Thermal stability of alcohol dehydrogenase enzyme determined by activity assay and calorimetry[1]

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Abstract

The thermostability of pure yeast alcohol dehydrogenase was investigated at various temperatures, in the presence and absence of sucrose, by both activity assay and differential scanning calorimetry. The thermal inactivation exhibited nonlinear biphasic behavior. The thermal inactivation rate constants and the magnitude of the heat-stable and heat-labile fractions of the enzyme were quantified. The values of the denaturation temperature were experimentally measured by calorimetry. It was found that although activity assay and calorimetry are based on different principles, they yield results that agree well with each other. However, each technique provides unique data (e.g. enzyme activity vis-a-vis basic thermodynamic properties, such as the denaturation enthalpy) and the two techniques may be considered complementary to each other. © 1998 Elsevier Science B.V.

1. Introduction

Thermal processes (e.g. sterilization, pasteurization, and blanching) are important in a number of contexts in the food and pharmaceutical industry and hence thermal inactivation and thermal stabilization of enzymes and proteins have received much attention [1–15]. In most studies, the reaction rate constants and thermodynamic parameters are determined on the basis of the assumption that the inactivation process obeys first-order kinetics [3,4,11]. However, in general, large deviations from first-order kinetics are observed in the log(residual enzyme activity)–time curve upon thermal treatment [4–6,8,13–15] and nonlinear enzyme activity–time relationships have recently been observed for various enzymes: horseradish peroxidase [4]; Bacillus licheniformis α-amylase [5]; acid phosphatase [8]; and alcohol dehydrogenase from baker's yeast [15]. This nonlinear thermal inactivation behavior could be due to the formation of enzyme groups with different thermal stabilities or the presence of stable/labile isoenzymes [4,5,15] or due to a series-type enzyme inactivation kinetics [8,10,13–15].

Calorimetry [16,17] is a powerful technique to evaluate the thermal stability of enzymes. However,

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although yeast alcohol dehydrogenase has a wide range of applications in fine organic synthesis, regeneration of coenzymes and analytical determination of alcohol in various media [18], the thermal stability of yeast alcohol dehydrogenase, in the presence or absence of sugars, has not been studied before using both activity assay and calorimetry. In this work, we investigate the thermal stability of pure alcohol dehydrogenase from baker's yeast by both the activity assay and differential scanning calorimetry and show that although the two techniques are based on different principles (kinetic in the former case as opposed to thermodynamic in the latter), they yield results that agree well with each other.

2. Theory

2.1. Analysis of thermal inactivation data

For thermal inactivation comprising two different, independent first-order inactivation processes, the fractional residual enzyme activity can be expressed as [15]

$$E/E_0 = A_1\exp(-k_1t) + A_s\exp(-k_st) \qquad (1)$$

where E_0 is the initial enzyme activity at zero time, E the enzyme activity after thermal processing at a constant temperature T for time t, k_1 and k_s the inactivation rate constants of the heat-labile and heat-stable groups, and A_1 and A_s the heat-labile and heat-stable fractions of the initial enzyme activity. The kinetic parameters can be determined either by nonlinear regression or by simple deconvolution methods, details of which can be found in the paper by Nath [15].

3. Experimental

3.1. Materials

Pure yeast alcohol dehydrogenase (E.C. 1.1.1.1) was purchased from Calbiochem Novabiochem (Lucerne, Switzerland). NAD was purchased from Spectrochem (Bombay, India). Sodium dihydrogen phosphate and disodium hydrogen phosphate were bought from Qualigens, Glaxo (Bombay, India) and tetrasodium pyrophosphate from Loba (Bombay,

India). Ethanol was purchased from Merck (Darmstadt, Germany) and sucrose from BDH (Poole, England). All reagents were of analytical grade.

3.2. Thermal treatment

Pure yeast alcohol dehydrogenase was dissolved in 10 mM phosphate buffer, pH 7.5, to yield a concentration of 0.75–1 mg ml^{-1}. Sucrose was added to the buffer-enzyme solution. The concentration of sucrose after mixing with the buffer-enzyme solution measured 44.44%(w/w). The thermal treatment was performed in a constant temperature water bath. The samples were placed in the bath for a fixed period of time, taken out and immediately cooled in ice water. The residual enzyme activity was then measured. The sucrose percentage referred throughout this work is percentage (w/w). The sample size measured 1 ml in each case.

3.3. Enzyme activity measurement

The activity of alcohol dehydrogenase was measured spectrophotometrically at 340 nm, 25°C in pH 8.8 tetrasodium pyrophosphate buffer. The procedure is based on progressive oxidation of ethanol, with NAD continuously becoming reduced to NADH. The 50 μl of diluted enzyme solution was introduced into a 1.5 ml assay mixture. NAD concentration was maintained at 8.3 mM and that of ethanol at 0.7 M. The extinction coefficient value is 6.22 mM cm^{-1}. Enzyme activity was calculated by linear regression of absorbance at 340 nm vs. time data. In all the cases, both the initial and residual activity were measured. The least count of the measurement technique was on the order of 0.1 U ml^{-1}.

3.4. Differential scanning calorimetry

Differential scanning calorimetry experiments were performed with a Setaram (Lyons, France) DSC calorimeter. The sensitivity used was 0.3 mJ s^{-1} while the heating rate measured 0.6°C min^{-1}. The pure yeast alcohol dehydrogenase sample was dissolved in cold phosphate buffer, pH 7.5. The enzyme concentration used was 8 mg ml^{-1}. The sample volume measured 0.8 ml. Buffer was used as a reference in all the experiments.

4. Results and discussion

4.1. Kinetic parameters for thermal inactivation of yeast alcohol dehydrogenase

The kinetic parameters of pure yeast alcohol dehydrogenase, based on the activity assay for the range 50–70°C [15] in the presence and absence of sucrose, are tabulated in Table 1. The sum of A_s and A_l is unity in all the cases (Table 1). This, along with the high k_l/k_s value, justifies the method used to determine the kinetic parameters. Note that both A and k parameters need to be considered simultaneously (and not singly) for a correct interpretation. A decrease in temperature at constant sucrose concentration or addition of sucrose at constant temperature has the effect of increasing A_s and altering k_l and k_s and the method of analysis clearly differentiates between these two effects.

4.2. Denaturation temperature and denaturation enthalpy of yeast alcohol dehydrogenase by differential scanning calorimetry

The thermal stability of yeast alcohol dehydrogenase (concentration 8 mg ml^{-1}, pH 7.5) was also experimentally evaluated by differential scanning calorimetry. The DSC thermograms in the absence and presence of 44.44% sucrose are shown in Fig. 1. The values of the denaturation temperature (T_d) and the denaturation enthalpy (ΔH_d) for pure enzyme, in the absence and presence of 44.44% sucrose, are tabulated in Table 2. A T_d of 63°C was determined
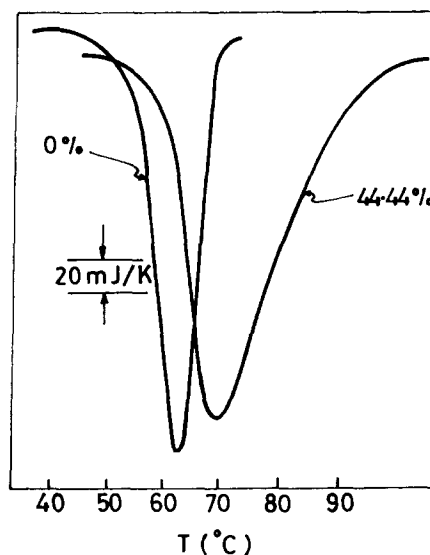

Fig. 1. DSC curves for pure yeast alcohol dehydrogenase in the absence and presence of 44.44% sucrose. Enzyme concentration, 8 mg ml^{-1}; heating rate, 0.6°C min^{-1}.

Table 2
Values of the denaturation temperature and the denaturation enthalpy for thermal inactivation of alcohol dehydrogenase in the absence and presence of 44.44%(w/w) sucrose

Sucrose (%(w/w))	T_d (°C)	ΔH_d (J g^{-1})
0	63	4.2
44.44	70	6.9

for yeast alcohol dehydrogenase in the absence of sucrose; addition of 44.44%(w/w) sucrose yielded a higher (70°C) value of T_d (Fig. 1, Table 2).

The results of Table 1 clearly show that in the absence of sucrose, the value of the heat-stable enzyme fraction, A_s, becomes zero at (and beyond) 63°C, while the heat-labile rate constant, k_l increases more than sixfold from 0.19 min^{-1} at 60°C to 1.25 min^{-1} at 63°C, i.e. the enzyme gets denatured at 63°C in the absence of additives. In the presence of 44.44% sucrose, the corresponding temperature for denaturation of the enzyme is 70°C and the A_s value almost reaches zero at this temperature (Table 1). These results agree well with the denaturation temperature measurements on yeast alcohol dehydrogenase by differential scanning calorimetry (Fig. 1, Table 2), which show that the enzyme is denatured

Table 1
Kinetic parameters for thermal inactivation of pure yeast alcohol dehydrogenase

T (°C)	Sucrose (%(w/w))	A_s	$k_s \times 10^2$ (min^{-1})	A_l	k_l (min^{-1})
50	0	0.86	1.30	0.14	0.10
55	0	0.85	4.70	0.15	0.12
57.5	0	0.83	7.40	0.17	0.23
60	0	0.31	8.60	0.69	0.19
63	0	0.00	—	1.00	1.25
62.5	44.44	0.93	2.10	0.07	0.06
65	44.44	0.82	2.30	0.18	0.16
67.5	44.44	0.55	8.30	0.45	0.19
70	44.44	0.04	11.20	0.95	0.58

at 63°C and 70°C in the absence and presence of 44.44% sucrose, respectively. Note that, at the denaturation temperature T_d, the kinetic results should be interpreted to imply that the stable part of the enzyme activity has decayed; it does not imply that the enzyme activity measures zero. In fact, a substantial part of the enzyme would be active *at short times* even at the denaturation temperature. In other words, active molecules would be present from both the kinetic and thermodynamic points of view at $T=T_d$. Thus, it is found that two different techniques, one kinetic and the other thermodynamic, yield similar results for the thermostability of alcohol dehydrogenase. Finally, calorimetric experiments carried out at different temperature scanning rates should help in throwing more light on the nature of the irreversibility of enzyme inactivation.

Regarding the role of sucrose in stabilization of enzymes, several hypotheses have been constructed in the literature. According to Arakawa and Timasheff [1], the addition of sucrose increases the chemical potential of the enzyme and this increase is proportional to the surface of the enzyme, so that the native, folded state is favored over the denatured one. As a general rule, sucrose and other stabilizing solutes are preferentially excluded from the vicinity of the enzyme. This exclusion of sugars is attributed to their water surface tension increasing effect. Monsan and Combes [12] suggest that the addition of sucrose depresses the water activity and that stabilization against denaturation arises from the increased degree of water organization induced by the solutes. Back et al. [2] assert that stabilization by sugars is due to the strengthening of hydrophobic interaction in the presence of solutes that reinforce the hydrogen-bonded organization of water.

5. Conclusions

The thermal stability of yeast alcohol dehydrogenase was investigated by two techniques, i.e. activity assay and differential scanning calorimetry. The kinetic parameters of the nonlinear thermal inactivation process were determined at various temperatures, both in the absence and presence of sucrose. The values of the denaturation temperature and the denaturation enthalpy were also quantified for the enzyme inactivation process. The results of thermal stability obtained by the two techniques agree well with each other. Each technique provides unique data (e.g. enzyme activity vis-a-vis basic thermodynamic properties, such as the denaturation enthalpy) and the two techniques may be considered complementary to each other.

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