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Cofactor recycling in a coupled enzyme oxidation–reduction reaction: conversion of ω-oxo-fatty acids into ω-hydroxy and dicarboxylic acids

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Aldehydes are reduced to alcohols by the enzyme alcohol dehydrogenase (ADH), whereas the enzyme aldehyde dehydrogenase (AldDH) oxidizes aldehydes to carboxylic acids. ADH and AldDH require, respectively, the reduced and oxidized forms of the cofactor NAD (NAD^+/NADH). By combining both oxidation and reduction reactions into one process, it is possible to produce alcohols and carboxylic acids simultaneously from aldehydes by continuous recycling of the NAD^+/NADH cofactor. However, both enzymes need to be active within the same pH region and buffer system. To test this hypothesis, the pH profile (V_{max} and V_{max}/K_m) as well as the pK_a of the prototropic groups involved in catalysis for both dehydrogenases were determined using (*Z*,*Z*)-nona-2,4-dienal as a model substrate. The pH profile (V_{max} and V_{max}/K_m) of both enzymes overlapped in the pH range of 6–8 in potassium phosphate buffer. When the coupled enzyme system was used at pH 7 with 10% NAD^+ cofactor, over 90% of the starting aldehyde was converted to its corresponding acid and alcohol derivatives in a 1:1 ratio. The sequential action of the enzymes lipoxygenase and hydroperoxide lyase converts polyunsaturated fatty acids to aldehydic fatty acids. The products arising from the oxidation or reduction of the aldehydic functionality are of industrial interest. It was found that 13-oxo-9-(*Z*),11-(*E*)-tridecadienoic acid, the product of the sequential reaction of soya bean lipoxygenase and hydroperoxide lyase from *Chlorella pyrenoidosa* on linoleic acid, is also a substrate in this coupled enzyme system.

Introduction

The major fatty acid found in several vegetable oils, linoleic acid **1**, is oxygenated by the enzyme lipoxygenase (LOX, EC 1.13.11.12) to give the hydroperoxy derivative, 13(*S*)-hydroperoxy-9-(*Z*),11(*E*)-octadecadienoic acid, **2** (Scheme 1). Hydroperoxide **2** can be cleaved by the enzyme hydroperoxide lyase (HPLS) to an ω-oxo-fatty acid and a short volatile fragment [1]. For example, HPLS from microalgae, such as *Chlorella*, cleaves **2** into the C_{13} ω-oxo-fatty acid **3a** and a C_5 fragment (pentane) [1–4], whereas HPLS from higher plants cleaves **2** into the C_{12} ω-oxo-fatty acid **3b** and a C_6 fragment (hexanal) [5,6]. The oxo-fatty acids **3a** and **3b** can be converted to their ω-hydroxy and ω-dicarboxylic acid derivatives **4** and **5**, respectively, by chemical or enzymic methods. These materials have potential application in the production of surfactants, polyesters, and polyamides similar to nylon 13,13. Moreover, the presence of double bonds in products **4** and **5** allows for additional derivatization or cross-linking. However, enzymic methods are needed that can reduce or oxidize **3a** and **3b** in an environmentally safe and cost-effective manner compared with chemical procedures.

Aldehydes are reduced to alcohols by the enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1) using reduced NADH as the cofactor. On the other hand, the enzyme aldehyde dehydrogenase (AldDH, EC 1.2.1.5) and the oxidized cofactor NAD^+ convert aldehydes to carboxylic acids. In general, the need for cofactors in enzymic processes increases their cost to prohibitive levels. However, by combining the oxidation and reduction steps into a single process, it is possible to produce alcohols and carboxylic acids from aldehydes by recycling small amounts of cofactor. Such a redox combination, however, requires enzyme compatibility. In particular, both enzymes must be active under similar reaction conditions and pH [7].

The aldehydic HPLS product **3** contains an ionizable carboxylic acid group, which affects its solubility and binding capacity with an enzyme. Thus the use of oxo-acids **3a** or **3b** might complicate a pH study of ADH and AldDH. Therefore a model substrate without an ionizable group was sought for this study. The model substrate (*Z*,*Z*)-nona-2,4-dienal **6** (Scheme 2) was chosen because it is commercially available,

Abbreviations used: ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; GC/MS; gas chromatography–MS; HPLS, hydroperoxide lyase; LOX, lipoxygenase.

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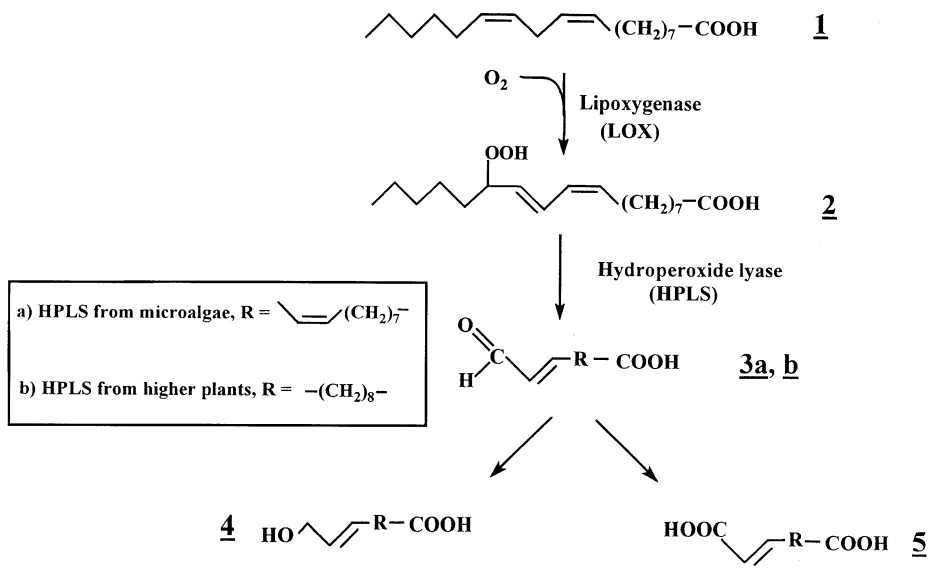
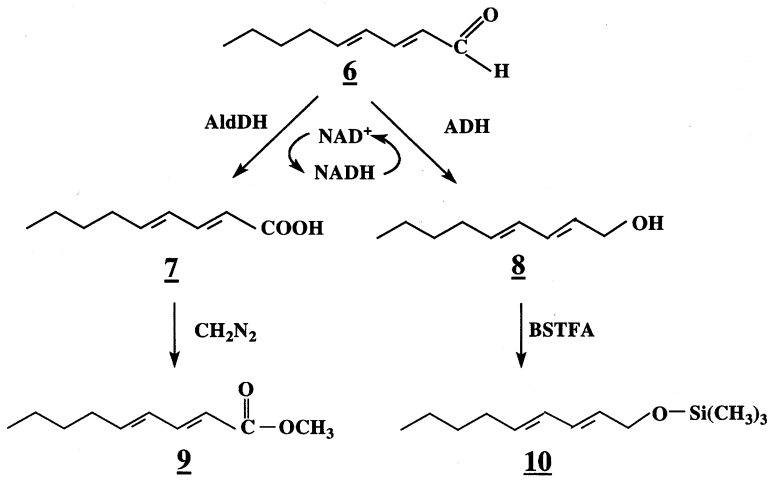


Scheme 1 Enzymic cleavage of linoleic acid to ω-hydroxy and dicarboxylic acids

Linoleic acid **1** is converted into hydroperoxide **2** by lipoxygenase. Hydroperoxide **2** is converted into aldehydes **3a** and **3b** by hydroperoxide lyase. The aldehydes **3a** and **3b** could be chemically or enzymically transformed into the corresponding ω-hydroxy carboxylic acid **4** and the dicarboxylic acid **5**.



Scheme 2 Enzymic transformation of aldehydes to hydroxy and carboxylic acids

The oxidized product **7** and reduced alcohol **8** can be obtained by the action of the enzymes AldDH and ADH with cycling of the cofactors NAD^+ and NADH. Products **9** and **10** are the derivatized products for GC/MS analysis (BSTFA; *N,O*-bis(trimethylsilyl)trifluoroacetamide).

and because it is contains allylic conjugated double bonds similar to the oxo-acids **3a** and **3b**. Using model aldehyde **6**, the pH profiles of ADH and AldDH were determined in a potassium phosphate buffer system, which was compatible with both enzymes. After conditions were found in which both enzymes were active, coupled oxidation–reduction was conducted with catalytic amounts of NAD^+ cofactor. The coupled assay also was conducted with HPLS product **3a**, and the structure of the resulting ω-hydroxy and ω-dicarboxylic fatty acid products confirmed by gas chromatography–MS (GC/MS).

Materials and methods

Chemicals and materials

Linoleic acid, NAD^+ and NADH were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Yeast AldDH (20 units/mg

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of enzyme protein) and yeast ADH (400 units/mg of enzyme protein) were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Water was purified to a resistance of 18 M-cm using a Barnstead (Dubuque, IA, U.S.A.) NANOpure system. (Z,Z)-Nona-2,4-dienal, **6** (Scheme 2), was purchased from Aldrich Chemical (Milwaukee, WI, U.S.A.). All other reagents were of the highest purity available. Hydroperoxide **2** and oxo-acid **3a** (Scheme 1) were produced and purified as described previously [4].

Kinetics

Initial rate determinations of the dehydrogenase reactions were performed as a function of the concentration of **6** at constant concentration of the cofactor at 25 °C. A 10 μl aliquot of a solution containing NAD^+ (7 mM), AldDH (113 μg of protein/ml), dithiotreitol (1 mM) and KCl (1 mM), kept at 0 °C, was added to 990 μl of a solution of **6** in Tris/K_2HPO_4/HCl buffer (50 mM). A 0.5 ml aliquot of the mixture was placed into a 0.5 ml quartz fluorescence cell, and the initial oxidation rate determined at the corresponding pH by monitoring the formation of NADH fluorimetrically in a Perkin–Elmer Luminescent Spectrometer LS50B (Perkin–Elmer, Norwalk, CT, U.S.A.). Assays were performed with right-angle excitation at 340 nm and emission at 464 nm. V_{max} values for this reaction also were determined spectrophotometrically at substrate concentration 100-fold greater than K_m with a Hewlett–Packard (San Fernando, CA, U.S.A.) 8452A Diode Array Spectrophotometer monitoring the formation of NADH ($\varepsilon = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$) at 340 nm. Similarly, initial rates for the reaction of NADH/ADH with **6** were determined spectrophotometrically at 340 nm. For this reaction a 20 μl aliquot of a solution of NADH (6 mM)/ADH (39.4 μg of protein/ml)/KCl (0.1 mM), kept at 0 °C, were added to a 980 μl solution of **6** in citrate/K_2HPO_4/HCl buffer (50 mM).

Product isolation and characterization

Enzymic reaction products were separated from the aqueous medium by solid-phase extraction using a C_{18} bonded phase cartridge (60 cm^3/10 g) from Varian (Harbor City, CA, U.S.A.). Organic products were isolated by elution with ether. After removal of the solvent, the isolated products were methylated or silylated and analysed by GC/MS with a Hewlett–Packard 5890 Series II Plus GC equipped with a Hewlett–Packard 5972 Series mass detector set to scan from m/z 35 to m/z 400 at 1.2 scans per s and with a HP-5MS (30 m × 0.25 mm) column; 50 °C (5 min) to 230 °C (10 min) at 10 °C/min. All injections were splitless with the injector and detector set at 250 °C, using He as a carrier gas at 1 ml/min.

Results and discussion

Enzyme kinetics

A standard model used to analyse the enzyme kinetics is described by the Michaelis–Menten eqn. (eqn. 1) where V_0 is the instantaneous rate, [S] is the substrate concentration, K_m is the apparent substrate dissociation constant and V_{max} is the rate when the enzyme is completely saturated by the substrate:

$$V_0 = V_{max}[S]/(K_m+[S]) \quad (1)$$

Eqn. (1) can be rearranged to eqn. (2) (see below), where K is equal to $1/K_m$. Non-linear fitting of the plot of the initial rate versus substrate concentration using eqn. (2) with an iterative method provides the estimates for V_{max} and K_m. Eqn. (2) can also be obtained by the general linked functions or the thermodynamic linkage theory of Wyman [8], as described by Rusling and Kumosinski [9]. These authors have shown that the application of a nonlinear fitting method to eqn. (2) gives better results than Lineweaver–Burk double-reciprocal plots [9,10]:

$$V_0 = K[S]V_{max}/(1+K[S]) \quad (2)$$

In general, for the NAD^+-dependent dehydrogenase reaction, V_0 can be determined by following the changes in NADH concentration spectrophotometrically at 340 nm. Alternatively, changes in low concentrations of NADH can be followed by its luminescent fluorescence emission at 464 nm with excitation at 340 nm. Figure 1 shows a typical non-linear fit of the data points using eqn. (2) with the program Abacus, which is based on a Gauss–Newton iterative method, for the initial rate of oxidation of **6** with AldDH/NAD^+. The low K_m for this enzymic reaction required the

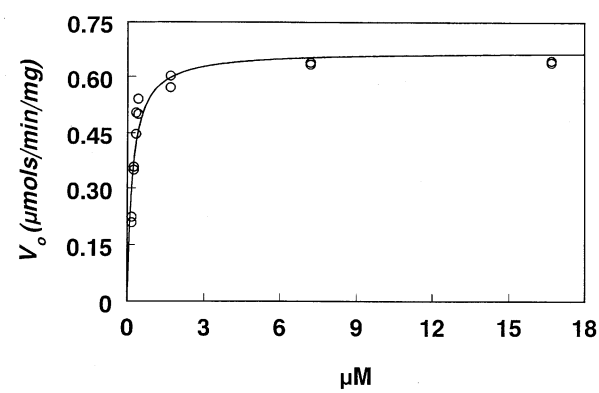

Figure 1 Influence of the substrate concentration on the initial rate of yeast AldDH/NAD^+ oxidation of 6 in Tris/K_2HPO_3/HCl buffer (50 mM, pH 9.0)

Points are experimental data; line computed as best fit using eqn. (2). Non-linear regression analysis gave K_m and V_{max} (±S.D.).

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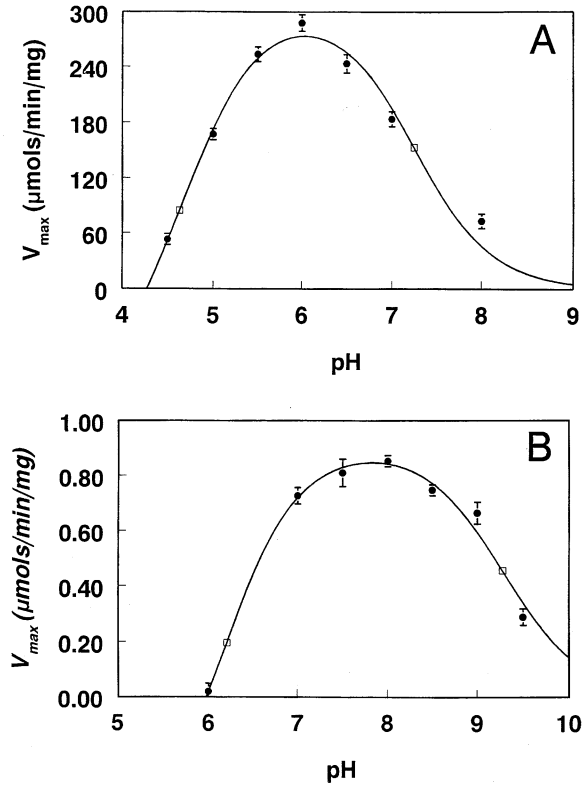

Figure 2 V_{max}±S.D. (●) determined with eqn. (2) for the reaction of 6 with ADH/NADH (A) and NAD^+/AldDH (B) as function of pH

Computer nonlinear regression using eqn. (6) allowed the determination pK_1 and pK_2 (□), and fitting of the data points.

sensitivity of fluorimetric detection of NADH to obtain the initial rate at low concentration of **6**. However, since the fluorescence efficiency of NADH changes with binding conditions, V_{max} values at different pH values could not be determined with fluorescence detection alone [11]. Consequently, V_{max} values as function of pH were determined spectrophotometrically at a substrate concentration 100-fold greater than K_m. This procedure is feasible since substrate **6** exhibited no substrate inhibition at high concentrations. With ADH/NADH both K_m and V_{max} could be determined by following the initial rate spectrophotometrically. As with AldDH/NAD^+, the ADH/NADH reaction exhibited simple kinetics with no substrate inhibition and no co-operativity evident.

Influence of pH on enzyme activity

Figures 2(A) and 2(B) show the pH profiles for the enzymic oxidation and reduction of **6** by AldDH and ADH, respectively. For both enzymes, V_{max} decreases at low and high pH values, yielding a bell-shaped curve. This bell-shape-type plot shows that an initial protonation of ionizable groups of the enzyme is needed to activate the system, whereas a

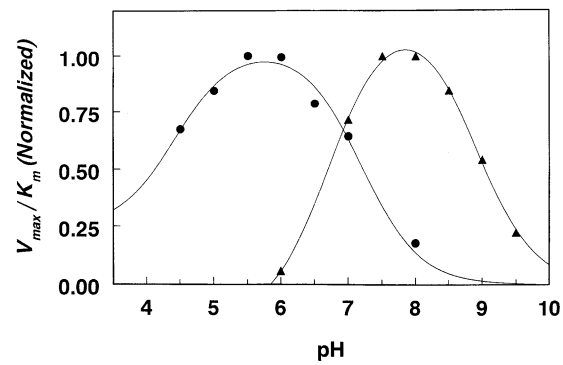

Figure 3 Normalized V_{max}/K_m versus pH for the reaction 6 with ADH/NADH (●) and AldDH/NAD^+ (▲)

Data points were fit using non-linear regression.

second protonation of the active form de-activates the enzyme (E). In general this can be represented as shown in eqns. (3) and (4):

$$E+H^+ \leftrightarrow EH^+ K_1 = [EH^+]/[E][H^+] \quad (3)$$
$$EH^+ + H^+ \leftrightarrow EH_2^{2+} K_2 = [EH_2^{2+}]/[E^+][H^+] \quad (4)$$

This is a stepwise or co-operative model where K_1 and K_2 represent the association or protonation constant. According to the linked function model, the activity (V_{obs}) is given by eqn. (5):

$$V_{obs} = f_E V_1 + f_{EH} V_2 + f_{EH2}(V_3 - V_2) \quad (5)$$

where f_E, f_{EH} and f_{EH2} are the protein fractional components and V_1, V_2 and V_3 are the activities of E, EH^+ and EH_2^{2+}. Accordingly, by expressing the protein fractional component as a function of the protonation constants, regression eqn. (6) can be obtained [9,12]:

$$V_{obs} = [V_1/(1+K_1C_H)] + [K_1C_HV_2/(1+K_1C_H)] + [K_2C_H(V_3 - V_2)/(1+K_2C_H)] \quad (6)$$

In eqn. (6), V_1, V_2, V_3, K_1 and K_2 are the regression parameters, C_H is the proton concentration (10^{-pH}), and V_{obs} is V_{max}. This equation allowed the nonlinear fitting of the V_{max} versus pH using the Abacus program as depicted in Figure 2(A) for ADH/NADH. The K_1 and K_2 values obtained with eqn. (6) are related to the pK_a of the prototropic groups involved in catalytic activity [13]. From Figure 2(A) a pK_2 of 4.6±0.3 is obtained, which suggests that a β- or γ-carboxylic acid (pK_a 3–5) is necessary for catalytic activity. A value of 7.2±0.2 was obtained for pK_1, which is indicative of the involvement of an amino group of imidazolium in histidine (pK_a 5.5–7) [13]. This conclusion is in accordance with the model presented by Ganzhorn et al. for yeast ADH, where an atom of zinc is ligated by two cysteine residues and one histidine in the

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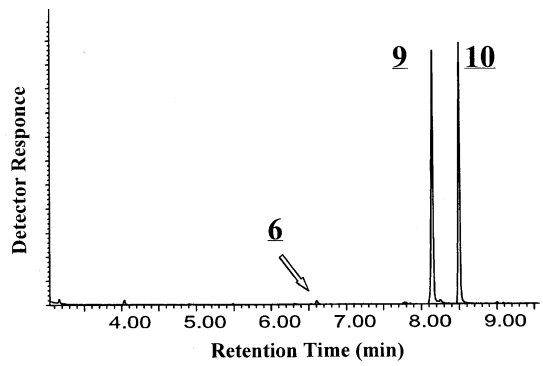


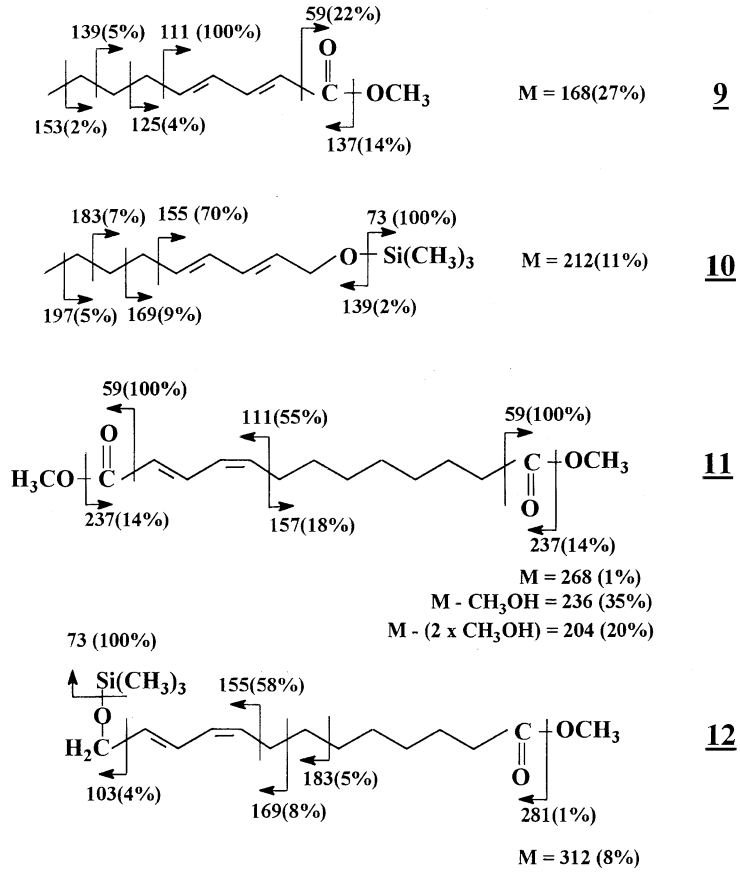
Figure 4 GC/MS chromatogram (total ion current) for the reaction of 6 with AldDH/ADH and NAD^+ at 10% of substrate concentration (see Scheme 2)

The mass analysis indicates the formation of products **9** and **10** with an area ratio near to 1 : 1 and conversion to product over 90%.

catalytic site [14]. The optimal pH for ADH activity with substrate **6** is near pH 6 as determined from $(pK_1+pK_2)/2$.

The plot of V_{max} versus pH for the reaction of **6** with AldDH/ NAD^+ is shown in Figure 2(B). From this plot pK_1 and pK_2 were determined to be 9.3±0.1 and 6.2±0.4, respectively. Earlier studies with yeast AldDH have shown that thiol groups play an essential role in its activity [15]. Since the pK_a for a thiol group in cysteine is near 9, the pH profile is consistent with the participation of SH groups for catalytic activity [13]. A pK_2 of 6.2±0.4 indicates a role for histidine in AldDH catalytic activity [13]. Overall, Figure 2(B) shows that AldDH/NAD^+ has best activity with substrate **6** at pH near 8.0. For both AldDH and ADH the best fits of the data points in Figure 2 were obtained when the first term in eqn. (6) is dropped, which requires that $V_1 = 0$. This indicates that only the protonated forms of the enzymes are active (i.e. EH^+ and EH_2^{2+}), according to eqn. (5).

The influence of pH on the binding capabilities of the enzyme for the substrate is reflected in the pH profile of K_m/V_{max}. As Figure 3 shows, the shape of the plot of the V_{max}/K_m versus pH was nearly identical with the plot of V_{max}, and the pK_1 and pK_2 remained the same within experimental error. This demonstrated that substrate binding is not an important factor in the pH profile of either enzyme.



Scheme 3 Mass-fragmentation pattern of products

The Scheme shows GC/MS analyses of the derivatized products formed by the action of AldDH and ADH and recycling of NAD^+ and NADH on substrates **3a** and **6**.

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Recycling procedures

Model aldehyde. A successful recycling procedure requires that both ADH and AldDH be active in the same pH region and buffer system. As Figure 3 shows, both enzymes have activity in the pH range 6–8 in potassium phosphate buffer. Therefore, the coupled enzymes were tested with 150 μmol of substrate and 15 μmol of cofactor at pH 7.0. After methylation and preparation of the silylated derivatives, the products were analysed by GC/MS. The resulting ion chromatogram showed two major peaks with a retention time of 8.15 and 8.50 min, respectively, with an area ratio near 1:1 (Figure 4). As shown in Scheme 3, the mass-fragmentation pattern of the peak at 8.15 min is consistent with structure **9**, the methyl ester of product **7** (Scheme 2). The second peak had a mass-fragmentation pattern consistent with structure **10**, the silylated derivative of **8** (Scheme 2). For both products **9** and **10**, no alteration of the double bonds were observed in the enzymic reactions and the yield of products was 90%.

Aldehyde **3a** *from HPLS.* HPLS from the microalga *Chlorella pyrenoidosa* was used to produce **3a** from hydroperoxide **2** [4]. Product **3a**, obtained as described previously [4], was treated with ADH/AldDH/NAD^+ in potassium phosphate buffer at pH 7.5, in which **3a** has good solubility. The isolated products were methylated, silylated and analysed by GC/MS. As in Figure 4, analysis showed the formation of two products, **11** and **12** (Scheme 3), in a ratio close to 1:1 (results not shown). The mass spectrum of product **11** is consistent with the formation of a methylated C_{13} ω-dicarboxylic ester with a molecular ion, M^+, at m/z 268. The fragment at m/z 204 is indicative of the loss of two methanol molecules from the methyl esters. The fragment at m/z 111 is characteristic of a methyl ester with two conjugated double bonds [16]. The base peak of the spectrum is at m/z 59, and is attributed to the methoxycarbonyl group [16]. Another significant fragment at m/z 94 (80%) was attributed to the formation of a phenol ion after cyclization followed by the loss of methanol and $CH_2{=}CH(CH_2)_4CO(OCH_3)$ [17]. The mass spectrum of the second product **12** is consistent with the formation of a ω-hydroxycarboxylic acids **11**, and the spectrum is identical with that previously described for the product obtained by the chemical reduction of **3a** [3,18].

Conclusion

This study demonstrated that AldDH and ADH can be used for the simultaneous oxidation and reduction of the model aldehyde **6** using continuous regeneration of NAD^+ cofactor. It also was demonstrated that HPLS product **3** is a substrate for the dehydrogenases. This result is significant because it demonstrates the feasibility of converting fatty acid **1** into industrially useful products using enzyme technology.

Acknowledgment

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Received 12 August 1998/27 October 1998; accepted 10 November 1998

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