

# Characterizing NAD- and NADP-dependent Alcohol Dehydrogenase Enzymes of Strawberries

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**Abstract.** The NAD-dependent and NADP-dependent alcohol dehydrogenase activities of strawberries (*Fragaria xananassa* Duch.) were found to have broad substrate specificities including those alcohols and aldehydes responsible for strawberry aroma and flavor either directly or through their ester products. NAD-dependent activities were greatest against short-chained alcohols, whereas the NADP-dependent activities were most active against aromatic and terpene alcohols. Differences were seen in substrate specificity between receptacle and achene alcohol dehydrogenase activities. Alcohol dehydrogenase activities were found to be developmentally regulated in receptacle tissue and increased during the period of fruit maturation and ripening. Isoelectric focusing of NAD-dependent ADH activities showed that several isozymes of this enzyme exist, that they differ between receptacle and achene tissues, and that they vary among specific genotypes. Our results suggest that NAD- and NADP-dependent ADH activities are integral components of flavor and fragrance volatile production in ripening strawberries.

NAD-dependent (NAD-ADH; E.C. 1.1.1.1) and NADP-dependent alcohol dehydrogenases [NADP-ADH (cinnamyl alcohol dehydrogenase); E.C. 1.1.1.2] exist in a wide variety of higher plant tissues (Davies et al., 1973; Freeling and Bennett, 1985; Hatanaka et al., 1973; Kanellis et al., 1991; Noueiry, 1989; Roe et al., 1984; Tressl and Albrecht, 1986; Yamashita et al., 1978). Putative roles attributed to plant alcohol dehydrogenases include surviving periods of hypoxia (Freeling and Bennett, 1985; Kanellis et al., 1991; Roe et al., 1984), regulation of development (Mitchell and Jelenkovic, 1992; Roe et al., 1984), production of cinnamyl alcohols for the lignification of cell walls (Davies et al., 1973), protection from chilling (C. Frenkel, personal communication), and biogenesis of flavor and fragrance volatiles (Hatanaka et al., 1973; Noueiry, 1989; Tressl and Albrecht, 1986; Yamashita et al., 1978).

Biosynthetic pathways of alcohols, aldehydes, and esters implicated in flavor and fragrance production have been tentatively determined for many fruit (Tressl and Albrecht, 1986). In strawberries, exogenously applied fatty acids, aldehydes and alcohols (Yamashita et al., 1977; Yamashita et al., 1979) were metabolized to their corresponding alcohols and esters. These results suggested that endogenous alcohols and aldehydes are interconverted by ADH in the pathway for producing the complex mix of volatile compounds (Honkanen and Hirvi, 1990) responsible for the characteristic flavor and fragrance of strawberries.

The present work examines the substrate specificity of strawberry ADH activities, the isozyme pattern of the NAD-dependent ADH, and changes in ADH activities that occur during fruit ripening and development. Our results support the hypothesis that NAD-dependent and NADP-dependent ADH enzymes are important components of the in vivo biosynthetic pathway of strawberry flavor and fragrance volatiles.

## Materials and Methods

**Plant material.** Primary fruit were freshly harvested from strawberry plants that were either grown in a greenhouse (genotype NJ 8343-6) or field (NJ 8336-1, NJ 8611-1, 'Earliglow', 'Raritan').

Four developmental stages of strawberries were used in this study. Stage 1 and stage 2 fruit had fully white receptacles and green achenes. Stage 3 fruit had receptacles with pink and white sections and green achenes. Stage 4 fruit were fully red, fragrant, and had brown achenes. Average fresh weights of stage 1 through stage 4 fruit were  $0.315 \pm 0.056$  g,  $1.204 \pm 0.106$  g,  $6.492 \pm 1.236$  g, and  $8.302 \pm 2.015$  g, respectively.

**Protein extraction from achenes.** Protein extractions from achenes and receptacles were performed at 4°C. Achenes were removed from ripe strawberries, washed to remove debris, and air dried. Achene tissue was ground in 1.5-ml microfuge tubes with tapered glass rods in an extraction buffer composed of 100 mM Tris-HCl, pH 7.5, 2% w/v soluble polyvinylpyrrolidone (PVP) as a phenolic scavenger, 1 mM ZnSO<sub>4</sub>, 10 mM β-mercaptoethanol (BME) as a reducing agent, and the protease inhibitors 0.8 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidinium hydrochloride (BHC), and 5 mM ε-amino-n-caproic acid (ACA). 100 μl of buffer was used per 10 mg of fresh weight. The extract was centrifuged for 15 min at 13,600× g and the supernatant was collected as the enzyme source.

**Protein extraction from receptacles.** Strawberries were harvested and achenes were removed from the receptacles by peeling the epidermis away with razor blades. Achene-free receptacles were then homogenized in an extraction buffer composed of 100 mM Tris-HCl (pH 8.5), 2% w/v PVP, 20 mM CaCl<sub>2</sub>, 0.1 mM ZnSO<sub>4</sub>, 0.8 mM PMSF, 1 mM BHC, 5 mM ACA, and 10 mM BME in a food blender. The extract was filtered through one layer of Miracloth (Calbiochem) and the pH of the supernatant was adjusted to 7.5. The extract was then stirred for 15 min and centrifuged at 30,000× g to remove insoluble residues. The supernatant was made to 80% saturation in ammonium sulfate, stirred for 30 min, and centrifuged 20,000× g for 15 min to precipitate protein. The pellet was resuspended in a minimal volume of a buffer composed of 100 mM Tris-HCl (pH 7.5), 0.1 mM ZnSO<sub>4</sub>, 0.8 mM PMSF, 1 mM BHC, 5 mM ACA, and 10 mM BME and dialyzed against 50 mM Tris-Cl (pH 7.5), 0.1 mM ZnSO<sub>4</sub>, 0.8 mM PMSF, 1 mM BHC, 5 mM ACA, and 10 mM BME.

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The protein extraction method for developmental studies was simpler. Achenes were removed from receptacles as above and the achene-free receptacles were homogenized in a Potter-Elvehjem tissue grinder, in an extraction buffer composed of 100 mM Tris-HCl (pH 8.5), 2% w/v PVP, 0.1 mM ZnSO<sub>4</sub>, 0.8 mM PMSF, 1 mM BHC, 5 mM ACA, and 10 mM BME. The extract was adjusted to pH 7.5 and centrifuged at 13,600× g for 15 min to pellet insoluble debris. The supernatant was used as the enzyme source.

**ADH assays.** All ADH assays were carried out at 24°C. Oxidations of alcohols by ADH were assayed in reaction mixes containing 100 mM glycine-KOH (pH 9.5) and 1.3 mM NAD or NADP, while reductions of aldehydes were assayed in reaction mixes containing 100 mM Tris-HCl (pH 7.5) with 1.3 mM NADH or NADPH. Substrate concentrations of 100 mM were used to developmental course assays. Substrate concentrations of 40 mM were

used when comparing relative activities for different substrates (Tables 2 and 3), except for the substrates cinnamyl alcohol (20 mM) and geraniol (5 mM), which have relatively poor solubility in aqueous solutions. Calculation of NAD(P) formed was assessed by the change in A<sub>340</sub> (Worthington, 1988). Preliminary results with strawberry NAD-dependent ADH gave a K<sub>m</sub> with ethanol as substrate of 6.96 ± 0.32 mM, and strawberry NADP-dependent ADH gave a K<sub>m</sub> with benzyl alcohol of 24.82 ± 1.28 mM. Our K<sub>m</sub> values were similar to those of other plant NAD- and NADP-dependent ADH enzymes (Bicsak et al., 1982; Davies et al., 1973; Roe et al, 1984; Yamashita et al., 1978).

**Native isoelectric focusing.** Native isoelectric focusing (IEF) was performed on a horizontal flat bed Fisher Biotech FN 1001 IEF system according to manufacturers instructions. Gels 1 mm thick were composed of 1% (w/v) agarose, 13% (w/v) sorbitol, 6.7% (v/v)

Table 1. ADH activities of stage four strawberries.<sup>z</sup>

Type of ADH activity	Activity/fresh wt (μmol·min <sup>-1</sup> ·g <sup>-1</sup> )		Activity/fruit (nmol·min <sup>-1</sup> )	
	Achenes	Receptacles	Achenes	Receptacles
NAD-ADH	4970	75.8	467	680
NADP-ADH	1272	11.0	120	99

<sup>z</sup>Activities were determined for primary fruit of genotype NJ 8343-6. NAD-ADH activity was measured using 100 mM ethanol as substrate, and NADP-ADH activity was measured using 100 mM benzyl alcohol as substrate.

Table 2. Substrate specificity of strawberry NAD-ADH.<sup>z</sup>

Substrate	Specific activity (nmol·min <sup>-1</sup> ·mg <sup>-1</sup> )	
	Receptacles	Achenes
Benzyl alcohol	0.0 ± 0.0	14.9 ± 8.6
1-Butanol	295 ± 27	289 ± 25
2-Butanol	48.8 ± 2.8	53.4 ± 5.7
Cinnamyl alcohol	0.0 ± 0.0	9.9 ± 5.7
Ethanol	531 ± 6	621 ± 66
Geraniol	196 ± 33	82.0 ± 9.5
1-Hexanol	46.8 ± 5.4	90.0 ± 4.0
cis-3-Hexen-1-ol	104 ± 6	169 ± 4
Isoamyl alcohol	123 ± 29	119 ± 13
Methanol	0.0 ± 0.0	8.1 ± 4.7

<sup>z</sup>Specific activity for NAD-ADH (genotype NJ 8336-1) was measured using 40 mM substrate except in the cases of cinnamyl alcohol and geraniol, where 20 mM and 5 mM were used, respectively. Values are ± SE.

Table 3. Substrate specificity of strawberry NADP-ADH.<sup>z</sup>

Substrate	Specific activity (nmol·min <sup>-1</sup> ·mg <sup>-1</sup> )	
	Receptacles	Achenes
Benzyl alcohol	64.7 ± 6.7	137 ± 3
1-Butanol	17.6 ± 0.3	32.0 ± 1.3
2-Butanol	0.0 ± 0.0	26.1 ± 1.0
Cinnamyl alcohol	105 ± 3	269 ± 11
Ethanol	0.9 ± 0.3	5.6 ± 0.5
Geraniol	88.3 ± 3.5	103 ± 20
1-Hexanol	50.4 ± 0.6	117 ± 5
cis-3-Hexen-1-ol	29.0 ± 0.3	84.6 ± 3.8
Isoamyl alcohol	11.6 ± 0.6	4.3 ± 0.2
Methanol	0.0 ± 0.0	0.0 ± 0.0

<sup>z</sup>Specific activity for NADP-ADH (genotype NJ 8336-1) was measured using 40 mM substrate except in the cases of cinnamyl alcohol and geraniol, where 20 mM and 5 mM were used, respectively. Values are ± SE.

v) ampholines (pH 4 to 6.5), and 1% (v/v) Triton X-100 (Fisher). The electrode buffers were 0.05 N H<sub>2</sub>SO<sub>4</sub> and 1 N NaOH. Electrophoresis was carried out with 15 W constant power for 2 h at 9C. After electrophoresis, gels were stained for NAD-dependent ADH activity, with ethanol as substrate as by Noueiry (1989).

Staining IEF gels for NADP-ADH activity using benzyl alcohol as substrate was unsuccessful. Eggplant fruit and seed NADP-dependent ADH enzymes were stainable with NADP cofactor and various substrates (including benzyl alcohol and coniferyl alcohol) after nondenaturing polyacrylamide gel electrophoresis (unpublished data). This leads us to believe that NADP-dependent ADH is unstable during IEF.

The pI measurements of histochemically localized ADH isozymes were determined by directly measuring the pH gradient in the gels (Garlin, 1990).

## Results and Discussion

ADH activities in fruit. NAD- and NADP-dependent ADH activities of stage four strawberries are compared in Table 1. NAD-dependent ADH activity was 65.6-fold greater and NADP-dependent ADH was 116-fold greater for achene tissue than for receptacle tissue on a fresh-weight basis. The total NAD-dependent ADH activity of achenes on a stage 4 fruit was 31.3% less than the activity found in receptacles, while the total NADP-dependent ADH activity of achenes was 2.12% greater than the activity found in receptacles. NAD-dependent ADH activity was found to be 3.9- and 6.9-fold greater than the NADP-dependent activities found in achene and receptacle tissues respectively.

The NAD-dependent ADH activity of receptacles (Table 2) was highest against ethanol and showed considerable activity against other unbranched primary alcohols. The receptacle enzyme also showed high activity against terpene (e.g., geraniol), branched (e.g. isoamyl alcohol), and unsaturated six-carbon (e.g., cis-3-hexen-1-ol) alcohols.

Strawberry receptacle NADP-dependent ADH (Table 3) showed greatest activity against aromatic (e.g., cinnamyl alcohol and benzyl alcohol) and terpene alcohols and a lesser activity against four- and six-carbon primary alcohols. A weak correlation existed between the hydrophobicity of each substrate and the activity of the NADP-dependent enzyme for each substrate (e.g., the activity against aromatic alcohols and geraniol > four and six carbon primary alcohols > ethanol).

The reduction of an aliphatic and an aromatic aldehyde by receptacle ADH was assessed. NAD-dependent ADH reduced acetaldehyde at twice the rate it reduced benzaldehyde, whereas, NADP-dependent ADH reduced benzaldehyde at nearly six times the rate of acetaldehyde.

The specificity of the NAD-dependent ADH of achenes (Table 2) was similar to that found in receptacles, except that the achene enzyme showed less relative activity for terpene alcohols and more relative activity for unsaturated six carbon alcohols and aromatic alcohols. Achene NADP-dependent ADH (Table 3) showed lesser affinity for branched alcohols and higher affinity for secondary straight-chained alcohols compared to receptacle NADP-dependent ADH.

Figure 1 shows a zymogram of strawberry achene and receptacle NAD-dependent ADH isozymes after separation by native IEF. Strawberry receptacles exhibited three to six isozymes depending on the genotype. Noueiry (1989) had previously documented the presence of strawberry achene NAD-dependent ADH isozymes. Differences in isozymes might confer differences in the substrate specificities of the enzymes from different sources.

Isozyme differences may account for the differences in substrate specificity we have seen between our achene NAD-dependent ADH and that of Yamashita et al. (1978) since the plants were genetically different.

Developmental control of ADH activity. The hypothesis that NAD-dependent and NADP-dependent ADH activities play important roles in flavor and fragrance production would be disproved if ADH activities did not increase before or during the time of fruit volatile production, that is, before or during the ripening process (stages 3 and 4). The ADH activities of entire strawberry receptacles were determined by enzyme assays for the four growth stages described in materials and methods. Total NAD-dependent ADH activity increased dramatically between stages 2 and 3 and afterward remained at relatively high levels (Fig. 2), whereas total NADP-dependent ADH activity increased gradually from stage 2 to stage 4 (Fig. 3). Specific activities of strawberry receptacle NAD- and NADP-dependent ADH (Figs. 2 and 3) showed their greatest increase between stages 3 and 4. Our hypothesis about fruit ADH has therefore not been violated by the time course of ADH inductions during fruit growth and ripening. Our results agree with the observed developmental increases of NAD-dependent ADH in oranges and tomatoes (Chen and Chase, 1993; Roe et al, 1984) and increases of the NAD- and NADP-dependent ADH of eggplants (unpublished data).

*Functions of fruit ADH.* The well-documented role of NAD-dependent ADH's alleviating hypoxic stress through anaerobic respiration (Freeling et al., 1985) and the role of some NADP-dependent ADH enzymes (cinnamyl alcohol .dehydrogenases), which synthesize cinnamyl alcohols and thereby allow lignification of woody tissues, seem to be inappropriate roles in strawberries. Strawberries have large surface to volume ratios, large intercellular air spaces and lack a thick cuticle (Darrow, 1966), characteristics that allow free gas exchange and reduce the likelihood of fruit hypoxia; their vasculature is unlikely to require significant lignification.

A putative role for strawberry NADP-dependent ADH in chilling protection can not be ruled out. Work on a possible stimulatory role for ADH in strawberry ripening (Mitchell and Jelenkovic, 1992) remains inconclusive.

Alcohols and aldehydes contribute to the characteristic flavor and fragrance of ripe fruit (Tressl and Albrecht, 1986). The alcohols that contribute to the flavor of ripe strawberries (Honkanen

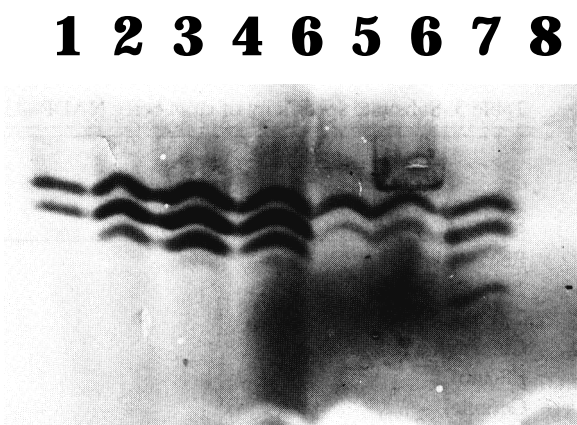


Fig. 1. Native IEF of stage four strawberry fruit NAD-ADH isozymes. Protein from 10 mg of achenes or 250 mg of receptacles were separated and histochemically developed on isoelectric focusing gels. The cathode is at the top of the figure. Lanes from left to right are 1) 'Raritan' achenes, 2) 'Earliglow' achenes, 3) NJ 8336-1 achenes, 4) NJ 861 I-1 achenes, 5) 'Raritan' receptacles, 6) 'Earliglow' receptacles, 7) NJ 8336-1 receptacles, 8) NJ 861 I-1 receptacles.

Fig. 2. Developmental changes in NAD-dependent ADH activities in whole strawberry receptacles (genotype NJ 8336-1) over time. Assays used 100 mM ethanol as substrate as described under materials and methods. Bars represent the SE of the means.

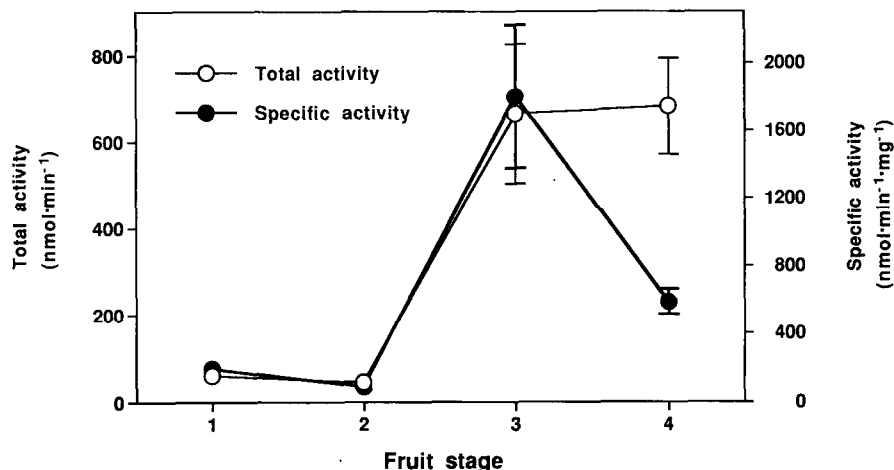
and Hit-vi, 1990) were good substrates for one or both of the ADH activities characterized in this paper. In the future, we may be able to engineer strawberry flavor by selecting for specific fruit ADH isozymes. Genetic transformation of strawberry plants with a previously isolated gene for NAD-dependent ADH (Wolyn and Jelenkovic, 1990) could change the ADH present in the fruit and thereby allow direct analysis of the effect of an added ADH isozyme on fruit flavor and fragrance.

Our present work has indicated that 1) strawberry NAD- and NADP-dependent ADH could use the kinds of alcohols and aldehydes found in ripe strawberries as substrates (NAD-dependent ADH was more active against terpene and aromatic substrates), 2) receptacle NAD- and NADP-dependent ADH substrate specificities differed slightly, from those of the achene, suggesting a difference in ADH isozymes between tissues involved in flavor production (receptacle) and those not involved in flavor production (achenes); 3) NAD-dependent ADH was composed of polymorphic isozymes as shown by native IEF; 4) isozyme differences were seen between receptacle and achene tissues of the same genotype, showing additional ADH isozymes in the flavor production tissue (receptacle); and 5) the level of fruit ADH enzymes increased before and during ripening when they would be needed for flavor and fragrance volatile production. These results strengthen the hypothesis that NAD- and NADP-dependent ADH enzymes function as important components of the *in vivo* biosynthetic machinery of strawberry flavor and fragrance volatiles.

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Fig. 3. Developmental changes in NADP-dependent ADH activities in whole strawberry receptacles (genotype NJ 8336-1) over time. Assays used 100 mM benzyl alcohol as substrate as described under materials and methods. Bars represent the SE of the means.



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