic acid and NADP as substrates. NADP could not be replaced by NAD. The tomato enzyme is competitively inhibited by protocatechuic acid with a K_i value of 7.7×10^{-5} M. On the other hand, cinnamic acid derivatives and 2-hydroxybenzoic acid were ineffective. At 50° for 5 min the SDH is inactivated by 85%. The activity was inhibited by *p*CMB and *N*-ethylmaleimide, suggesting a requirement for SH groups. The inactivation plot of oxidation by *p*CMB was biphasic, and NADP decreased the reactivity of sulphydryl groups to the reagent. The activation energy was found to be 14.2 kcal/mol. The properties of the SDH are discussed in relation to the enzymes from other sources.

INTRODUCTION

The shikimate pathway leading to the biosynthesis of aromatic amino acids has been elucidated for microorganisms and evidence obtained by tracer experiments indicates that this pathway is also operative in higher plants [1]. Shikimate dehydrogenase (EC 1.1.1.25; SDH) catalyses the reduction of 3-dehydroshikimic acid by NADPH to shikimic acid, an intermediate in the biosynthesis of the aromatic amino acids. Its distribution in higher plants has been studied by Balinski and Davies [2]. SDH was present in *Pisum sativum* [3] and its activity is stimulated in response to wounding [4]. Rhodes *et al.* [5] have shown a 2–3 fold increase in the activity of SDH during the ageing of swede roots discs. A similar rise in enzyme activity occurs in sweet potato root discs after 24 hr incubation [6]. Kojima *et al.* [7] studied SDH from sweet potato root, and Nandy and Ganguly [8] isolated this enzyme from *P. aureus*, describing its biochemical properties. Feierabend *et al.* [9] and Rothe [10] have reported different subcellular localizations of SDH in peas. Balinsky and Davies [2] obtained evidence that the enzyme from cauliflower buds occured mainly in the supernatant fraction.

This paper deals with the purification of the SDH from tomato fruit, describing some of its biochemical properties.

RESULTS AND DISCUSSION

Purification and properties

Earlier works has shown that SDH from peas and *P. mungo* seedlings [10, 11] consists of two isoenzymes.In this report the enzyme purified from tomatoes was resolved into two active fractions after hydroxyapatite chromatography. The SDH was eluted as a major peak (I) (fractions 18–30) containing *ca* 90% of the total activity applied on the column, and a minor peak (II) with *ca* 10% of the activity (fractions 40–60). The properties so far studied are distinct, indicating that this enzyme exist in the tomato tissue in two forms differing in substrate specificity, MW, dependence on pH and behaviour towards inhibitors. In this report only the results obtained with peak I are shown. Table 1 summarizes the results of the purification procedure described in detail in the Experimental. Treatment of the crude extract with ammonium sulphate as 30–85% saturation, desalting on Sephadex G25, DEAE-cellulose and hydroxyapatite columns resulted in 11.5-fold purification with a 4% recovery of the total activity and a sp. act. of 4.71 nkat/mg protein. The enzyme was unstable during the entire purification procedure, mainly at the DEAE-cellulose chromatography step when the activity decreased by 85% of the original level, even with the addition of 2 mM cysteine, 8 mM 2-mercaptoethanol (2-ME) and 2% glycerol to the eluting buffer. On the other hand, this stage was quite effective to remove inert protein, yielding a preparation with *ca* 5% of applied protein. The purity of SDH after the last purification step was checked by analytical polyacrylamide disc electrophoresis and the procedure adopted did not yield a homogenous enzyme. The electrophoretic pattern revealed two protein bands after staining with Coomassie Blue and the MW of SDH performed on a Sephadex G-200 calibrated column was estimated to be 73 000 which is somewhat larger than the values found for the *Q. pedunculata* [12] and *P. mungo* [11] enzymes.

In this study it is shown that the tomato SDH has several properties in common with the SDHs from other

*Present address: Department of Food Science and Nutrition, Faculty of Pharmaceutical Sciences, UNESP, Araraquara, S. Paulo, Brasil.

Table 1. Purification of shikimate dehydrogenase

Purification steps	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Recovery (%)	Purification
1. Crude extract	175.5	72.0	8.41	100	1
2. $(NH_4)_2SO_4$ 30–85%	150.6	69.5	0.46	96.5	1.1
3. Sephadex G-25	72.0	59.5	0.83	82.6	2.0
4. DEAE-cellulose	3.8	9.3	2.45	12.9	6.0
5. Hydroxyapatite	0.67	2.8	4.71	3.8	11.5

sources [7, 13, 14]. The SDH is not inhibited significantly by quinic acid (5 μM), L-try (5 μM) L-phe (5 μM), trans-cinnamic acid (5 μM), caffeic acid (0.5 μM), ferulic acid (0.5 μM), hydroxybenzoic acid (5 μM), chlorogenic acid (0.5 μM) or EDTA (5 μM). Among the compounds tested for inhibitory activity protocatechuic acid was the strongest inhibitor (397-activity at 0.5 μM). The kinetic studies revealed a competitive type inhibition, and a K_i value of 7.7×10^{-5} M was obtained by a Dixon plot of $1/v$ against $[i]$ at two levels of substrate [15]. Since the K_i value is higher than the K_m it is unlikely that protocatechuic acid could act as a regulator for SDH activity. Balinsky and Davies [16] also found a competitive inhibition for the pea enzyme.

The effect of several mono and divalent cations on the enzyme activity was tested. The enzyme was completely inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+}. Mg^{2+}, Ca^{2+} and Fe^{3+} had little or no effect on the activity whereas NH_4^+ and borate were inhibitors at high concentration. Chelating agents such as EDTA had no effect on the activity and this seems to suggest that there is no requirement for divalent ions. The Cu^{2+} inhibition is of a non-competitive type, with a K_i value of 6.0×10^{-5} M. The purified enzyme was specific for NADP and no activity was detected when NADP was replaced by NAD in the reaction. The enzyme is also specific for shikimic acid, showing no activity towards quinic acid.

The effects of pH on the catalysis show a range of activity between pH 6 and 11; below 6 and above pH 11 there was no detectable activity. The pH–activity profile for SDH showed that the enzyme exhibited a curve with a maximum at pH 9.1 in Tris–glycine buffer. The pH optimum of the SDH from peas [13], tea plant [14] and *P. mungo* [11] was estimated to be 10.0.

The time course of the heat inactivation was followed by incubating the SDH in the temperature ranging from 30 to 50°. At various time intervals 0.5 ml of the heated enzyme was withdrawn, cooled and assayed for the remaining activity at 30°. The SDH is heat labile; at 50° it is inactivated by 85% after 5 min with a half-life of 3.6 min. The apparent reaction rate of 0.0011/min and 0.192/min was calculated from the plot of activity vs time, for the inactivation at 40° and 50° respectively. The activation energy (E_a) calculated from the slope of the Arrhenius plot was estimated to be 14 200 kcal/mol; the plot shows no discontinuity over a temperature range from 25 to 3°.

The K_m values of the enzyme for shikimic acid and NADP were 3.8×10^{-5} M and 1×10^{-5} M respectively. This result indicated that the enzyme has a higher affinity for NADP. Higher affinity for NADP was also found for the sweet potato root [7], tea plant [14] and bamboo shoot [17] SDHs.

The requirement of SH-groups for the catalytic activity was tested. The enzyme was dialysed against 10 mM K-Pi buffer, pH 7.5, and assayed in the presence of *p*CMB, *N*-ethylmaleimide and iodoacetamide at various concentrations. Both *p*CMB and *N*-ethylmaleimide were inhibitory at 10^{-6} M. This sulphydryl reagents inhibition seems to be characteristic of SDH [7, 11, 13]. The time course of the enzyme inactivation by *p*CMB was followed at 25° over 10 min. The plot of remaining activity vs time (Fig. 1) was biphasic with an initial rapid phase when 75% of the enzyme was inactivated within 1 min, and a slow inactivation followed. The inactivation rate increased with the concentration of the reagent and this finding indicated that the sulphydryl groups of the native protein became inacessible to the substrates. Since the reagent yielded a completely inactive enzyme after 10 min; the sulphydryl group is probably at the active site. On the other hand, the reactivity of the sulphydryl groups to the reagent decreased in the presence of NADP. Nagainis *et al.* [18] reported a low reactivity to the reagent of sulphydryl groups of the fumarylacetoacetate fumarylhydrolase from beef liver when assayed in the presence of the substrate.

EXPERIMENTAL

Tomatoes (*Lycopersicon esculentum* Mill. var. Santa Cruz) grown under controlled conditions in a experimental farm at the

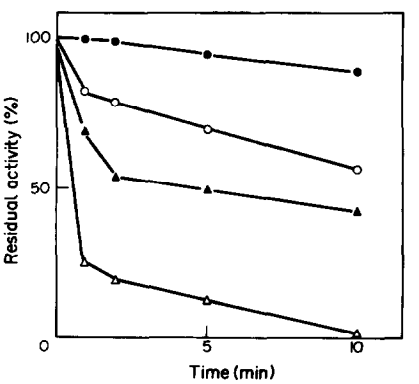

Fig. 1. Time course of enzyme inactivation by *p*CMB. The enzyme was incubated with *p*CMB at various concentrations and the remaining activity was determined at 1, 2, 5 and 10 min. (●——●) control; (○——○) 0.25 μmol; (▲——▲) 0.5 μmol; (△——△) 1 μmol of *p*CMB.

State of São Paulo, Brasil, were harvested at mature green stage.

Enzyme purification. Aliquots (100–150 g) of frozen wall material of the tomatoes were homogenized in 0.2 M K-Pi buffer, containing 0.5 mM DTE, 8 mM 2-ME, 2 mM cysteine, 2 mM EDTA and 0.4 g Polyclar AT/10 g of tissue. The extract was filtered through four layers of cheesecloth and centrifuged at 40 000 *g* for 40 min. The clear supernatant obtained was treated with $(NH_4)_2SO_4$ at 30–25% satn. After precipitation the suspension was centrifuged at 40 000 *g* for 40 min. The $(NH_4)_2SO_4$ ppt was dissolved in a minimal vol. of 5 mM K-Pi buffer pH 7.5, 0.5 mM DTE, 8 mM 2-ME, 2 mM cysteine and desalted on a column of Sephadex G-25 (30 × 2.5 cm) equilibrated with the same buffer. The eluate was applied to a column of DEAE-cellulose (15 × 2.5 cm) previously equilibrated with the same buffer. The enzyme was eluted with a linear gradient of KCl (0–0.4 M) in 5 mM K-Pi buffer pH 7.5 containing 0.5 DTE, 8 mM 2-ME and 2 mM cysteine. The active fractions were combined, and $(NH_4)_2SO_4$ added to give 85% satn. The resultant ppt was suspended in 10 mM K-Pi buffer containing 0.5 mM DTE, 8 mM 2-ME and 2 mM cysteine and the suspension dialysed against the same buffer. The dialysate was applied to a hydroxyapatite column (7 × 2 cm) equilibrated with the same buffer. The SDH was eluted with a linear gradient from 10 to 200 mM of K-Pi buffer, pH 7.5. The fractions with enzyme activity were pooled and used for the assays. All the steps were carried out at 0–4°.

SDH assay. This was as described previously in ref. [13], following the oxidation of shikimic acid to dehydroshikimic acid by the production of NADPH from $NADP^+$ at 340 nm. The incubation mixture at 30° contained; Tris–HCl buffer pH 8.6, 4 μmol of shikimic acid, 0.5 μmol of NADP, and enzyme in a total vol. of 3 ml. The reaction was initiated by the addition of $NADP^+$. The amount of product formed was calculated using a molar extinction coefficient of 6.22/cm/min for NADPH.

MW of SDH. This was determined by gel chromatography on Sephadex G-200 according to the method of ref. [19]. The column was calibrated with cytochrome *c* (12 500), soybean trypsin inhibitor (21 000), ovalbumin (45 000), bovine serum albumin (67 000) and alkaline phosphatase (100 000).

Polyacrylamide gel electrophoresis. This was performed according to the method of ref. [20]. The combined active fractions from hydroxyapatite were applied in 0.5 × 10 cm tubes filled with 7% acrylamide gels. The electrophoresis was carried out using Tris–glycine buffer, pH 8.3, and 2 mA/tube for 15 min followed by 3 mA/tube for 2 hr. The gels were stained for protein with Coomassie Blue.

Protein concentration was determined by the method of ref. [21] using BSA as standard. Protein in the column eluate was determined at 280 nm.

Acknowledgement—This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil.

REFERENCES

1. Yoshida, S. (1969) *Annu. Rev. Plant Physiol.* **20**, 41.
2. Balinsky, D. and Davies, D. D. (1962) *J. Exp. Botany* **13**, 414.
3. Attridge, T. H. and Smith, H. (1967) *Biochim. Biophys. Acta* **148**, 805.
4. Minamikawa, T., Kojima, M. and Uritani, I. (1966) *Arch. Biochem. Biophys.* **117**, 194.
5. Rhodes, M. J. C., Hill, A. C. R. and Wooltorton, L. S. C. (1976) *Phytochemistry* **15**, 707.
6. Minamikawa, T., Kojima, M. and Uritani, I. (1966) *Plant Cell. Physiol.* **7**, 583.
7. Kojima, M., Minamikawa, T. and Uritani, I. (1969) *Plant Cell Physiol.* **10**, 245.
8. Nandy, M. and Ganguli, N. C. (1961) *Biochim. Biophys. Acta* **48**, 608.
9. Feierabend, J. and Brassel, D. (1977) *Z. Pflanzenphysiol.* **82**, 334.
10. Rothe, G. M. (1974) *Z. Pflanzenphysiol.* **74**, 152.
11. Koshiba, T. (1978) *Biochim. Biophys. Acta* **522**, 10.
12. Boudet, A. (1971) *FEBS Letters* **14**, 257.
13. Balinsky, D. and Davies, D. D. (1961) *Biochem. J.* **80**, 292.
14. Sanderson, G. W. (1966) *Biochem. J.* **98**, 248.
15. Dixon, M. (1953) *Biochem. J.* **55**, 170.
16. Balinsky, D. and Davies, D. D. (1961) *Biochem. J.* **80**, 296.
17. Higuchi, T. and Shimada, M. (1967) *Plant Cell Physiol.* **8**, 61.
18. Nagainis, M. P., Pu, W., Cheng, B., Taylor, K. E. and *Schmidt, D. E.* (1981) *Biochim. Biophys. Acta* **657**, 203.
19. Andrews, P. (1964) *Biochem. J.* **91**, 222.
20. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.