Enzymatic determination of fatty acids using alcohol dehydrogenase from horse liver

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An enzymatic method for the determination of fatty acids using alcohol dehydrogenase from horse liver has been developed.

Alcohol dehydrogenases (ADHs) catalyse the first stage of ethanol oxidation, as well as the oxidation of some other alcohols.[1] ADHs isolated from bakers yeast (YADH) and horse liver (HLADH) are commercially available reagents. In contrast to the latter, YADH is used in chemical analysis already: highly sensitive and selective procedures for the determination of heavy metal ions, heterocyclic N-containing compounds and amino acids have been developed.[2,3] Data on the inhibition of HLADH catalytic activity in the presence of fatty acids were published.[1,4–7] Nevertheless, no indication on the systematic investigation of this phenomenon and its analytical application was pointed out. It should be mentioned that there is a lack of methods for fatty acids determination,[8,9] and that enzymatic ones are absent altogether.

The aim of this work was to study in detail the effects of fatty acids on the catalytic activity of HLADH and to develop a procedure for their determination.

Ethanol oxidation was chosen as the indicator reaction.† The rate of the reaction was monitored spectrophotometrically as the absorption of the reaction solution increased in the course of the reaction due to the formation of a coloured product (NADH).

In order to optimise conditions for carrying out the indicator reaction, the dependence of the reaction rate in the presence and absence of fatty acids on the medium pH (Figure 1), reagent concentrations (Figure 2) and the time of the enzyme-acid preincubation was studied. The conditions were considered to be optimum if the inhibition effect was clear and convenient for detection while the reaction rate was sufficiently high to monitor. The optimised conditions are as follows: Tris–HCl buffer solution, pH 9.5; LADH concentration, 4 μM; NAD^+ concentration, 0.6 mM and ethanol concentration, 0.03 M.

We found that the inhibition effects of various acids depended

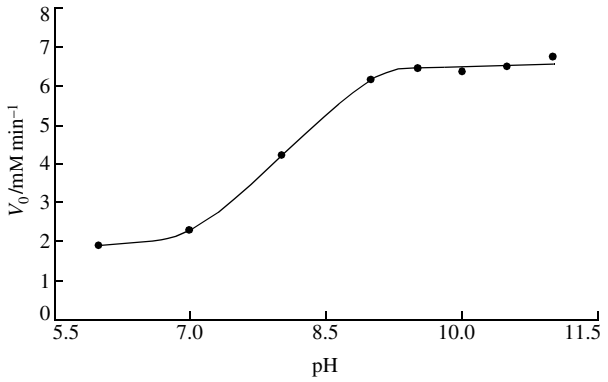

Figure 1 The pH dependence of the indicator reaction rate (Tris–HCl buffer, 0.4 M ethanol, 0.45 mM NAD^+ and 4 μM ADH).

† Deionised water purified with a Simplicity Proto system (Millipore) was used. Aqueous solutions of alcohol dehydrogenase from horse liver (Sigma) were prepared daily by dissolving a portion of the enzyme (E.C.1.1.1.1.) in a phosphate buffer solution (pH 7.6). Aqueous NAD (Sigma) solutions were prepared daily. Ethanol solutions were prepared using 96% rectified ethanol.

In the case of nonanoic and capric acids, the indicator reaction in the presence of 6 or 13 M ethanol, respectively, was performed as a blank experiment because of low solubility of these acids.

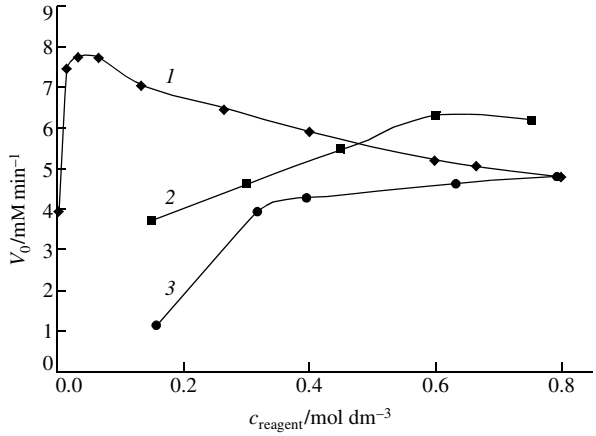

Figure 2 The dependence of the indicator reaction rate on the concentrations of (*1*) ethanol, (*2*) NAD^+ (×10^3) and (*3*) ADH (×10^5).

on the enzyme–acid preincubation time: the degree of ADH inhibition with butyric, heptanoic, octanoic, nonanoic and sebacic acids was nearly independent of their preincubation time with the enzyme; the inhibitory effect of propionic, capryc, palmitic, succinic and glutaric acids appeared to be maximum in the case of 5 min preincubation (in case of valeric acid, 30 min were required).

A number of saturated straight-chained monocarboxylic C_3–C_{18} and dicarboxylic (succinic, glutaric and sebacic) acids were chosen as the test substances. The catalytic activity of HLADH decreased in the presence of many studied acids. Data regarding such kind of the influence of several acids were published.[2] It has been supposed[2] that the inhibition effect of saturated acids increases simultaneously with the carbon chain length. The experimental data revealed more complicated dependence. Whilst propionic, butyric and valeric (C_3–C_5) acids exhibited rather clear inhibition effects, caproic, heptanoic and octanoic (C_6–C_8) acids had weak effects. Nonanoic and capric (C_9 and C_{10}) acids acted as more effective inhibitors than those mentioned above. Acids with C_{16} carbon chains or longer (palmitic and stearic) have no inhibitory effect at all. It was also shown that aromatic fatty acids, such as nicotinic acid, do not affect the activity of HLADH.

This fact can be justified when taking into account intermolecular interactions and polarization of acid molecules. Thus, saturated aliphatic straight-chained carboxylic acids with small alkyl groups are heavily polarised, and they can compete with an alcohol for the substrate-binding site of HLADH. This causes the inhibition of the enzyme. Acids with carbon chains containing 6–8 carbon atoms have only a weak effect because of an approximate balance in hydrophilic and hydrophobic interactions. Since both are weak, such acids fail to compete effectively for any enzymatic domain responsible for its catalytic activity. In the case of 9 and 10 carbon atoms in the acid chain (nonanoic and capric acids), hydrophobic interactions are predominant. These acids attack the so-called oleophilic site of an enzyme–coenzyme binary complex (the affinity to this site increases with the length of the carbon chain) instead of the

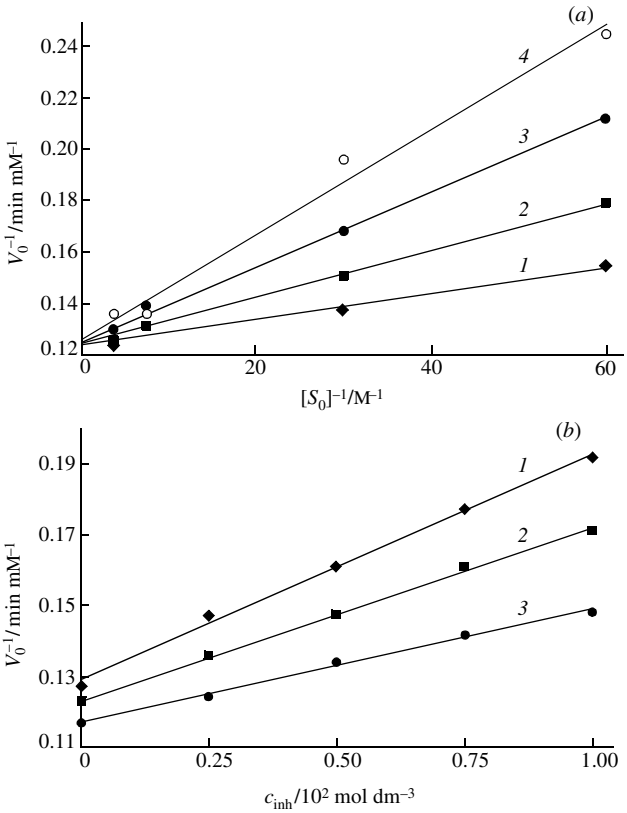

Figure 3 The dependence of the reverse initial rate of the indicator reaction (*a*) on the reverse ethanol concentration (Lineweaver–Burk coordinates) and (*b*) on the concentration of butyric acid with ethanol concentration varies (Dixon coordinates). Optimum conditions for the acid determination: (*a*) butyric acid concentrations/mM: (*1*) 0, (*2*) 2.5, (*3*) 5 and (*4*) 7.5; (*b*) ethanol concentrations/M: (*1*) 0.02, (*2*) 0.03 and (*3*) 0.13.

HLADH substrate-binding site and inhibit the enzyme to a higher extent than those competing with alcohol. Further chain growth leads to the appearance of a steric factor that forbids acids to come close to any site responsible for the enzymatic activity. The substitution of a straight-chained alkyl group with a branched or aromatic one will also result in steric hindrance.

Thus, it has been demonstrated that the fatty acid coupling to the oleophilic site of the HLADH–NAD$^+$ complex leads to more effective inhibition of HLADH than do acids competing for the substrate-binding site. Moreover, there is no simple correlation between the inhibitory effect and the amount of methylene (CH_2) groups.

The type of HLADH inhibition with the saturated aliphatic carboxylic acids was investigated by means of the Lineweaver–Burk, Dixon and Hanes[10,11] linearization methods using butyric acid as the model (Figure 3). It was found that the test fatty acid inhibited the enzyme competitively.

The inversely proportional dependence of the rate of the indicator reaction catalysed by HLADH in the presence of fatty acids under the optimum conditions allowed us to develop a sensitive enzymatic determination procedure with analytical characteristics presented in Table 1.

The determination of acid inhibitors in the systems containing those acids that revealed no effect on the catalytic activity of HLADH was also studied. A combination of butyric (inhibitor) and heptanoic (non-inhibitor) acids was chosen. Heptanoic acid in a tenfold amount caused no interference with the determination of butyric acid. Butyric acid (0.75 mM), provided the extent of inhibition (I) equal to 9%. Heptanoic acid in a concentration of 0.75 or 7.5 mM resulted in I = 11 or 10%, respectively.

Thus, the determination of fatty acids can be based on their inhibitory effects on the HLADH enzymatic activity in the presence of fatty acids that do not exhibit such an effect.‡

In conclusion, data on the effect of fatty acids on the catalytic activity of HLADH in the reaction of ethanol oxidation were confirmed and expanded. The reaction conditions were optimised, and the mechanism of the effect of fatty acids was specified. An enzymatic procedure for the determination of straight-chained saturated carboxylic acids was developed.

Table 1 Analytical characteristics for the determination of fatty acids using alcohol dehydrogenase from horse liver (P = 95%; n = 3).

Fatty acid	Analytical range/mM	c^a_{min}/M	RSD^b (%)	Calibration function
Propionic	0.5–10	1×10^{-4}	1	$V_0^c = 6.90 - 1.56x^d$
Butyric	0.75–10	4×10^{-4}	2	$V_0^c = 6.29 - 1.53x$
Valeric	0.01–10	2×10^{-6}	2	$V_0^c = 9.87 - 98.09x$
Nonanoic	0.01–1	2×10^{-6}	2	$V_0^c = 7.70 - 12.89x$
Capric	0.005–1	3×10^{-6}	2	$V_0^c = 6.88 - 160.22x$
Succinic	0.01–1	3×10^{-6}	1	$V_0^c = 8.70 - 17.72x$
Glutaric	0.05–1	2×10^{-5}	3	$V_0^c = 5.54 - 27.68x$
Sebacic	0.005–0.1	2×10^{-6}	3	$V_0^c = 5.37 - 242.92x$

[a]The detection limit was calculated using the 3S value. [b]The relative standard deviation was calculated at the lower limit of the applicable concentration range of fatty acid. [c]The initial rate of the indicator reaction/mM min^{-1}. [d]The inhibitor concentration/mol dm^{-3}.

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‡ A 0.05 M Tris–HCl buffer solution with pH 9.5 (1.1 ml), 0.1 ml of a 0.6 mM NAD$^+$ solution and 0.1 ml of a 0.45 M ethanol solution were placed sequentially in a glass tube fitted with a ground-glass stopper. A 0.1 ml portion of the test fatty acid solution was added to the mixture. Finally, 0.1 ml of a 60 μM ADH solution was added. The total volume was 1.5 ml (for cuvettes with l = 0.3 cm). At the moment the enzyme solution was added a stopwatch was started. The absorbance of the reaction mixture at 315 nm was measured for 2 min at 15 s intervals. Fatty acid concentrations in the test samples were determined from calibration curves plotted as V_0 versus the inhibitor concentration.

The initial rate of the indicator reaction (V_0/μM min^{-1}) was calculated as $V_0 = \Delta c/\Delta\tau = \Delta A l/\Delta t l\varepsilon = \mathrm{tg}\,\alpha/\varepsilon l$, where c is the product concentration, τ is the reaction time, ε is the molar absorbance coefficient of NADH, l is the optical path length, tg α is the slope of the absorbance *vs.* time kinetic curve.