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Kinetic Studies with Crude Tomato Alcohol Dehydrogenase

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Abstract: Tomato alcohol dehydrogenase (ADH) is very important in the formation of fresh tomato aroma volatiles. The kinetic characterization and isolation of this enzyme may promote some *in vitro* applications. Kinetic studies of crude tomato ADH associated with NAD^+ cofactor revealed Km values of 2.03 mM for ethanol and 0.064 mM for NAD^+, a Vmax value of 24.51 µM/min, and an activation energy of 0.0208 kcal/mole. Variable pH measurements indicated that the enzyme is optimally active in a narrow range above pH 8.5. Dalziel coefficients were consistent with a sequential but not ordered mechanism. Also the enzyme was found to be heat labile. Crude tomato alchol dehydrogenase can be a suitable enzyme for some biotechnological applications.

Key Words: tomato, alcohol dehydrogenase, Dalziel coefficients, enzyme kinetics

Ham Özüt Domates Alkol Dehidrojenazıyla Kinetik Çalışmalar

Özet: Domates alkol dehidrojenazı (ADH), taze domates uçucu aromatiklerinin oluşumunda çok önemlidir. Bu enzimin kinetik karakterizasyonu ve izolasyonu bazı *in vitro* uygulamalara önayak olabilir. NAD^+ kofaktörü gerektiren ham özüt domates alkol dehidrojenazı ile yapılan kinetik çalışmalar sonunda etanol için 2,03 mM ve NAD^+ için 0,064 mM Km değerleri ile 24,51 µM/min Vmax değeri, ve 0,0208 kcal/mol aktivasyon enerjisi değeri bulunmuştur. Çeşitli pH ölçümleri sonunda enzimin pH 8.5'in üzerindeki dar bir aralıkta optimum aktiviteye sahip olduğu belirlenmiştir. Dalziel katsayıları düzenli sıralı değil sadece sıralı mekanizma ile uyumludur. Ayrıca enzimin sıcaklığa karşı hassas olduğu bulunmuştur. Ham özüt domates alkol dehidrojenazı bazı biyoteknoloji uygulamaları için uygun bir enzim olabilir.

Anahtar Sözcükler: domates, alkol dehidrojenaz, Dalziel katsayısı, enzim kinetiği

Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is an important enzyme in tomato flavour development (Salunkhe et al., 1972). ADH has some important functions in the oxylipin pathway. The pathway is based on the sequential action of lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) on the free unsaturated fatty acids (Galliard et al., 1977; Gardner, 1995). Fatty acids released in the form of 1,4-pentadiene from tomato *acyl*-lipids serve as the primary substrate for LOX to yield the corresponding 9,13-hydroperoxides. These hydroperoxides can serve as substrates for several distinct pathways in addition to the HPL enzyme system. By the action of HPL, C6 and C9 aldehydes and aldoacids are produced (Salunkhe et al., 1972). These aldehydes, hexanal and *cis*-3-hexenal, are associated with characteristic "green or grassy" flavour notes. Primary HPL products are further converted by allylic isomerization into the *trans* isomers such as *trans* -2-hexenal, or by ADH-mediated reduction into hexanol, *cis*-3-hexenol and others (Galliard et al., 1977; Salunkhe et al., 1972). In addition, it has been shown that 3-methylbutanal, formed from α-ketisocaproic acid, can be converted by ADH into its corresponding alcohol, 3-methylbutanol (van der Hijden and Bom, 1996).

Although Stein (1983) has indicated the presence of ADH isozymes in both mature green and red ripe tomatoes, Chase et al. (1982) found that there is no activity in green tomatoes. On the other hand, Brady et al. (1990) has identified ADH activity both in mature green and red tomatoes, and determined that activity increases as fruit ripens. The highest ADH activity observed in overripe tomatoes may be responsible for the change of "fresh" flavour notes to the ones described as "processed" or "enzymic" (Buttery and Ling, 1993). The compilation of results suggests that ADH plays a role in

the formation of fresh tomato flavour. In fact, one study reports the use of tomato tissue in the production of green note aromas (Yilmaz, 2001).

Tomato fruit ADH is a dimer with a NAD^+ coenzyme and a molecular mass of 90-100 kDa. Atomic absorption studies have indicated the presence of 15-48 zinc atoms per enzyme dimer. Typical spectrophotometric assays are based on absorbance readings at 340 nm that arise from NAD^+ reduction (Chase et al., 1982; Brady et al., 1990). While NADPH mediated activity was not found, ADH can be made to reduce aldehydes upon substituting NADH for NAD^+. Ethanol and acetaldehyde are the best substrates for tomato ADH (Chase et al., 1982). While there is one study reporting some kinetic parameters of tomato ADH, more questions need to be addressed.

The objective of the present study is to characterize the kinetic parameters of crude tomato ADH.

Materials and Methods

Materials

Tomatoes of the cultivar Florida47 were supplied by a grower in Tifton, Georgia, USA, grown under standard commercial field conditions. Tomatoes were harvested at mature green, pink, and red stages of maturity. Mature green tomatoes were ripened at room temperature (20ºC) for 5 days. Analyses were done at the red stages of maturity. All measurements were done at the research laboratories of the Food Science and Technology Department of the University of Georgia, Athens, Georgia, USA.

Tween 20, EGTA, linoleic acid, sorbitol, $MgCl_2$, PVPP, DTT, PMSF, benzamidine, aminocaproic acid, and β-NAD^+ were purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol, KOH, and glycine were purchased from J.T. Baker (Philipsburg, NJ). Ethyl alcohol was from Aaper Alcohol and Chemical Co. (Shelbyville, KT).

Enzyme Extraction

The composition of homogenizing buffer was 150 mM Tris-HCl (pH 8.0), including 250 mM sorbitol, 10 mM $MgCl_2$, 1% glycerol (v/v), 0.2% PVPP (w/v), 5 mM DTT, and the following protease inhibitors: 0.1 mM PMSF, 0.1 mM benzamidine, and 5 mM aminocaproic acid. Isolated and cube cut pericarp tissue was blended at high speed with cold homogenizing buffer (1 g/ml) at a setting of 4 for 30 s with cooling intervals. After filtration through a nylon cloth, the homogenate was centrifuged for 20 min at 12,000 g at 4ºC, and the supernatant was collected as a crude enzyme source and assayed immediately.

Protein Determination

Protein determination of the extract was carried out according to the Bradford (1976) method (protein assay kit, Biorad, Hercules, CA), using a microtitre plate assay equipped with a computer accessory. The calibration curve was determined using IgG (immunoglobulin G) as standard.

ADH Assay

A HP 8451A Diode Array spectrophotometer (190-820 nm range with 2 nm bandwidh) was used to assay ADH activity. The assay method was adapted from Brady et al. (1990). The standard assay mixture contained 1 ml of 50 mM Glycine-KOH (pH 9.6) buffer, including 1 mM NAD^+, 1 ml of 1:8 diluted extract, and 1 ml of 0.5 M ethanol. The change in absorbance was followed at 340 nm at room temperature (20ºC) for 5 min. ADH specific activity is defined as the amount of enzyme required to produce 1 mmol NADH per minute at room temperature. All initial rate measurements were interpolated from the linear part of the graph, and data is presented as the mean value of duplicate measurements.

Initial velocities were examined after diluting the crude enzyme extract in the ratios of 1:2, 1:5 and 1:8. As a factor of 1:8 produced a conveniently measurable rate, this dilution was maintained for all subsequent kinetic determinations.

Dalziel coefficients were determined graphically according to the method of Engel (1996). Plots of [Eo]/Vo versus 1/[Ethanol] were made for 0.05, 0.5, and 1mM NAD^+ concentrations. Slopes and intercepts of these primary plots were replotted against $1/[NAD^+]$ to yield the Dalziel coefficients. The total protein concentration (mg protein / ml) in the crude extract ([Eo]) was assumed to be fixed.

Effect of pH

1 mM NAD^+ was dissolved in 150 mM MES (2-(N-Morpholino)ethanesulfonic acid) (pH 6.0), 0.2 M Hepes-KOH (pH 7.5), 150 mM Tris-HCl (pH 8.5), 50 mM Glycine-KOH (pH 9.6), 50 mM CAPS (3-(Cyclohexylamino)propanesulfonic acid) (pH 10.4), and 50 mM Piperidine (pH 11.12). The assay was carried out

using 0.5 M ethanol as substrate for each assay buffer under standard Michaelis-Menten conditions.

Temperature Stability

The crude extract was held in a preset waterbath at 60, 65, 70 and 80ºC for 1, 2, 3, 4 and 5 min before carrying out each assay.

Determination of Activation Energy

Enzyme activities were measured at several temperatures from –10 to 25ºC. An Arrhenius plot of the initial rate data was performed to determine the activation energy.

Results and Discussion

Crude enzyme extract, diluted by a ratio of 1:8, was selected as the most convenient concentration for the kinetic studies. At this level of dilution, the enzyme concentration was optimum for spectroscopic analysis in terms of precluding lengthy assay times while still maintaining measurement accuracy. Furthermore, this dilution better assured that [Eo] << [So], one consideration required to model initial rate data using the Michaelis-Menten theory.

The initial-rate parameters were measured at pH 9.6 by making initial-rate measurements at 0.05-0.5 M ethanol at a series of fixed NAD^+ concentrations ($[Bo]_1$ = 0.05, $[Bo]_2$ = 0.5, and $[Bo]_3$ = 1 mM). [E]/Vo was plotted versus 1/[Ethanol], and slopes and intercepts were determined by non-linear least-squares analysis of the Michaelis-Menten equation (Figure 1). The slope and intercept data from these primary plots were re-plotted against $1/[NAD^+]$ (Figure 2), and the Dalziel coefficients were calculated (Table 1). Φ_A and Φ_{AB} are the intercept and slope of the Primary slopes vs. $1/[NAD^+]$, and Φ_O and Φ_B are the intercept and slope of the Primary Intercepts vs. $1/[NAD^+]$ (plots, Figure 2), respectively. These kinetic coefficients were determined for ethanol oxidation only with the crude enzyme. The Km values for ethanol and NAD^+ were 2.03 mM and 0.064 mM respectively, and the Vmax was 24.51 µM/min. The value of $\Phi_A\Phi_A$ / Φ_{AB} is found to be greater than Φ_O; hence, the results contradict the findings of Chase et al. (1982) in that the mechanism may not be an ordered sequential one. The difference in the findings may be due to the level of enzyme purity as well as the assay pH (9.6 versus 7.6). Further investigations have been implemented to resolve the dispute concerning the reaction mechanism.

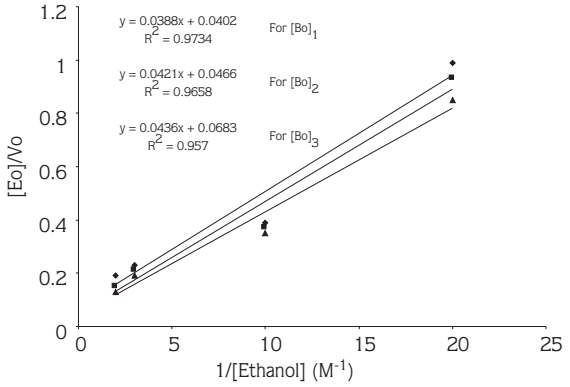

Figure. 1. Primary Plots of the Kinetic Determination at the Three Fixed Concentrations of NAD^+ (◆ - $[Bo]_1$= 0.05, ■ - $[Bo]_2$= 0.5 and ▲ - $[Bo]_3$= 1 mM).

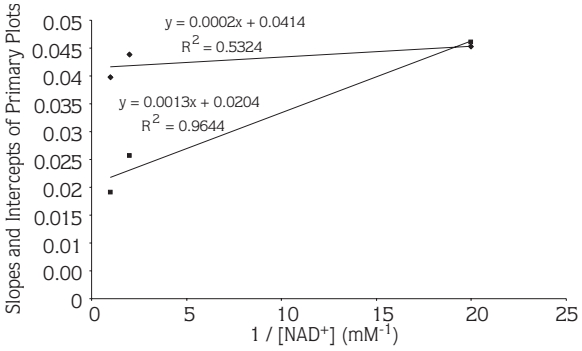

Figure 2. Replots of the Slopes and Intercepts of the Primary Plots vs. $1/[NAD^+]$ (◆ - slopes, ■ - intercepts).

Table 1. Dalziel Coefficients and Kinetic Parameters Determined for Crude Tomato ADH with Ethanol Substrate.

Φ_O (10^{-3} min)	Φ_A (µM min)	Φ_B (µM min)	Φ_{AB} (μM^2 min)	V_{max} (µM min^{-1})	Km^A (mM)	Km^B (mM)	Ks^A (mM)	Φ (min mM^{-1})	$\Phi_A\Phi_B$ / Φ_{AB} (10^{-3} min)
0.0204	0.0414	0.0013	0.0002	24.51	2.03	0.064	0.15	0.041	0.27

The effect of different assay buffers prepared at different pH values on the ionization and activity of the crude tomato ADH is shown in Figure 3. The middle line in the figure shows the log (Vo) versus pH, which indicates slope changes in the enzyme-substrate complex's pK values. Similarly, the bottom plot [log (Vo / Km) versus pH] shows ionizations of the free enzyme, yielding corresponding pK values. It should be pointed out that we have taken the pH of the assay buffer rather than pH of the reaction mixture, so that the estimate must be interpreted accordingly. The three-dimensional structure of horse liver alcohol dehydrogenase at 2.4 Å resolution shows that the enzyme folded into two separate domains, one of which, the dinucleotide-binding domain, binds the cofactor NAD^+. The second domain mediates substrate binding and also harbours the catalytic groups. It was noted that NAD^+ is the first substrate to be bound and NADH is the last product to leave, the dissociation of NADH being the rate-limiting step of the overall reaction; this is one reason why NAD^+ is regarded as a coenzyme rather than a substrate (Branden and Tooze, 1991). These details support our findings of the two dissociation points determined for this enzyme. The enzyme's pKE1 and pKE2 values are 8.5 and 9.6, respectively, according to the slopes. Similarly, ionization constants for the enzyme-substrate complex are 7.5 and 10.4, respectively. Therefore, it may be concluded that an assay buffer pH of 8.5 is an optimum value for activity.

The activation energy (Ea) for catalysis was determined by assaying the enzyme at different temperatures and constructing an Arrhenius plot (Figure 4). From the slope of the plot, the value of Ea was determined to be 0.0208 kcal.mole-1. Heat inactivation data are shown in Figure 5. As seen, while almost all enzyme activity is diminished at 80ºC within 2 min, marginal activity for up to 2 min can be seen at 70ºC. On the other hand, at 60ºC, some activity (12% residual) can be observed after even 4 minutes. It would appear therefore, that the enzyme is heat labile and is inactivated rapidly at higher temperatures.

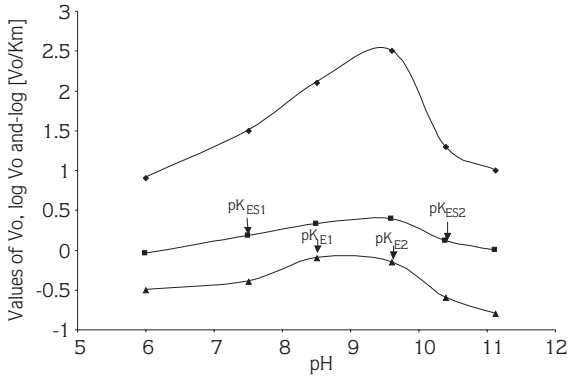

Figure 3. Effect of Ionization State on the Activity of Crude Tomato ADH (◆ - Vo vs. pH, ■ - Log Vo vs. pH, ▲ - log [Vo/Km] vs. pH).

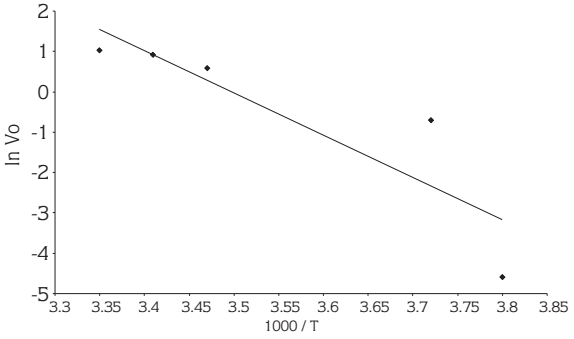

Figure 4. Arrhenius Plot of the Crude Tomato ADH (y = -10.489x + 36.685).

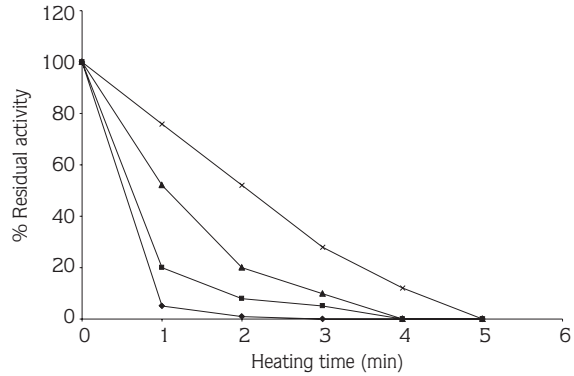

Figure 5. Heat Inactivation of Crude Tomato ADH (◆ - +80ºC, ■ - +70ºC, ▲ - +65ºC, x - +60ºC).

Conclusion

In summary, crude tomato ADH is a sequential-order-of-binding, heat-labile enzyme that optimally functions in a narrow pH range above 8.5. Further purification and isolation studies are envisioned. As tomato ADH is used as a catalyst in aroma bioproduction, mature red tomato tissue represents a cheap and available source of this enzyme. The level of enzyme activity can be adjusted by an appropriate choice of harvesting time or by implementing appropriate genetic modifications.

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