# Purification and Properties of Shikimate Dehydrogenase from Cucumber (*Cucumis sativus* L.)

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Shikimate dehydrogenase (SDH, EC 1.1.1.25) extracted from cucumber pulp (*Cucumis sativus* L.) was purified 7-fold by precipitation with ammonium sulfate and elution from columns of Sephadex G-25, DEAE-cellulose, and hydroxyapatite. Two activity bands were detected on polyacrylamide gel electrophoresis at the last purification step. pH optimum was 8.7, and a molecular weight of 45 000 was estimated on a Sephadex G-100 column. SDH was inhibited competitively by protocatechuic acid with a $K_i$ value of $2 \times 10^{-4}$ M. $K_m$ values of $6 \times 10^{-5}$ and $1 \times 10^{-5}$ M were determined for shikimic acid and $NADP^+$, respectively. The enzyme was completely inhibited by $HgCl_2$ and *p*-(chloromercuri)benzoate (PCMB). NaCl and KCl showed partial protection against inhibition by PCMB. Heat inactivation between 50 and 55 °C was biphasic, and the enzyme was completely inactivated after 10 min at 60 °C. Incubation of SDH with either $NADP^+$ or shikimic acid protected the enzyme against heat inactivation.

## INTRODUCTION

The biosynthetic pathway of aromatic amino acids is now completely elucidated in microorganisms. Tracer experiments indicate that biosynthesis in higher plants is essentially the same as that found in microorganisms. Higher plants synthesize aromatic amino acids from phosphoenolpyruvate and erythrose 4-phosphate through dehydroshikimic acid. Aromatic amino acids thus formed in the shikimate pathway are thought to be utilized for protein synthesis in higher plants. Alternatively, a considerable amount of these amino acids is converted in the phenylpropanoid metabolism to secondary metabolites such as cinnamic acids, lignin, flavonoids, and alkaloids (Yoshida, 1969). Shikimate dehydrogenase catalyzes the conversion of dehydroshikimic acid, a precursor of phenylalanine and tyrosine. This enzyme has been studied in purified and unpurified preparations. Some experiments have demonstrated its physical association with 3-dehydroquinate hydrolyase in a multifunctional enzyme aggregate (Boudet, 1971; Koshiba and Yoshida, 1976). The presence of isoenzymic forms has been reported in peas and in *Phaseolus mungo* (Feierabend and Brassel, 1977; Koshiba, 1978). Rothe (1974) reported different cellular localization of the enzyme in peas. Lourenço and Neves (1984) have also described some biochemical properties of the enzyme from tomatoes, and Lemos Silva et al. (1985) have also shown the inhibitory effect of aromatic compounds on shikimate dehydrogenase activity.

The effects of environment conditions in the activity of enzymes of the shikimate pathway and the phenylpropanoid pathway have been investigated by several workers. It has been shown that the metabolism of phenolic compounds is often enhanced in plant tissue under stress conditions such as mechanical damage (Rhodes and Wooltorton, 1978a), low-temperature storage (Rhodes and Wooltorton, 1977, 1978b), or infection (Friend et al., 1973; Legrand et al., 1976). Among the enzymes of the shikimate pathway the activities of shikimate dehydrogenase and dehydroquinate dehydratase markedly increase in response to wounding of sweet potato root (Minamikawa et al., 1969), and this increase was followed by active synthesis of polyphenols. Rhodes et al. (1976), when studying a range of enzymes of phenolic metabolism, found that SDH activity increased during ageing of swede root disks, accompanied by the accumulation of a lignin-like polymer. Among the studies on the metabolic changes that occur in fruits stored at low temperatures, there are some reports on the possible role of phenylpropanoid metabolism in tomatoes, potatoes, and sweet potatoes under chill stress conditions (Rhodes and Wooltorton, 1977, 1978b). These authors reported an increase in activity of a range of enzymes of phenylpropanoid metabolism, and this was correlated with chilling injury and phenolic synthesis. In a previous study in our laboratory on the changes in the activity of enzymes of shikimate pathway in tomatoes stored at various temperatures, it was shown that shikimate dehydrogenase activity increased markedly when the fruit was stored at 2–10 °C (Neves and Lourenço, 1982). This large increase in activity occurred at temperatures below the threshold temperature (12–13 °C) for chilling injury, and it was coincident with the most visible symptoms of chilling injury. Although it is generally agreed that SDH activity from a number of sources increases under stress conditions, reports on the enzyme properties from chilling-sensitive plants are few. Possibly this is partially due to the fact that the enzyme is often unstable and hence difficult to obtain as a purified preparation with a high specific activity.

The present work forms part of a study of the mechanism of activation of SDH activity which can be induced by low-temperature storage of chilling-sensitive vegetables. To increase our knowledge of SDH, we purified and characterized the physicochemical properties of the enzyme.

## MATERIALS AND METHODS

**Materials.** Cytochrome *c*, soybean trypsin inhibitor, ovalbumin, bovine serum albumin, alkaline phosphatase, PCMB, shikimic acid, $NADP^+$, DEAE-cellulose, Sephadex G-100 and G-25, and hydroxyapatite were purchased from Sigma Chemical Co., St. Louis. Cucumber fruit (*Cucumis sativus* L.) cv. Sikkimensis, grown in a local farm, were picked at the end of their growing period in November 1988. Fruits were peeled, and the pulp (mesocarp) was ground in liquid nitrogen.

**Methods.** *Purification of Shikimate Dehydrogenase.* Portions (40–60 g) of frozen pulp were homogenized in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM L-cysteine, and 8 mM 2-mercaptoethanol. The homogenate was filtered and centrifuged at 25000*g* for 40 min. The

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supernatant (crude extract) was brought to 30–90% saturation with $(NH_4)_2SO_4$. The precipitate was separated by centrifugation as described above and dissolved in 5 mM potassium phosphate buffer, pH 7.5, containing 1 mM L-cysteine and 8 mM 2-mercaptoethanol. The SDH purification was performed essentially as described by Lourenço and Neves (1984) using a combination of gel filtration on Sephadex G-25 and elution from columns of DEAE-cellulose and hydroxyapatite.

*Measurement of SDH Activity.* The enzyme activity was measured by following the increase in absorbance at 340 nm (30 °C) during the oxidation of shikimic acid to 3-dehydroshikimic acid as described by Balinsky and Davies (1961). One unit of enzyme activity (nkat) is the amount of enzyme that causes the convertion of 1 nmol of substrate to product in 1 s under the experimental conditions used.

*Protein Determination.* Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

*Molecular Weight.* Molecular weight was determined on a Sephadex G-100 column (2.5 × 60 cm) using cytochrome *c* (12 000), soybean trypsin inhibitor (21 000), ovalbumin (43 000), bovine serum albumin (68 000), and alkaline phosphatase (100 000) as molecular weight standards. The molecular weight was estimated by using a $V_e/V_0$ plot vs log MW of standard proteins according to the method of Whitaker (1963).

*Gel Electrophoresis.* Electrophoresis was performed on 7% polyacrylamide gel with Tris-HCl–glycine buffer, pH 8.9, according to the method of Davis (1964). After the electrophoretic run, the gels were stained for protein with Coomassie brilliant blue R-250. SDH activity was detected by incubating the gels in a reaction mixture containing 0.1 M glycine–NaOH buffer, pH 8.6, 4 mM shikimic acid, 1 mM $NADP^+$, 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Serva Feinbiochemical, Heidelberg, W. Germany), and 0.1 mg/mL phenazine methosulfate (Sigma).

*Kinetic Constants.* The apparent $K_m$ was determined according to the procedure described by Segel (1975) for an ordered bireactant systems. The type of inhibition and $K_i$ value were determined according to the method of Dixon (1953).

*Optimum pH.* The optimum pH of the enzyme was determined over the pH ranges 7.0–8.0 with 0.1 M potassium phosphate buffer and 8.5–11.0 with 0.1 M glycine–NaOH buffer.

*Inhibitor Effects.* To determine the effect of inhibitors, reactions were run at 30 °C in the presence and absence of inhibitor. To study the effect of mercurials on enzyme activity, enzyme solutions containing 0.08 mg of protein/mL were dialyzed against 10 mM potassium phosphate buffer, pH 7.6, and incubated at 30 °C with either $HgCl_2$ or PCMB at different concentrations in 50 mM potassium phosphate buffer, pH 7.6. At defined time intervals, aliquots of the incubated enzyme were withdrawn and immediately assayed for the remaining activity at 30 °C. The effect of inorganic ions on the inhibition of the enzyme by PCMB was determined by incubating dialyzed enzyme solution and PCMB in the presence and absence of inorganic ion.

*Heat Stability.* Enzyme solutions containing 0.08 mg of protein/mL were incubated in prewarmed test tubes at different temperatures. Aliquots of the heated enzyme solutions were taken at appropriate time intervals and immediately assayed for remaining activity at 30 °C. To study the effect of substrates on heat stability, the enzyme was incubated in the absence and presence of either $NADP^+$ or shikimic acid.

## RESULTS AND DISCUSSION

**Purification of Shikimate Dehydrogenase.** Typical protein and activity data for the various stages of purification of cucumber SDH are given in Table I. Results presented are representative of six separate purification trials. The specific activity increased approximately 7-fold by the purification procedure adopted with 2.7% recovery. Although the yield of pure enzyme afforded by the procedure was low, it provided sufficient material for characterization of the enzyme properties.

Various purification methods were tried to attain maximum purification with minimum loss of activity. The highest activity recovered was obtained by the method summarized in Table I. Our overall yield of 2.7% compares very favorably with the yield of 1.05% obtained by Koshiba (1978) using seven purification steps. The SDH was moderately stable in crude extracts and became unstable as the purification proceeded. This instability reached a maximum in the DEAE-cellulose step. There was a great loss of enzyme activity, with only 10% of the applied activity being recovered in the eluate. SDH activity could not be stabilized even by the addition to the elution buffer of the most protective agents normally used (DTE, L-cysteine, and 2-mercaptoethanol). Further purification was done by hydroxyapatite chromatography and similarly to the elution from the DEAE-cellulose column; the enzyme was eluted in a single peak, and about 34% of the activity applied was recovered in the fraction with the highest activity (Figure 1). This fraction was used for studies of activity characteristics. Electrophoresis of the purified enzyme showed two bands of SDH activity visualized by specific staining. On the basis of position on the gel these bands corresponded to the protein band stained by Coomassie brilliant blue (Figure 2). We may thus conclude that the enzyme consists of two isoenzyme forms with different electrophoretic mobilities and that the SDH preparation was free from contaminant protein. Examining a highly purified SDH from *P. mungo* seedlings, Koshiba (1978) also observed the heterogeneous nature of the enzyme. Similarly, two major bands of SDH were separated from crude extracts of peas by PAGE (Feierabend and Brassel, 1977; Rothe, 1974).

**Table I. Purification of Shikimate Dehydrogenase from Cucumber Fruit**

| procedure | total units, nkat | total protein, mg | sp act., units/mg of protein | purifn, factor |
|---|---|---|---|---|
| crude extract | 212.8 | 60 | 3.5 | 1 |
| $(NH_4)_2SO_4$ (30–90%) | 155.8 | 39.5 | 3.9 | 1.1 |
| Sephadex G-25 | 146.9 | 11 | 13.3 | 3.7 |
| DEAE-cellulose | 17.3 | 1.2 | 14.4 | 4 |
| hydroxyapatite | 5.8 | 0.23 | 25.4 | 7 |

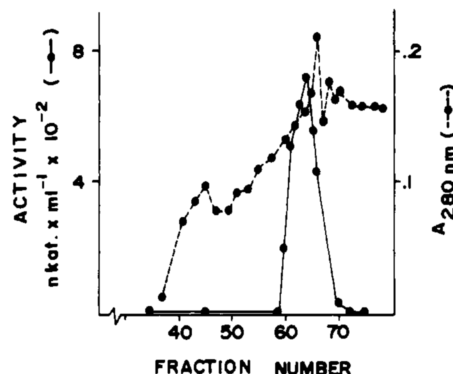


Figure 1. Hydroxyapatite elution of SDH from cucumber. The column was eluted with a linear gradient of potassium phosphate buffer (0.01–0.2 M), pH 7.5, containing 1 mM EDTA, 1 mM L-cysteine, and 8 mM 2-mercaptoethanol. The eluate was collected in 3-mL fractions. (●–●) Enzyme activity; (●- - -●) absorbance at 280 nm.

**Physicochemical Properties.** The native molecular weight of the cucumber enzyme, 43 000, is close to the value of 48 000 reported for the enzyme from *Physcomitrella patens* (Polley, 1978) and somewhat lower than the value of 57 000 found for *P. mungo* enzyme (Koshiba, 1978). The enzyme was eluted in a single peak from a Sephadex G-100 column, thus indicating the homogeneity of the SDH with respect to MW. The optimal activity occurred at pH 8.7. Almost all plant SDHs have optimum

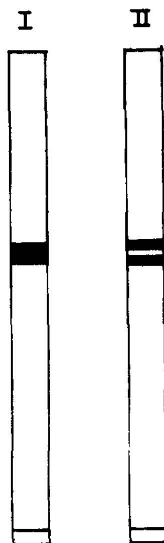


**Figure 2.** Polyacrylamide gel electrophoresis of SDH after chromatography on hydroxyapatite column. (I) Protein band stained by Coomassie brilliant blue R-250; (II) shikimate dehydrogenase activity bands.

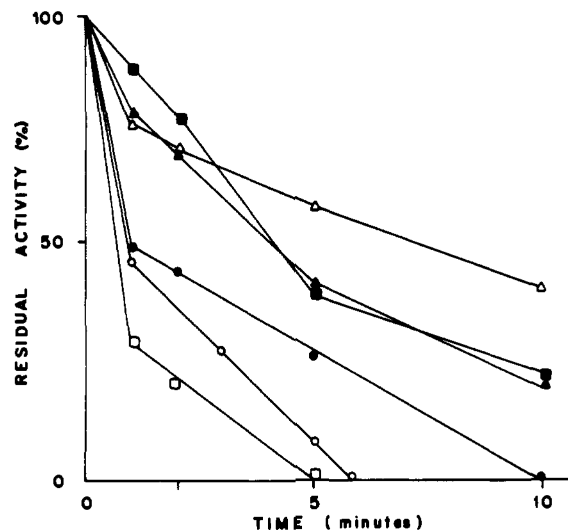


**Figure 3.** Kinetics of SDH inhibition by $HgCl_2$ and PCMB. The enzyme solution (0.08 mg of protein/mL) was incubated in 50 mM potassium phosphate buffer, pH 7.6, at 30 °C with the inhibitors. (△) 0.025 mM HgCl; (○) 0.05 mM HgCl; (●) 0.25 mM PCMB; (□) 0.5 mM PCMB. Kinetics of SDH inhibition by PCMB in the presence of NaCl or KCl. (●) Control with inhibitor (0.25 mM); (■) inhibitor with 160 mM NaCl; (▲) inhibitor with 160 mM KCl. The experimental conditions were the same as described above.

activities at pH 8.0–10.0 (Nandy and Ganguly, 1961; Sanderson, 1965; Dowsett et al., 1972; Lourenço and Neves, 1984). The optimum temperature was determined between 20 and 60 °C. The activity increased with temperature from 20 to 50 °C, with the optimum occurring at 50 °C. Above 50 °C, the enzyme became unstable with a great loss of activity.

The $K_m$ values of SDH for shikimic acid and $NADP^+$ were $6 \times 10^{-5}$ and $1 \times 10^{-5}$ M, respectively. The ability of several phenolic acids (quinic, ferulic, cinnamic, and protocatechuic acids) at different concentrations (0.1–10 mM) to inhibit the enzyme was examined. Protocatechuic acid was the only compound that displayed strong inhibitory activity. The inhibition kinetic study revealed a competitive-type inhibition, and a $K_i$ value of $2 \times 10^{-5}$ M was obtained from a Dixon (1953) plot at two substrate concentrations. These $K_i$ values compare favorably with those reported for pea and heart-of-palm enzymes (Feierabend and Brassel, 1977; Lemos Silva et al., 1985). In addition, the enzyme activity was also inhibited by $Zn^{2+}$ (5 $\mu$M) and $Cd^{2+}$ (0.5 $\mu$M), the latter having the highest inhibitory effect.

Although it has been recognized that SDH from a variety of sources is inactivated by SH inhibitors, additional research is needed to determine the kinetic behavior of the enzyme toward these reagents. On the basis of this observation the kinetics of SDH inhibition with two SH inhibitors, $HgCl_2$ and PCMB, were studied. The kinetics of reaction of the enzyme with the reagents is shown in Figure 3. The inhibition by PCMB apparently occurs in two phases: a rapid inhibition leading to a 70% loss of activity and a slower inhibition phase causing an additional 30% loss of activity after 5 min of incubation. Similarly, the inhibition of $HgCl_2$ was biphasic and resulted in a complete loss of activity at 50 $\mu$M, and it was noticed that the time course of inactivation was dependent on the concentration of the reagents as shown in Figure 3. Despite the observation that PCMB was less effective than $HgCl_2$, PCMB was employed in the subsequent experiments since $HgCl_2$ can inhibit enzymes by binding to protein groups other than SH. The effect of PCMB on the apparent $K_m$ value of the enzyme was determined. The Lineweaver–Burk plots obtained with or without inhibitor were linear and intersected at a single point on the $Y$ axis, indicating a competitive-type inhibition. The $K_m$ value obtained with shikimic acid as substrate in the absence and presence of inhibitor increased significantly from $6 \times 10^{-5}$ to $5 \times 10^{-3}$ M, indicating that the blocking of sulfydryl group(s) by PCMB reduced the access of the substrate to the enzyme.

Since the enzyme was completely inhibited by increasing concentration of the reagents (Figure 3), it seems probable that sulfydryl groups act as a functional group in the catalysis of this enzyme, and the time course of reaction suggests that there are two different classes of SH groups in the enzyme: one class that lost its activity rapidly and a second class with a much lower inhibition rate. Another possibility is that the biphasic inhibition curves of SDH might be due to its composition of isoenzyme species.

It has been assumed that the increase in the concentration of any ligand capable of complexing with the mercurials should reduce the inhibition of SH enzymes, but this possibility has not been investigated in depth. Recently, Nakano (1982) noted that the inhibition of renal trehalase by $HgCl_2$ was suppressed by concentrations of inorganic ions. These observations led us to investigate the effects of NaCl and KCl at high ionic strength on SDH inhibition by PCMB. When the enzyme was assayed in the presence of inhibitor and NaCl, the activity loss decreased by comparison to controls without the anion (Figure 3). To clarify whether this partial protective effect was caused by $Na^+$ or $Cl^-$, we examined the effect of KCl on the inhibition. The results in Figure 3 show that KCl had a similar effect, suggesting that $Cl^-$ is the "active ion". On the other hand, when the mercurial was incubated with the enzyme for 2 min followed by NaCl or KCl addition to the incubation mixture, the salts did not reverse the inhibition caused by PCMB. Despite the paucity of experimental data available in the literature, on the basis of the present results it is not unreasonable to speculate that the protective effect of the salts on SDH activity may be due to (1) a direct complexing of the free inhibitor by $Cl^-$, (2) an ionic strength effect, or (3) a competition of inhibitor and salt for a common binding site on the enzyme.

In the present study, it was also observed that the inhibition of the enzyme by PCMB was partially reversed by thiols. When DTE and L-cysteine were added at 50

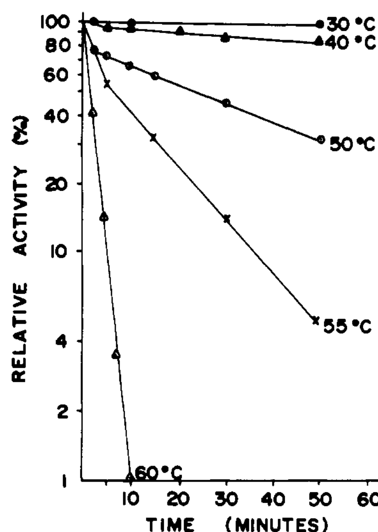


**Figure 4.** Heat inactivation of SDH at different temperatures. Enzyme solutions containing 0.08 mg of protein/mL were incubated in 0.1 M glycine–NaOH buffer, pH 8.7. (●) 30 °C; (▲) 40 °C; (⊙) 50 °C; (×) 55 °C; (▲) 60 °C.

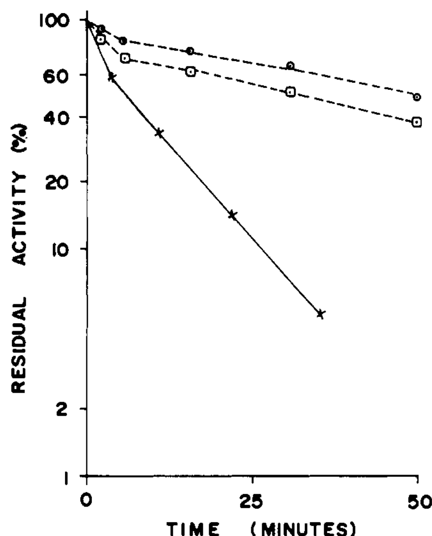


**Figure 5.** Heat inactivation of SDH in the presence of substrates. The enzyme solutions containing 0.08 mg of protein/mL were incubated at 55 °C in 0.1 M glycine–NaOH buffer, pH 8.7, with either shikimic acid or NADP+. (×) Control; (⊙) 4 mM shikimic acid; (⊡) 1 mM NADP+.

mM concentration to an enzyme sample that had been reduced to 50% of its original activity, the enzyme regained 25 and 20% of activity, respectively, within 2 min. However, when the thiol concentration was further increased to 100 mM, the reversal remained unchanged. Also, increasing the reversal time of the inhibited enzyme did not increase the recovery of activity.

Data showing the effect of heating time and temperature on the enzyme activity are presented in Figure 4. The heat inactivation plot at temperatures below 60 °C was biphasic, probably due to the heterogeneity of SDH present in the preparation as demonstrated in Figure 2. The enzyme was active and reasonably stable at temperatures between 40 and 55 °C. After heat treatment at 50 and 55 °C for 5 min, the enzyme retained 70 and 65% of the original activity, respectively, but the activity decreased by 95% when heated at 55 °C for 50 min. When the heat treatment was increased to 60 °C, the enzyme was fully inactivated within 10 min. The effect of substrates on heat inactivation of SDH was investigated (Figure 5). There was a marked protective effect on the enzyme activity when SDH was held at 55 °C and pH 8.7 for 50 min in the presence of either shikimic acid or NADP+. The results shown in Figure 5 indicate that both substrates did influence the stability of SDH, with shikimic acid appearing to be only slightly more effective in protecting the enzyme, and it should be noted that inactivation reached only 40–45% after 50 min of incubation, a value much lower than those obtained in the absence of substrates. It appears that the specific binding of each substrate to the enzyme increased its heat stability as measured by loss of activity. It is conceivable that the binding of the substrates brings about conformational changes, thereby increasing the protein structure resistance to heat inactivation.

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**Registry No.** SDH, 9026-87-3; $NADP^+$, 53-59-8; PCMB, 138-85-2; $HgCl_2$, 7487-94-7; shikimic acid, 138-59-0.