Detection and Kinetic Properties of Alcohol Dehydrogenase in Dormant Corm of *Crocus sativus* L.

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Abstract

An extract from dormant *Crocus sativus* L. corm was obtained which exhibited high alcohol dehydrogenase activity at optimum pH 8.5. The enzyme obeyed Michaelis-Menten kinetics with either ethanol or NAD^+ as variable substrate and there appeared to be no cooperative interaction between the two active sites of this protein. For ethanol, K_m was 13 ± 1 mM and V_{max} was 6.2 ± 0.5 nmol.min^{-1} (mg protein)$^{-1}$. For NAD^+, K_m was 1.12 ± 0.04 mM and V_{max} was 8.2 ± 0.6 nmol.min^{-1} (mg protein)$^{-1}$. Catalytic efficiency (calculated per mg protein in the extract) was (5 ± 0.5) x 10^{-4} min^{-1} with ethanol as the variable substrate, and it was (7.3 ± 0.6) x 10^{-3} min^{-1} with NAD^+ as the variable substrate. When ethanol concentration was above 0.5 M, substrate inhibition was observed, while when NAD^+ concentration was 0.005 M, substrate inhibition was also observed. Acetaldehyde inhibited ethanol oxidation rapidly; 50% inhibition was observed with 0.016 M acetaldehyde. *C. sativus* alcohol dehydrogenase did not use methanol, 2-propanol, nor isoamylalcohol as substrate, but used glycerol (2.8 ± 0.2 nmol.min^{-1} (mg protein)$^{-1}$ and butanol (3.8 ± 0.3 nmol.min^{-1} (mg protein)$^{-1}$). The enzyme being bifunctional, acetaldehyde and NADH could also be used as substrates. For acetaldehyde, K_m was 1.9 ± 0.2 mM and V_{max} was 30 ± 0.5 nmol.min^{-1} (mg protein)$^{-1}$. For NADH, K_m was 0.3 ± 0.03 mM and V_{max} was 20 ± 1 nmol.min^{-1} (mg protein)$^{-1}$. Catalytic efficiency (calculated per mg protein in the extract) was (1.6 ± 0.5) x 10^{-2} min^{-1} with acetaldehyde as the variable substrate, and it was (6.7 ± 1) x 10^{-2} min^{-1} with NADH as the variable substrate.

INTRODUCTION

Alcohol dehydrogenase catalyzes the oxidation of alcohol to aldehyde. This reaction requires the transfer of a hydride ion from the alcohol substrate to the NAD^+ coenzyme that is reduced to NADH. The reaction can occur in both directions and, depending on the initial conditions, one can make the reaction run in the direction of aldehyde reduction rather than alcohol oxidation.

$$RCH_2OH + NAD^+ \leftrightarrows RCHO + NADH + H^+$$

Studies suggest a link between active site structure , enzyme conformation and the catalytic hydride transfer (Bahnson et al., 1997; Colby et al., 1998). Human alcohol dehydrogenases are dimeric metalloenzymes and have been grouped into five classes. Four classes, I-IV, were defined according to different electrophoretic properties and different substrate specificities (Eklund et al., 1976; Smith, 1986; Yokoyama et al., 1990; Moreno and Pares, 1991); class V of human alcohol dehydrogenases was deduced from genomic and cDNA clones (Yasunami et al., 1991).

Alcohol dehydrogenase isozymes occur widely in higher plants. All alcohol dehydrogenase isozymes in plants are dimeric (Scandalios, 1971). In plants such as maize, tomato and barley, alcohol dehydrogenase synthesis is induced by anoxia during flooding (Tanksley and Jones, 1981). The ADH_1 isozyme is present during germination (Scandalios, 1971; Tanksley and Jones, 1981) while ADH_2 is only synthesized during anoxia and hypoxia in flooding (Loveday et al., 1983; Tihanyi et al., 1989). The close link between development and enzyme activity suggests that plant hormones also control

the synthesis of alcohol dehydrogenase (Efron and Schwartz, 1968; Kimmerer, 1987).

Besides functioning in carbohydrate and alcohol metabolism, alcohol dehydrogenase is also involved in fatty acid oxidation and cholesterol biosynthesis.

MATERIALS AND METHODS

Extract Preparation

Dormant corms used throughout these studies were without roots and shoots. Extracts were prepared from corms weighing each between 3 to 6 g, by homogenization in phosphate buffer 0.01 M, pH 7.00, containing 0.02% phenylmethanesulfonyl fluoride as protease inhibitor. After centrifugation at 3,000 g for 10 min, a pellet was obtained which was discarded; the supernatant was recentrifuged at 35,000 g for 30 min. A clear, transparent supernatant termed "crude extract" was obtained and used for our studies. Protein determination was done by the Lowry method.

Enzymatic Activity Assay

Alcohol dehydrogenase activity was assayed in corm extract for the reaction of alcohol oxidation to aldehyde, with concomitant reduction of NAD^+ to NADH, and for the reaction of aldehyde reduction to alcohol, with concomitant oxidation of NADH to NAD^+.

All results presented are averages of three separate experiments.

1. Alcohol → Aldehyde. Alcohol dehydrogenase activity was determined by following spectrophotometrically the rate of reduction of NAD^+ at 340 nm. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate the amount of NADH produced. The reaction, conducted in phosphate buffer 0.1 M, pH 8.5, with ethanol and NAD^+ as substrates, was initiated by the addition of corm extract. When ethanol was the variable substrate, concentrations from 0.002 to 0.75 M ethanol were used with NAD^+ 3 mM; when NAD^+ was the variable substrate, concentrations from 0.4 to 25 mM NAD^+ were used with ethanol 0.3 M. The enzymatic activity was also assayed using methanol, 2-propanol, isoamylalcohol or glycerol instead of ethanol.

2. Aldehyde → Alcohol. For the reverse reaction, the same procedure as that described above was followed except that acetaldehyde and NADH were used as substrates and that, this time, the amount of NADH consumed was measured at 340 nm. When acetaldehyde was the variable substrate, concentrations from 0.18 to 18 mM acetaldehyde were used with NADH 0.3 mM; when NADH was the variable substrate, concentrations from 0.01 to 0.33 mM NADH were used with acetaldehyde 9 mM.

3. pH Activity Profile. pH activity profiles were determined by measuring the activity at various pH according to the methods described above but using a citrate-phosphate-borate buffer system (range 3-12) at a concentration of 0.1 M; ethanol was 0.3 M and NAD^+ was 0.3 mM.

4. Effect of Temperature. To study the thermal stability of the enzymatic activity, aliquots of corm extract were incubated at various temperatures for either 5 or 15 min and their activity was then measured at room temperature after brief cooling in ice. The control was the activity measured without preincubation.

RESULTS AND DISCUSSION

Substrate Specificity

Under our experimental conditions, dormant corm extracts showed alcohol dehydrogenase activity using ethanol as a substrate in the presence of NAD^+ at the optimum pH of 8.5. In addition, other alcohols were also used as substrate in the presence of NAD^+, although with less efficiency than ethanol, in the following order:

Ethanol > Butanol > Glycerol

However, alcohol dehydrogenase activity was not detected in *Crocus sativus* corm extract under our experimental conditions, using either methanol, 2-propanol or isoamylalcohol as substrate in the presence of NAD^+.

Like other alcohol dehydrogenases, the enzyme present in dormant corm was bifunctional, using acetaldehyde as well as ethanol as substrate in the presence of either NADH or NAD^+, respectively.

Effect of pH

Figure 1 shows the rate of oxidation of ethanol to acetaldehyde by dormant *C. sativus* corm extract in the presence of NAD^+ at different pHs ranging from 3.0 to 10.0. Two peaks were found at pH 5.5 and 8.5, respectively, suggesting the presence of two isoenzymes in dormant corms. The pH range where activity was detected was in agreement with data reported for liver alcohol dehydrogenase (McFarland and Chu, 1975; Eftink and Byström, 1986).

Figure 2 shows the rate of reduction of acetaldehyde to ethanol by the corm extract in the presence of NADH at different pHs ranging from 4.5 to 10.0. Again two peaks were found, one at pH 5.5 and the other at pH 7.0. The shift to lower pH for the main peak was to be expected for this reverse reaction.

Ethanol

Figure 3 shows the variation in the rate of NAD^+ reduction as a function of ethanol concentration at pH 8.5, in the presence of 3 mM NAD^+. The maximum rate was observed for a substrate concentration of 200 mM. Thereafter a plateau was reached up to a concentration of 500 mM. Subsequent increase in substrate concentration produced an inhibition of the enzymatic activity so that the activity was reduced by 60 % for a substrate concentration of 750 mM.

NAD^+

Figure 4 shows the variation in the rate of reduction of NAD^+ as a function of NAD^+ concentration at pH 8.5, in the presence of 0.3 M ethanol. The maximum rate was observed for a NAD^+ concentration of 3 mM. Thereafter a small plateau was reached up to a substrate concentration of 5 mM. Then a biphasic substrate inhibition was observed: a rapid phase up to NAD^+ concentration of 8.3 mM, followed by a slower phase. Thus, for NAD^+, the activity was reduced by 70 % for a substrate concentration of 25 mM.

Acetaldehyde and NADH

Table 1 shows the K_m, V_{max} (calculated per mg extract protein), catalytic efficiency (calculated per mg extract protein) and the pseudo-first order rate constant for alcohol dehydrogenase activity found in dormant *C. sativus* corm at pH 8.5 for ethanol, NAD^+, acetaldehyde and NADH used as substrates. *C. sativus* alcohol dehydrogenase exhibited the highest affinity for NADH (K_m = 0.30 ± 0.03 mM) and the lowest for ethanol (K_m = 13 ± 1 mM); affinity for NAD^+ and acetaldehyde were similar (K_m = 1.12 ± 0.04 mM and K_m = 1.9 ± 0.2 mM, respectively).

Effect of Temperature

1. Alcohol → Aldehyde. Figure 5 and 6 show the effect of temperature on the alcohol dehydrogenase activity found in *C. sativus* dormant corm extract with ethanol 0.3 M and NAD^+ 0.3 mM used as substrates. When the extract was incubated for 5 min at temperatures between 25ºC and 40ºC, and then tested at room temperature, the activity remained unaffected. It decreased slightly (by 15 to 20 %) when incubated at 45ºC or 50ºC but showed enhance activity (up to 115 % of the control) when incubated at 55ºC. After incubation at 60ºC to 70ºC, the extract lost 70 % of its alcohol dehydrogenase activity. Further decrease in the activity was observed after incubation at 75ºC (85 % loss) or 80ºC (95 % loss) (Figure 5).

When the incubation at various temperatures lasted 15 min, results were similar to

those obtained after 5 min incubation except that an enhancement of activity was observed after incubation at 50ºC instead of 55ºC. The activity dropped to 5 % of the control after incubation at 75ºC instead of 80ºC (Figure 6).

2. Aldehyde ➔ Alcohol. Figures 7 and 8 show the effect of temperature on the alcohol dehydrogenase activity found in *C. sativus* dormant corm extract with acetaldehyde 9 mM and NADH 0.3 mM used as substrates. When the extract was incubated for 5 min at temperatures between 25ºC and 50ºC, and then tested at room temperature, the activity remained unaffected. Thereafter, the activity decreased steadily as the preincubation temperature increased with a sharp drop at 70ºC (from 65 % activity after incubation at 65ºC to 14 % activity after incubation at 70ºC). No alcohol dehydrogenase activity was detected after incubation at 75ºC (Figure 7).

When the incubation at various temperatures lasted 15 min, results were similar to those obtained after 5 min incubation except that the activity remained unaffected until a preincubation temperature of 45ºC instead of 50ºC. Thereafter, the activity decreased steadily as the preincubation temperature increased from 45ºC to 70ºC when no activity was detected (Figure 8).

CONCLUSIONS

Under anaerobic conditions, alcohol dehydrogenase plays a key role in maintaining the continuous flow of glycolysis. The enzyme is commonly found in plants and the synthesis of various isozymes has been related to various stages of the plant development as well as to stress responses. Alcohol dehydrogenase was detected in dormant *C. sativus* corm extract. Like alcohol dehydrogenases from other sources such as human, yeast, or other alcohol dehydrogenases, the enzyme was bifunctional. It oxidized ethanol to acetaldehyde with concomitant reduction of NAD^+ to NADH and it reduced acetaldehyde to ethanol with concomitant oxidation of NADH to NAD^+. Besides ethanol, *C. sativus* alcohol dehydrogenase also used glycerol and butanol as substrates in the presence of NAD^+ but did not use methanol, 2-propanol or isoamylalcohol. Kinetic parameters such as K_m, V_{max}, catalytic efficiency and pseudo-first order rate constant were calculated and reported. Moreover, the pH activity profile ranging from pH 3.0 to 11.0 for the oxidation of ethanol to acetaldehyde in the presence of NAD^+ was obtained and reported; a pH activity profile was also obtained and reported for the reduction of acetaldehyde to ethanol in the presence of NADH. Finally, the effect of temperature on the enzymatic reaction in the direction of ethanol oxidation as well as in the direction of acetaldehyde reduction was investigated and the results reported.

ACKNOWLEDGEMENTS

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Tables

Table 1. Kinetic parameters for the alcohol dehydrogenase activity detected in dormant *Crocus sativus* L. corm extract.

Substrate	K_m (μM)	V_{max} [nmol.min^{-1}(mg prot)^{1}]	V_{max}/K_m [(1)] (min^{-1})	$k^{(2)}$ (min^{-1})
Ethanol	13 ± 1	6.2 ± 0.5	(5.0 ± 0.4) x 10^{-4}	(2.7 ± 0.4) x 10^{-4}
NAD^+	1.12 ± 0.04	8.2 ± 0.6	(7.3 ± 0.5) x 10^{-3}	(4.0 ± 0.6) x 10^{-3}
Acetaldehyde	1.9 ± 0.2	30.0 ± 0.5	(1.6 ± 0.5) x 10^{-2}	(3.6 ± 0.4) x 10^{-1}
NADH	0.30 ± 0.03	20 ± 1	(6.7 ± 1.0) x 10^{-2}	(1.2 ± 0.04) x 10^{-2}

(1)Expressed per mg extract protein; (2)Pseudo-first order kinetic constant

Figures

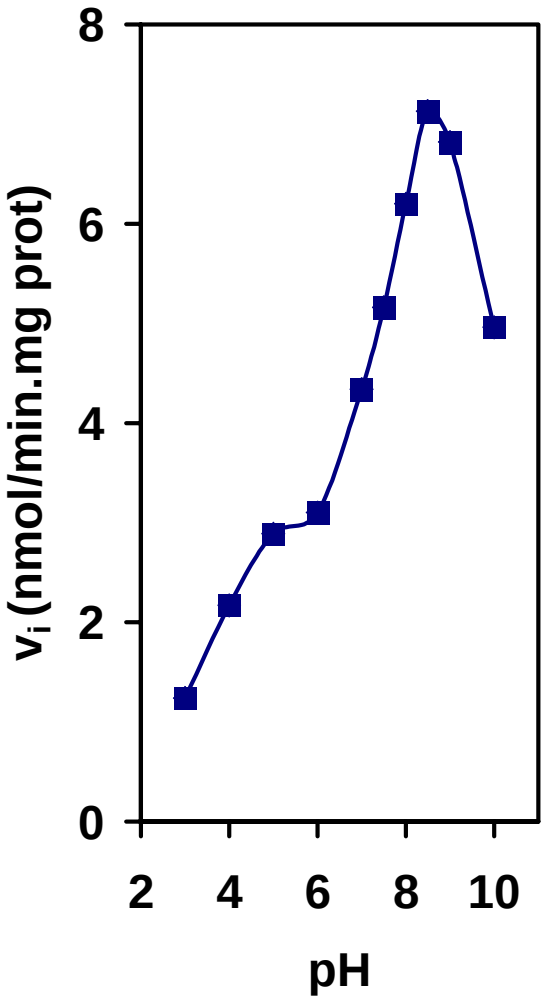

Fig. 1. pH activity profile of the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with ethanol and NAD^+ as substrates.

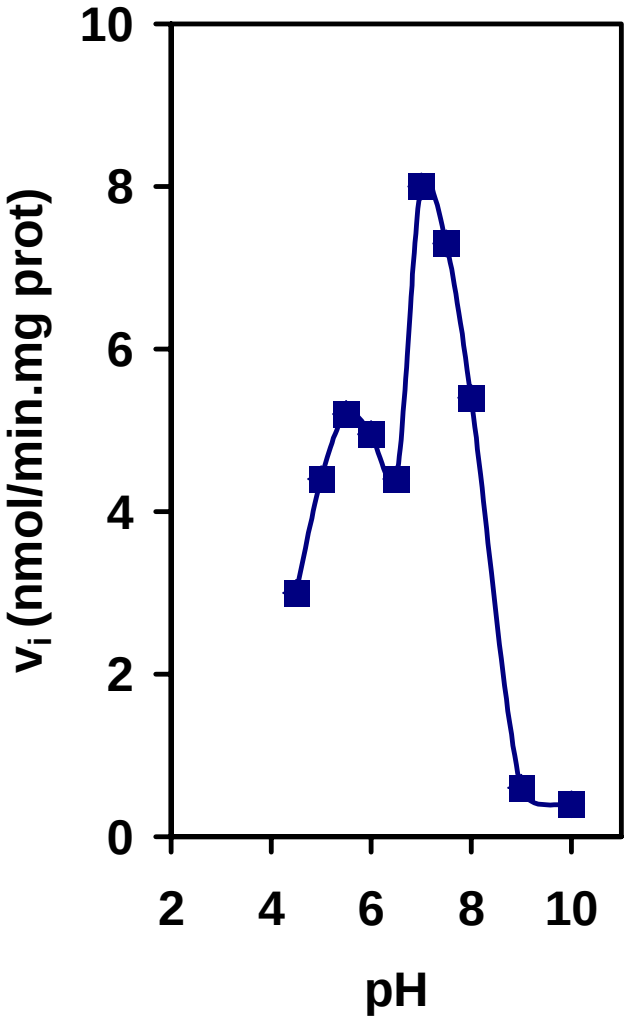

Fig. 2. pH activity profile of the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with acetaldehyde and NADH as substrates.

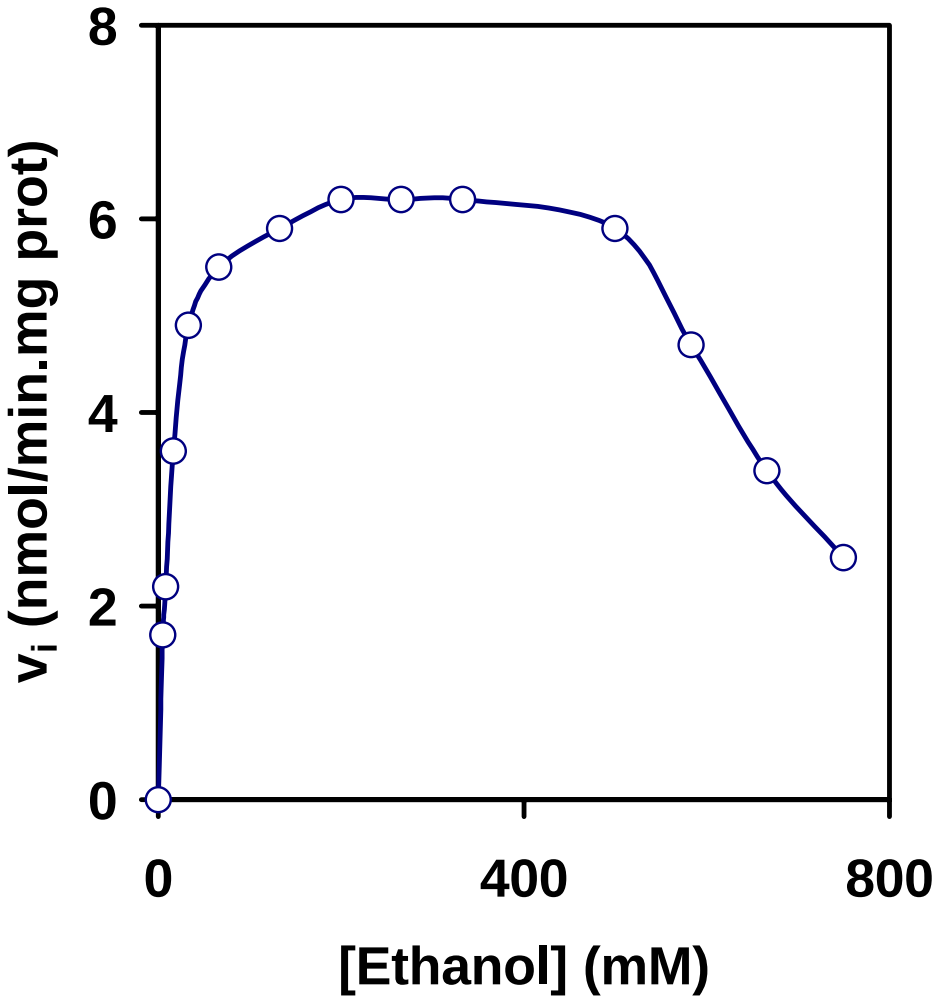

Fig. 3. Michaelis-Menten plot of *C. sativus* alcohol dehydrogenase activity detected in dormant corm extract as a function of ethanol concentration. NAD^+ concentration was kept at 3 mM.

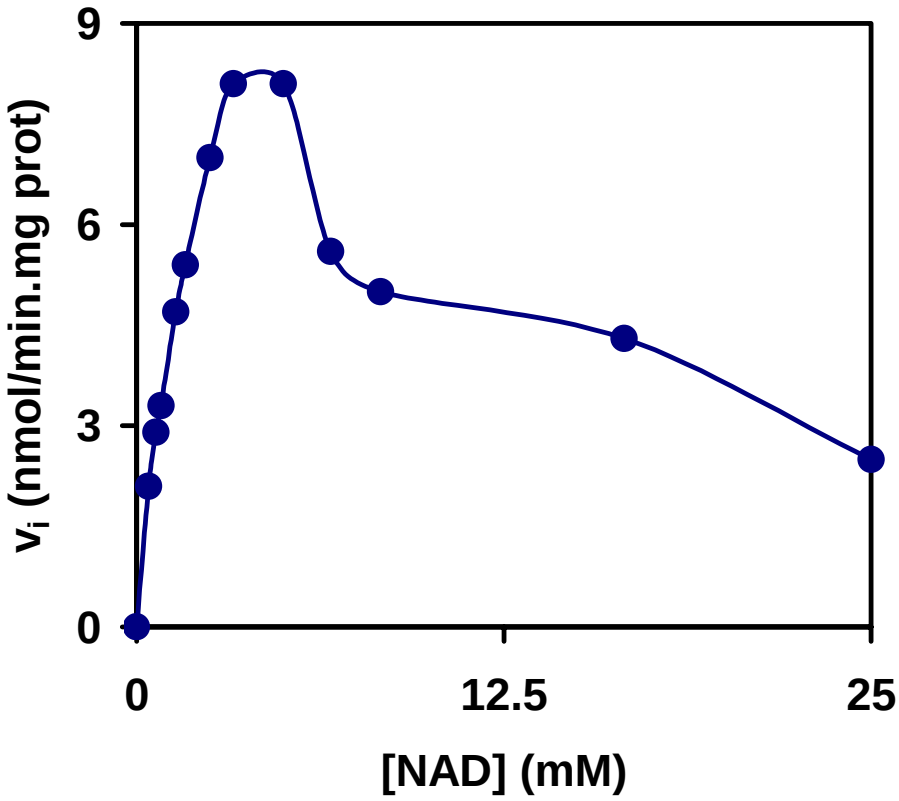

Fig. 4. Michaelis-Menten plot of *C. sativus* alcohol dehydrogenase activity detected in dormant corm extract as a function of NAD^+ concentration. Ethanol concentration was kept at 0.3 M.

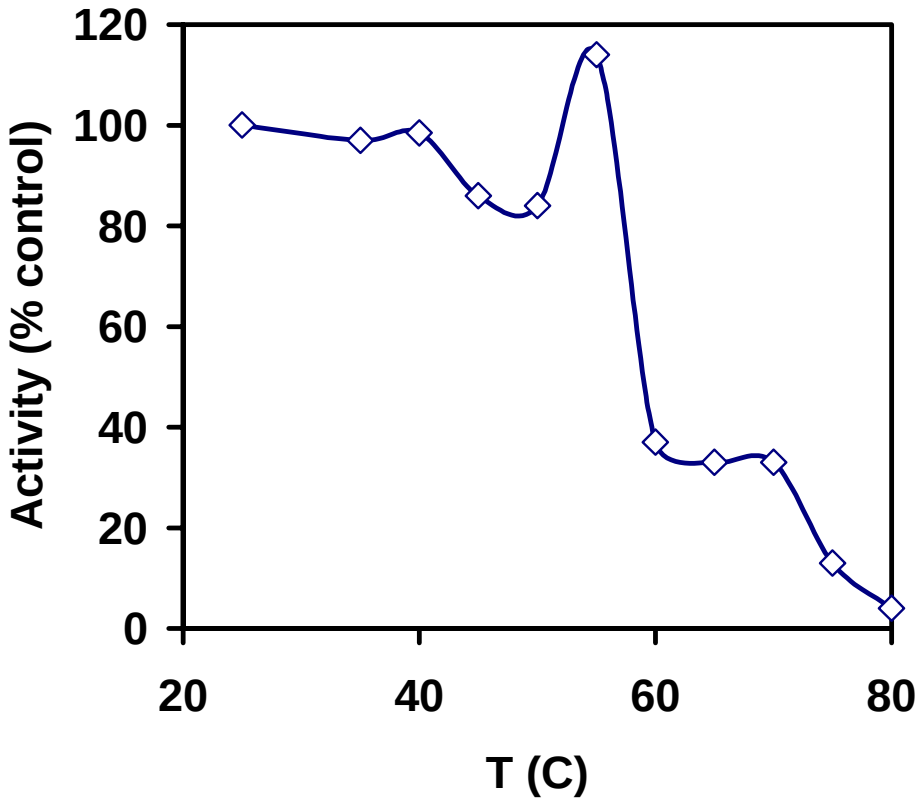

Fig. 5. Effect of temperature on the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with ethanol and NAD^+ as substrates. The extract was preincubated for 5 min at the indicated temperature.

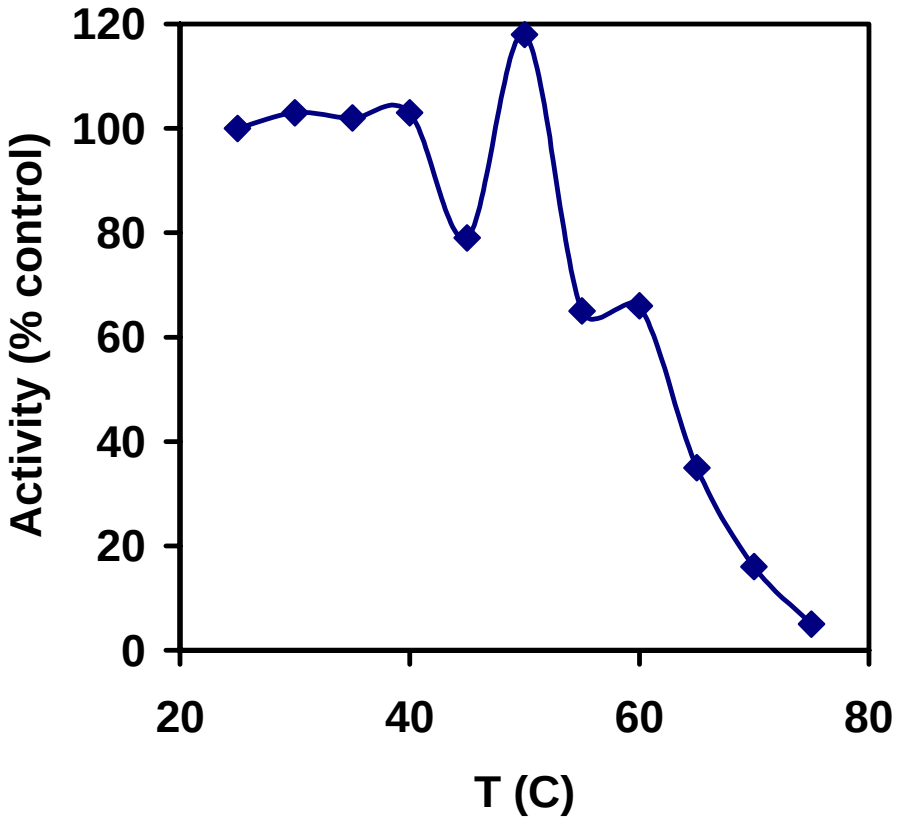

Fig. 6. Effect of temperature on the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with ethanol and NAD^+ as substrates. The extract was preincubated for 15 min at the indicated temperature.

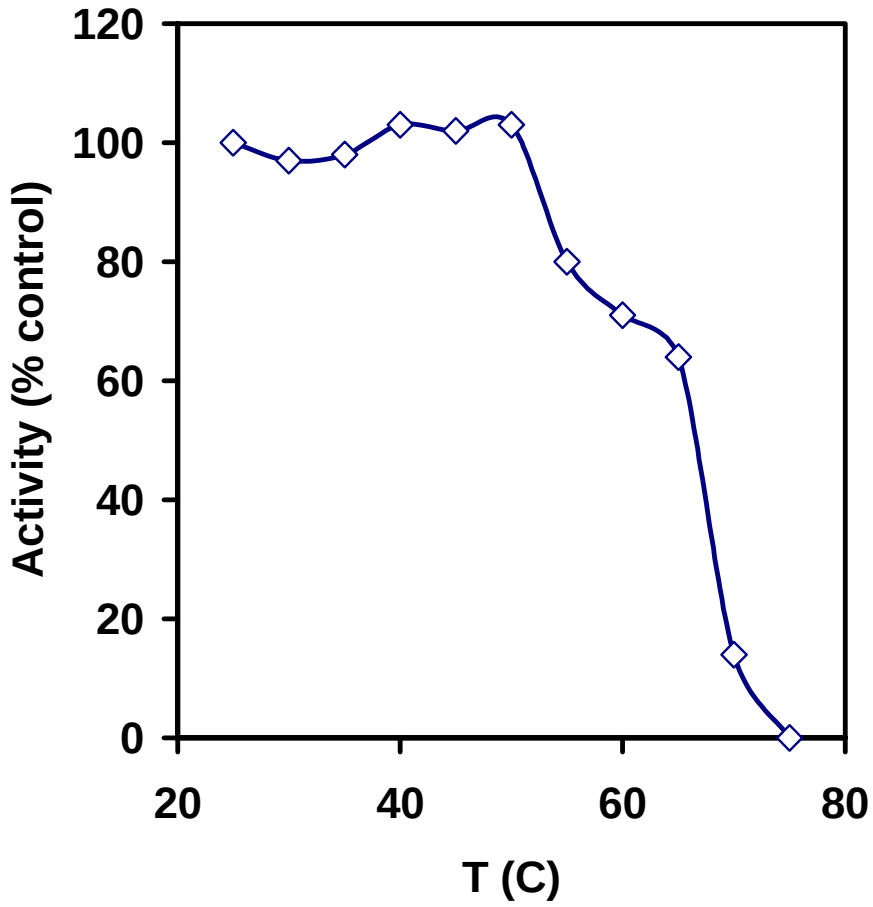

Fig. 7. Effect of temperature on the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with acetaldehyde and NADH as substrates. The extract was preincubated for 5 min at the indicated temperature.

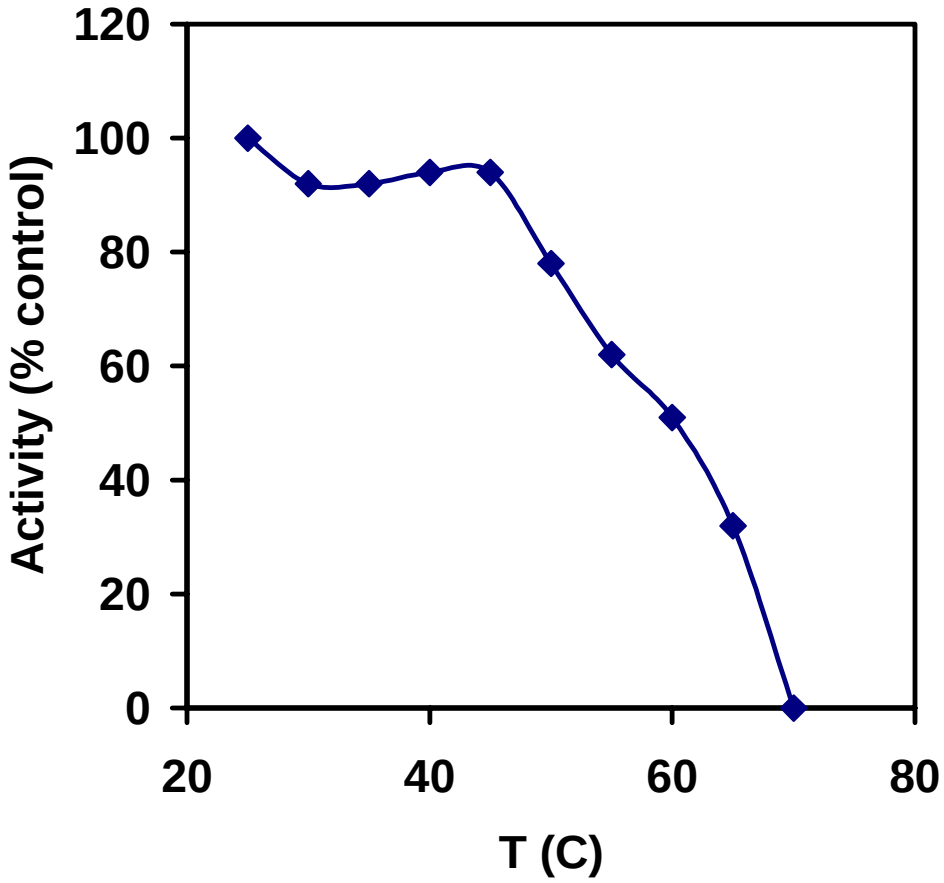

Fig. 8. Effect of temperature on the alcohol dehydrogenase activity detected in *C. sativus* dormant corm extract with acetaldehyde and NADH as substrates. The extract was preincubated for 15 min at the indicated temperature.