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Thermal stability and activity of *Candida cylindracea* lipase

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Abstract

Thermal analysis combined with other experimental techniques has shown how the activity and stability of lipase from *Candida cylindracea* are related to protein hydration and how the water is bonded with varying strength and energy to the enzyme surface. It was observed by DSC (differential scanning calorimetry) that the thermal denaturation is the result of two simultaneous events, dehydration and unfolding, and that this endothermic process becomes reversible at temperatures less than T_m (T_m = 86°C, maximum peak temperature) and irreversible at temperatures higher than T_m, leading to a partially or totally deactivated physico-chemical aggregate.

Keywords: *Candida cylindracea*; Differential scanning calorimetry; Lipase; Thermal analysis

1. Introduction

Lipases, triacylglycerol acylhydrolases (EC 3.1.1.3), are water soluble enzymes which in vivo catalyze the hydrolysis of triglycerides, insoluble substrates, by a heterogeneous process. In vitro they may also hydrolyse soluble esters, but the presence of a water–lipid interface significantly increases their lipolytic activity [3,4,10,16,22].

In recent years, the studies on the lipase from *Candida cylindracea*, commonly known as *C. rugosa*, have become more intensive for its potential industrial applications. In fact, this enzyme catalyzes esterifications and transesterifications in organic phases [2,19] with low content of water [11,12] and is also used in enantioselective reactions [6,8,9].

Lipase from *Candida rugosa* [20], shares the α/β-hydrolase fold and acts through a catalytic triad Ser–His–Glu covered by a superficial polypeptide lid. The face of the flap directed toward the active site is hydrophobic, while the face opposite is hydrophilic. At an interface, this loop undergoes important conformational changes, opening up to reveal a larger hydrophobic area, owing to interaction with the interface itself, and bonding with the substrate [8].

The water seems to have a two-fold effect on the enzymatic system: (i) it plays an essential role in the acquisition and maintenance of the catalytically active conformation of the enzyme to its optimal pH value, (ii) it plays a very important role in many inactivation processes, in particular those related to thermal inactivation.

It is therefore important to study water–enzyme interactions [1,5,14,17,18], particularly

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with regard to the optimization of enzymatic processes at the industrial level.

In this sense, thermoanalytical techniques allow us to obtain detailed information concerning the various water–biological matrix interactions [15]. By means of thermogravimetry (TG), derived thermogravimetry (DTG), TG–FTIR coupling (i.e. Fourier transform infrared spectroscopy coupled with thermobalance) and differential scanning calorimetry (DSC) it has been possible to evaluate the quantity of water present in the enzyme samples, the different types of water bonded to them (in species possessing different interaction energies) and their interrelation with the conformational changes associated with reversible and irreversible denaturation of the lipolytic enzyme. The effect of the quantity of water present on enzymatic activity was also investigated by lyophilization processes to separate the dehydration effect from the thermal effect, which occur simultaneously in thermoanalytical treatment.

2. Experimental procedures

2.1. Materials

The enzymes used in this work were purchased as dry powders from Sigma Chemical Co. (St. Louis, MO, USA). 2 lipase preparations were employed:

Lipase (type VII) from *Candida cylindracea* (EC 3.1.1.3) with a specific activity of 905 U/mg solid and 4570 U/mg protein (Biuret) using olive oil as substrate; this enzyme powder contains ≈ 30% lactose as extender.

Lipase from *Candida cylindracea* (EC 3.1.1.3) with a specific activity of 60000 U/mg solid and 300000 U/mg protein (Biuret) using olive oil as substrate; this enzyme powder contains ≈ 20% protein and traces of calcium but does not contain lactose. D-Lactose monohydrate was also purchased from Sigma Chemical Co.; tributyrin and Tris (trihydroxymethylaminomethane) were obtained from Merck. All other chemicals were of the purest grade available, and double-distilled water was used throughout this study.

2.2. Enzyme activities

Enzyme activity was assayed by Crison micro TT2022 pH-stat. In a typical experiment, 5 mg of lipase was dissolved in 5 ml of 50 mM Tris–HCl buffer at pH = 7.4 then 5 ml of tributyrin were added. The mixture was vigorously shaken for 2 min and then rapidly stirred. After 30 min of incubation at 37°C, the reaction was quenched with 5 ml of an acetone–ethanol (1:1) mixture. Blanks were prepared using the same solutions under the same experimental conditions but without enzyme. Each trial was replicated three times.

2.3. Thermal analysis

2.3.1. Differential scanning calorimetry (DSC)

For the DSC analysis, 10–20 mg samples of lipase powder were placed into aluminum sample pans, open and sealed, and then submitted to thermal treatment using pure nitrogen (99.9%) as carrier gas at a flow rate of 50–100 ml/min and a temperature scan rate of 10°C/min. The measurements were performed using a Perkin Elmer DSC-7 instrument.

2.3.2. Thermogravimetry and derived thermogravimetry (DT and DTG)

A Perkin Elmer thermogravimetric analyser (TGA-7) was used for TG and DTG analysis. 10–20 mg samples of lipase were assayed for qualitative and quantitative water determination. The carrier gas was nitrogen (99.9%) at a flow rate 50–100 ml/min and a temperature scan rate of 10°C/min was employed.

2.3.3. Infrared spectroscopy coupled with thermogravimetry (FTIR–TG)

The characterization of gases evolved during thermal treatment was performed using a Fourier transform infrared spectrometer (FTIR Perkin

Elmer model 1760X) coupled by transfer-line to a TGA-7 thermobalance (Perkin Elmer series 7). The IR spectra were collected in the 25–150°C temperature range, again using nitrogen as carrier gas.

3. Results

3.1. Study of thermostability of Candida cylindracea lipase with lactose

At first, lipase from *Candida rugosa*, containing 30% lactose as an 'extender', was investigated.

The TG and DTG curves of this lipase (Fig. 1) display an initial weight loss between room temperature and 118°C. This is followed by a second loss between 118°C and 130°C. The FTIR curves obtained for these two processes indicate (insert of Fig. 1) that only water is present in the decomposition gases.

The DSC curves (Fig. 2a) show an initial endothermic process that occurs between room temperature and 120°C, followed by a shoulder which turns into a sharp endothermic process with a maximum at 146°C. At 165°C there is a small exothermic process, followed by two consecutive endothermic processes with their maxima at 218°C and at 225°C respectively. The first endothermic process with maximum at 86°C, corresponds to the water loss and thermal transition of protein, while the subsequent processes are typical of α-lactose-monohydrate transformations, as is confirmed by the DSC curve for pure lactose (Fig. 2b) obtained under the same conditions: endothermic peak at 146°C correspond to the crystallization water; exothermic peak at 165°C is the recrystallization of small amorphous portions of lactose; endothermic peak at 218°C represent the melting process and endothermic peak at 225°C is the start of the decomposition process [7,13].

In order to evaluate the thermal effects on enzymatic activity, solid samples were placed in a DSC capsule and then heated to the temperatures of 45, 55, 86 and 125°C respectively, and then subjected to the enzymatic activity assay. In parallel, enzyme samples in a DSC capsule were heated to the above-mentioned tempera-

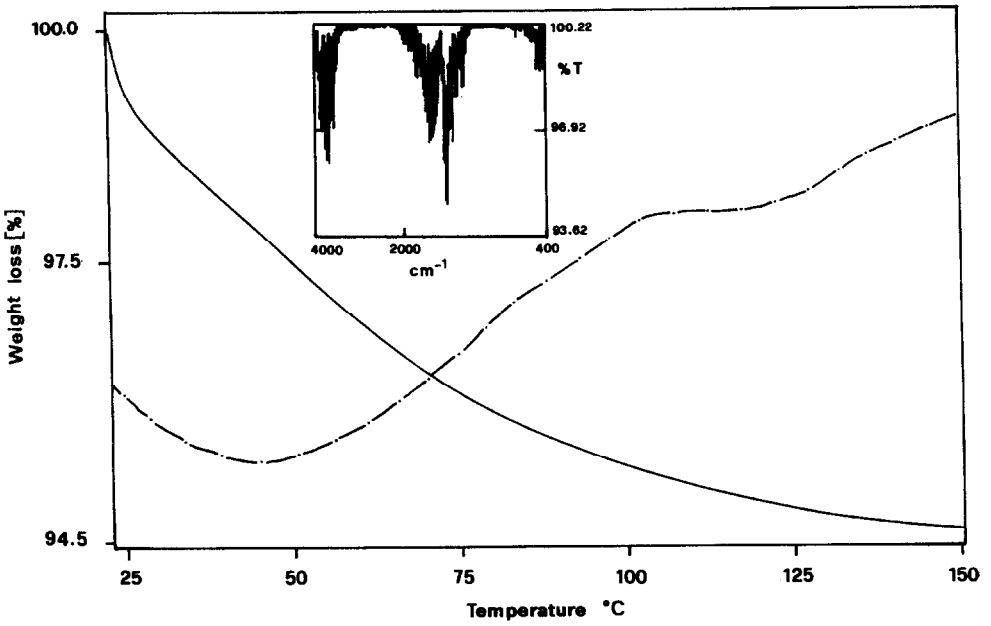

Fig. 1. TG (——) and DTG (– · –) curves of *Candida cylindracea* lipase with lactose. Heating rate 10°C/min, nitrogen (99.9%), flow rate of 50–100 ml/min. Insert: TG–FTIR spectrum of *Candida cylindracea* lipase with lactose.

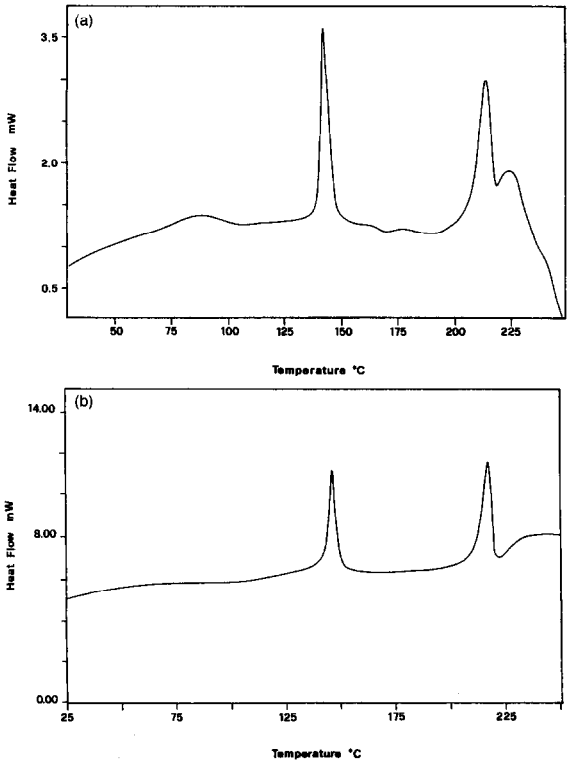

Fig. 2. (a) DSC curve of *Candida cylindracea* lipase with lactose. Heating rate 10°C/min; nitrogen (99.9%), flow rate of 50–100 ml/min. (b) DSC curve of α-lactose monohydrate standard. Heating rate 10°C/min, nitrogen (99.9%), flow rate of 50–100 ml/min.

tures, cooled at room temperature and resubjected to DSC scan. There was found to be a gradual flattening of the first endothermic process, in particular of the first part of this process up to 80°C, which corresponded to the elimination of the more superficial layers of water bonding to the sample.

Table 2
Lipase activity at different temperatures

T (°C)	Lipase activities (%) at different temperatures (maximum activity at 45°C is set as 100%).	
	lipase with lactose	lipase without lactose
30	85.7	82.2
35	90.4	85.4
40	96	90.3
45	100	100
50	89	87.6
55	78.5	75
60	55.3	41.4
65	37.2	28
70	26.6	22.5

Substrate: tributyrin, buffer: Tris–HCl pH = 7.4, incubation time: 30 min.

Lastly, aliquots heated to the above temperatures were stored at 4°C for 36 h and then, the activity was tested in order to verify whether rehydration could lead to recovery of enzymatic activity. The results are plotted in Table 1.

In conclusion, lipase activity was tested at different temperatures (Table 2) after 30 min incubation in order to determine the temperature at which lipase displays maximum activity.

3.2. Study of thermostability of lactose-free lipase from Candida cylindracea

This type of lipase contains no lactose according to the specifications of the manufacturer, and was confirmed by DSC analysis performed by us.

The TG and DTG curves of this lipase (Fig.

Table 1
Effect of 36 h storage at 4°C, in a closed vessel, on samples of lipase heated at different temperatures

T (°C)	Specific activities (U/mg solid) of			
	lipase with lactose		lipase without lactose	
	immediately after thermal treatment	after 36 h of storage at 4°C	immediately after thermal treatment	after 36 h of storage at 4°C
25	2.90 ± 0.05	2.80 ± 0.05	49.8 ± 0.2	50.9 ± 0.1
45	2.78 ± 0.05	2.90 ± 0.05	47.8 ± 0.2	51.8 ± 0.2
55	2.70 ± 0.05	2.95 ± 0.05	46.0 ± 0.2	52.3 ± 0.2
86	2.20 ± 0.05	2.5 ± 0.1	32.0 ± 0.2	35.0 ± 0.2
125	0.07 ± 0.05	0.25 ± 0.05	21.9 ± 0.1	25.2 ± 0.4
134			11.8 ± 0.5	7.9 ± 0.2
138			4.3 ± 0.2	4.6 ± 0.4

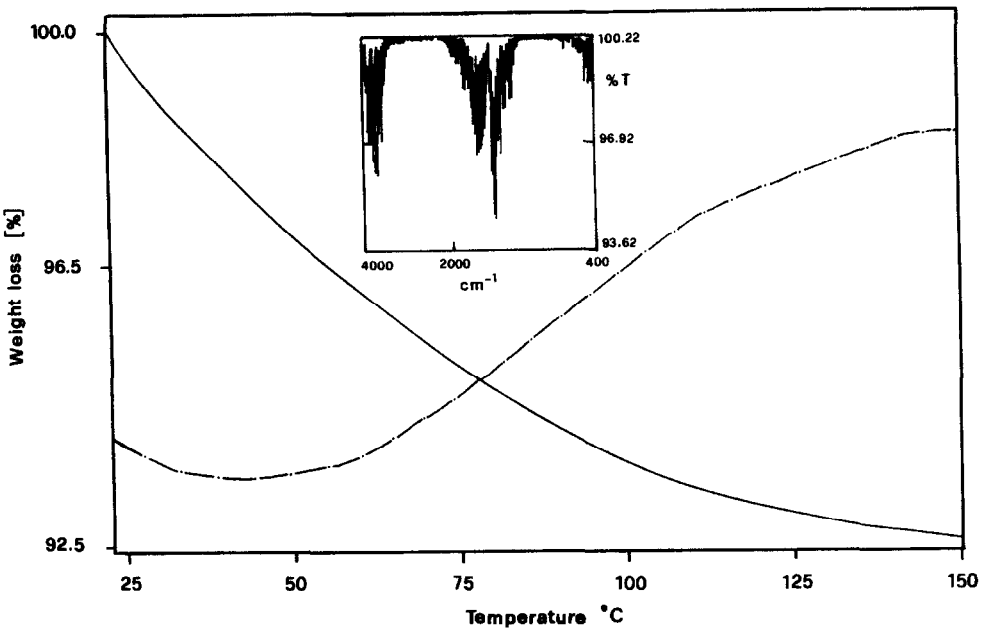

Fig. 3. TG (——) and DTG (— · —) curves of *Candida cylindracea* lipase without lactose. Heating rate 10°C/min, nitrogen (99.9%), flow rate of 50–100 ml/min. Insert: TG–FTIR spectrum of *Candida cylindracea* lipase without lactose.

3) show an initial weight loss between room temperature and 130°C.

The FTIR curves, corresponding to the process under investigation, indicate (insert of Fig. 3) that only water is present in the gases evolved from sample decomposition.

The DSC curves (Fig. 4) show an initial endothermic process localized between room temperature and 140°C. In the descending portion of the first endothermic process, there is a trace of a second endothermic process with maximum at 138°C. It was attempted to resolve these two processes and, to this end, DSC analysis was performed using sealed sample pan with holes. As shown in Fig. 5 there is a good deconvolution of the two processes with the appearance of a well resolved second endothermic peak with a maximum at 138°C.

Also in this case, in order to evaluate the thermal effects on enzymatic activity, aliquots

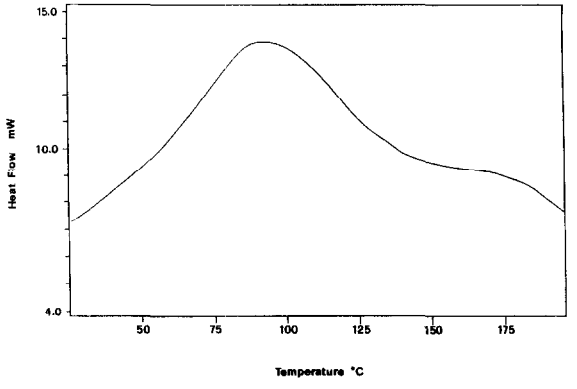

Fig. 4. DSC curve of *Candida cylindracea* lipase without lactose. Heating rate 10°C/min; nitrogen (99.9%), flow rate of 50–100 ml/min.

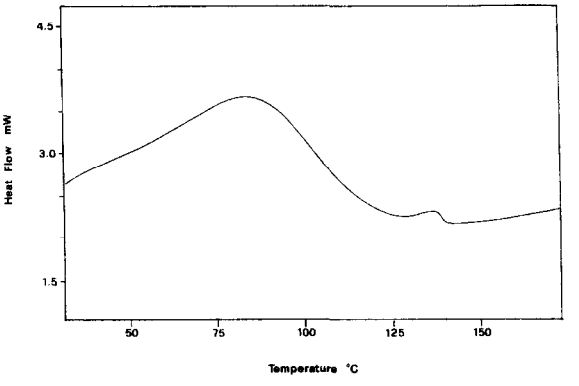

Fig. 5. DSC curve of *Candida cylindracea* lipase without lactose using a sealed sample and reference pans with holes. Heating rate 10°C/min, nitrogen (99.9%), flow rate of 50–100 ml/min.

of the enzyme stock were placed in DSC capsules and heated respectively to 45, 55, 86, 125, 134 and 138°C. In order to verify whether rehydration could produce any change in enzymatic activity, aliquots of the enzyme were heated to the above-mentioned temperatures and then stored at 4°C for 36 h. The plots of the activities measured after the above treatments are shown in Table 1.

The activity was also tested at different temperatures (Table 2) in order to determine the temperature at which maximum activity occurs.

4. Discussion

The DSC curves of the enzyme with and without lactose indicate that the lactose-free product is the more stable.

An examination of Table 1 clearly shows that at 125°C the enzyme containing lactose retains a residual activity of 2.4% of the initial activity, while the lactose-free enzyme, at the same temperature, retains 44% of its original activity and shows an 8.6% residual activity at 138°C, i.e. temperature corresponding to the second endothermic DSC peak (Fig. 5).

The maximum activity of both preparations, measured after 30 min incubation in a thermostated water bath under constant stirring, was found to occur around 45°C (Table 2), while the Merck Index (An Encyclopedia of Chemicals, Drugs and Biologicals, twelfth edition, S. Budavari, Ed., 1996) reports that the maximum activity occurs in the range 35–37°C. Furthermore, at 70°C the enzyme with lactose retains 27% of its maximum activity, while the lactose-free preparation retains 22.5% In both cases, the maximum decrease in activity occurs between 55 and 60°C.

In conclusion, the endothermic process under discussion may be said to be the result of two simultaneous processes dehydration and denaturation ('thermal unfolding').

A progressive activity decrease is also found parallel to the temperature increase, up to approximately zero level at the end of the peak.

The sample's rehydration, after thermal stress ($T < 86$°C), causes a full recovery of enzymatic activity, while it has virtually no effect on those treatments performed with $T > 86$°C temperatures, although a very little activity recovery can be detected. At the end of the peak a significant activity loss is observed.

It is also possible to hypothesize that, with $T < T_m$ (maximum peak temperature), thermal denaturation is a reversible process, while, with $T > 86$°C, an irreversible denaturation process take place, leading to a macroscopic aggregate with solubility loss.

Water loss, according to what was hypothesized by Klibanov [21], probably induces a more rigid enzymatic structure, with special reference to its active site.

Water layers weakly bound to the protein, were observed by means of cyclic DSC treatments ($T < 86$°C), which showed a flattening in the temperature range from 25 to 70°C of the endothermic process.

Parallel activity assay, carried out after rehydration, showed a full activity recovery, suggesting that the lost water is not essential to the above activity. This allows us to hypothesize that, once essential water (this means the water most closely bound to the enzyme) is lost, an irreversible denaturation process is likely to occur.

Acknowledgements

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