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THERMAL STABILITY OF INVERTASE IMMOBILIZED ON SUPERFINE FILAMENTS OF A POLY(VINYL ALCOHOL) DERIVATIVE *

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ABSTRACT

The thermal stability of invertase immobilized on dimethylaminated poly(vinyl alcohol) superfine fibres (PVA–SFF) was measured by calorimetry. The denaturation temperature T_d of invertase on the PVA–SFF was raised by about 4–6 °C on increasing the amount of enzyme from 0.08 to 0.5 g (g dry PVA–SFF)$^{-1}$. The values of T_d for invertase on PVA–SFF, as well as in aqueous solution, were affected by the pH of the solution into which samples were dipped. The values of T_d were raised and the denaturation enthalpy was reduced by immobilization. The cause of the stabilizing effect and the reversibility of the thermal denaturation are discussed.

INTRODUCTION

The physical properties of immobilized enzymes differ from those of the native enzymes owing to the change in the microenvironment caused by the polymer support. One of the most important physical properties which change on immobilization is the thermal stability. The following factors are thought to affect the thermal stability of immobilized enzymes: the high local concentration of enzymes, charged groups on the support, the change in ion concentration around immobilized enzymes compared with that in the bulk solution, the change in the water structure around immobilized enzymes compared with that in the bulk solution and the interaction of the polymer support with enzymes.

We have previously studied the reactivity of enzymes immobilized on dimethylaminated poly(vinyl alcohol) superfine fibres (PVA–SFF) [1] and in photocross-linked PVA films [2] and proved that the immobilized enzymes show high activity. Many investigations have been made on the thermal

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reversibility of immobilized invertase by measuring the enzymatic activity of the samples which was preserved after heat treatment at a certain temperature for a certain time [3–7]. To elucidate the thermal stability of immobilized enzymes, we have previously studied, using calorimetry, the thermal denaturation of invertase immobilized in photocross-linked PVA films [8], and also compared the thermal stability of immobilized invertase found by activity assay with that found by calorimetry [9].

In this paper, we report the calorimetric studies on the thermal denaturation of invertase immobilized on PVA–SFF. The effect of the amount of enzyme in grams per gram of PVA–SFF (enzyme loading), the effect of pH and the reversibility of the thermal denaturation were investigated. These results were compared with previous results obtained for invertase in aqueous solution, and the cause of the stabilizing effect is discussed.

EXPERIMENTAL

Materials

Invertase (EC 3.2.1.26) from *Candida utilis* (117 U/g, Seikagaku Kogyo Co.) was dialysed against distilled water for 24 to 48 h and then lyophilized. PVA–SFF was prepared by the method reported previously [1]. Its average degree of polymerization was 1200. The amount of the dimethylamino group is 1.1 mol per gram of total PVA weight. Phosphate buffer (10 mM) containing 1 mM ethylenediaminetetraacetic acid (EDTA) was used for adjusting the pH of the solution.

Preparation of immobilized invertase

Invertase was immobilized on PVA–SFF as reported previously [1]. The PVA fibre was put into an enzyme solution and shaken for 1.5–2 h at 37 °C. The amount of immobilized enzyme was estimated from the concentration change of the enzyme solution. The concentration of invertase was determined from the absorbance at 280 nm utilizing the value $E^{1\%}_{1cm} = 10.8$ [10]. The measurements were made for samples with various enzyme loadings from 0.0776 to 0.5065 g enzyme (g PVA–SFF)$^{-1}$ at pH 4.58 and 7.19. For the measurements of pH dependence, the samples with 0.2042 g enzyme (g PVA–SFF)$^{-1}$ were used.

Method

Calorimetric measurements of the immobilized invertase were carried out in a differential scanning calorimeter SSC/560U (Seiko Instrument & Electric Ltd.) at a sensitivity of 100 μV full scale and at a scan rate of 1 °C

min^{-1}. PVA–SFF with invertase (PVA–enzyme) was divided into small amounts of fibres with appropriate weights, and each sample was stored in a refrigerator separately. Silver cells with 70 μl capacity were used. PVA–enzyme samples were dipped in a buffer solution before DSC measurements for 1–2 h. The wet PVA–enzyme samples were centrifuged and immediately afterwards the wet samples were weighed and placed in the silver cells. The weight of the wet PVA–SFF in this condition was 4.093 times the weight of dry PVA–SFF. The average weight of the wet PVA–enzyme which was placed in the cell was 35 mg. A buffer solution with the same pH value as that used for wetting the PVA–enzyme was added. For each DSC measurement the total amount of the sample (PVA–enzyme + buffer solution) was regulated at about 50 mg to avoid the effect of the sample weight on the DSC results. The difference between the weight of the sample and that of the reference water was controlled so that it was within 2 mg. The sample was weighed before and after the DSC measurements in order to determine whether any water had evaporated. Water and gallium were used for the calibration of the calorimeter.

Calorimetric measurements for invertase in aqueous solutions were also made with a differential scanning adiabatic microcalorimeter DASM-1M (Privalov calorimeter [11]) with 1 ml cells and at a scan rate of 1°C min^{-1}. Degassing during heating was prevented by applying an extra constant pressure of 1.1 atm over the solutions in the cells. A known heat input of 50 μW was applied to calibrate the heat capacity scale of the recorder.

RESULTS AND DISCUSSION

The effect of enzyme loading on the thermal denaturation of invertase

The thermal denaturation of immobilized invertase for samples with various amounts of immobilized invertase per gram of PVA–SFF was measured at pH 4.58 (the optimum pH for the reaction) and at pH 7.19. The DSC curves for pH 4.58 are shown in Fig. 1. In the samples in Fig. 1, the enzyme loading was varied from 0.0776 g enzyme (g PVA–SFF)$^{-1}$ (curve D) to 0.5065 g enzyme (g PVA–SFF)$^{-1}$ (curve A). The figure shows that the peak area as well as the peak temperature T_d increases with increasing enzyme loading. Similar results were obtained with invertase at pH 7.19. The values of T_d obtained at these two pH values were plotted against the enzyme loading in Fig. 2. This figure shows that T_d increases with enzyme loading by about 5°C. The limiting values of T_d at a high enzyme loading are about 77.5°C and 77.0°C at pH 4.58 and pH 7.19 respectively. In a previous paper [8], a remarkable increase in T_d was reported for the aqueous solution when the concentration of invertase was increased to 1%. At a concentration higher than 1%, the increase in the values of T_d showed a

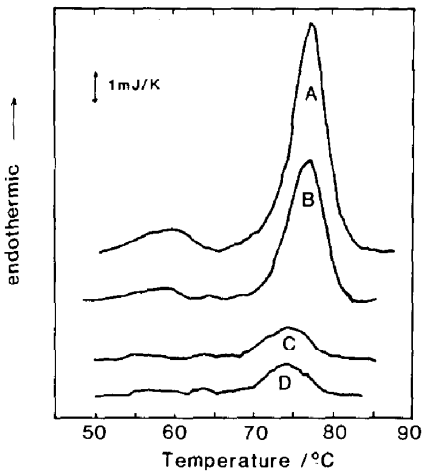

Fig. 1. DSC curves for invertase immobilized on PVA–SFF at various enzyme loadings (g enzyme (g PVA–SFF)$^{-1}$) at pH 4.58: curve A, 0.5065; curve B, 0.3232; curve C, 0.1016; curve D, 0.0776.

slight drop. The shape of the curves in Fig. 2 in this paper resembles that in aqueous solution in the concentration range between 1% and 2.2% [8], although the local concentration of invertase on PVA–SFF may differ from the concentration range in aqueous solution.

Invertase exists as an oligomer in the native state [8]. If the invertase solution is dilute enough so that the interaction between invertase oligomers can be neglected, and if the oligomeric invertase dissociates during the denaturation, then the value of T_d must increase with increasing concentration [12]. Since the local concentration of immobilized invertase is very high, the following effects are also considered to be important for invertase on PVA–SFF. Carbohydrates and polyhydric alcohols have a stabilizing effect

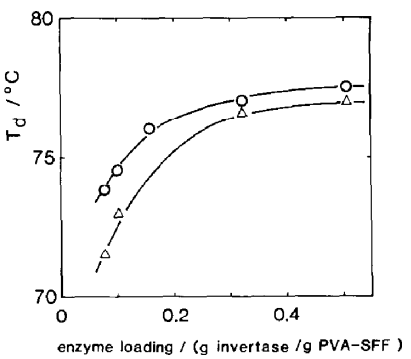

Fig. 2. The relation between enzyme loading and the denaturation temperature (T_d) of invertase on PVA–SFF: ○——○, pH 4.58; △——△, pH 7.19.

TABLE 1

Denaturation temperature T_d and enthalpy ΔH_d for the thermal denaturation of immobilized invertase on PVA–SFF

pH	Enzyme loading (g enzyme (g PVA–SFF)$^{-1}$)	T_d (°C)	ΔH_d (J g^{-1})
4.58	0.0776	73.8	11.2
	0.1016	74.5	9.5
	0.1640	76.0	9.9
	0.3232	77.0	10.4
	0.5065	77.5	12.2
5.41	0.2042	78.2	8.8
6.73	0.2042	74.9	11.7
7.19	0.0776	71.5	9.7
	0.1016	73.0	13.5
	0.3232	76.7	14.7
	0.5065	77.0	13.2
7.91	0.2042	65.7	9.2

on the enzyme [13–15]. Invertase from *Candida utilis* contains about 50% by weight of carbohydrate [10,16] and PVA is one kind of polyhydric alcohol. The results in Fig. 2 are believed to support the idea that the stabilization of immobilized invertase is mainly caused by the effect of the local high concentration of the carbohydrate in the invertase molecule. The limiting value of T_d at the extremely high concentration in aqueous solution is about 75 °C [8], which is about 2 °C lower than the value of T_d for immobilized invertase at a high enzyme loading. This difference suggests that the stabilization is also partly due to the interaction of PVA with invertase.

The enthalpy of thermal denaturation of invertase on PVA–SFF, ΔH_d, together with T_d at various enzyme loadings are shown in Table 1. ΔH_d does not vary much within the range of the enzyme loading. For comparison, the

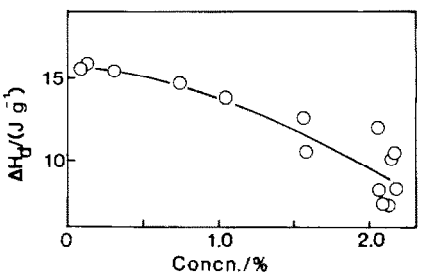

Fig. 3. Concentration dependence of denaturation enthalpy (ΔH_d) of invertase in aqueous solutions at pH 7.2.

values of ΔH_d for aqueous solution at various concentrations are shown in Fig. 3. The decrease in ΔH_d with increasing concentraion of enzyme in solution suggests that coagulation occurs at higher concentrations. The values of ΔH_d for immobilized invertase are close to the values of ΔH_d in aqueous solution at concentrations between 1% and 2%.

The effect of pH on the thermal denaturation

The relation between T_d and the pH values of the buffer solutions into which PVA–SFF with invertase was dipped is shown in Fig. 4. The concentration of invertase in aqueous solution is about 0.08%, and the value of the enzyme loading for immobilized invertase is 0.204 g enzyme (g dry PVA–SFF)$^{-1}$. It is seen from Fig. 4 that the T_d values for invertase on PVA–SFF as well as in aqueous solution are affected by pH but the effect is less than that in aqueous solution at an acidic pH. In all the ranges of pH, the values of T_d for the immobilized invertase are 5–13° C higher than those for invertase in aqueous solutions. As mentioned in the previous section, this difference in T_d is considered to be due to the difference in local concentration. The actual enzyme reaction in aqueous solution is carried out at an enzyme concentration which is more dilute than 0.005%. However, the reaction is usually carried out using the immobilized enzymes with an enzyme loading similar to that shown in Fig. 4. This means that the stabilizing effect of immobilization, which is shown in Fig. 4, can be expected under the conditions of practical enzyme reaction. An increase in thermal stability was also observed when invertase was immobilized in PVA film [8].

The reversibility of the thermal denaturation

To examine the reversibility of the thermal denaturation of invertase, the calorimetric measurements were made with the pre-heated invertase samples

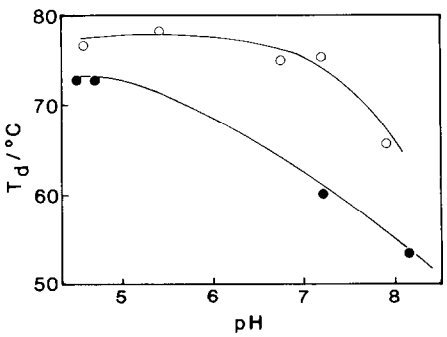

Fig. 4. The pH dependence of the denaturation temperature of invertase: ○——○, immobilized on PVA–SFF; ●——●, in aqueous solution.

TABLE 2

The reversibility of the thermal denaturation of invertase at pH 4.5. $f_N(T)$ denotes the remaining native fraction at T °C and $f_{rev}(T)$ the reversibility of the sample which was preheated from 25 to T °C at a heating rate of 1 °C min^{-1}

Temp. (°C)	$f_N(T)$ (%)	$f_{rev}(T)$ (%)
Invertase in aqueous solution		
60.0	100	97.4
70.0	77.3	58.0
72.0	36.5	22.0
72.6	20.3	10.9
76.0	0	0
Invertase on PVA–SFF		
70.0	96.7	103
73.0	81.4	68.4
77.0	39.1	43.0
82.5	0	11.2
94.0	0	0

at pH 4.5. Invertase solutions and invertase–PVA in buffer solutions were heated from 25 °C to various temperatures at a heating rate of 1 °C min^{-1} and then cooled rapidly in an ice bath. In the DSC curve for the invertase without heat treatment, the peak area beyond certain temperatures relative to the total peak area was assumed to be the native fraction $f_N(T)$ at that temperature. The peak area in the DSC curve for the heat-treated invertase relative to the total area of the invertase before the heat treatment, $f_{rev}(T)$, was used as a measure of the reversibility of thermal denaturation. The results are shown in Table 2. If the denaturation process is completely reversible, then the peak areas after and before heat treatment must be equal at all temperatures. If the denaturated fraction cannot be recovered and the remaining native fraction after the treatment is maintained, $f_{rev}(T)$ must be equal to $f_N(T)$. In this case, the results of the thermal reversibility of an enzyme reflect the thermal stability ($f_N(T)$) of the enzyme. The results in Table 2 show that the denaturation of invertase is not reversible. The ratio $f_{rev}(T)/f_N(T)$ for aqueous solution is less than unity and decreases with increasing temperature of the heat treatment. For invertase immobilized on PVA–SFF, the ratio does not decrease with the temperature of the heat treatment but is scattered around unity. The thermal reversibility of invertase was previously studied by activity assay [9] and the results agreed with those from calorimetry.

The thermal stability of invertase on PVA–SFF is higher than that of invertase in aqueous solutions with respect to the denaturation temperature and reversibility.

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