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Relationship between thermal inactivation and conformational change of *Yarrowia lipolytica* lipase and the effect of additives on enzyme stability

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ABSTRACT

The relationship between thermal inactivation and conformational changes of *Yarrowia lipolytica* lipase has been investigated. The enzyme loses activity over 40 °C, with a half-life of 0.325 h at 50 °C. The thermal inactivation kinetics fits with a first-order expression. The conformational transition from ordered to unfolded structures during thermal denaturation has been studied by fluorescence, circular dichroism (CD), ultraviolet (UV) spectra, and dynamic light-scattering (DLS). The thermal unfolding occurs in three stages where changes in tertiary and secondary structure, are accompanied by molecular aggregation. Additives such as span 85 can prolong the half-life of the lipase by a factor ca. 850 at 50 °C. The increase in denaturation temperature is confirmed by differential scanning calorimetry (DSC).

1. Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are important industrial enzymes and are used for production of foods, pharmaceuticals, biofuels, detergents and in waste water treatment, etc. [1]. However, lipases, as many other enzymes, cannot stand the effect of high temperature, extreme pH, high ionic strength, and organic solvents. Several methods, including immobilization [2–4], structural and chemical modification [5–7], and the use of additives [8–12] have been employed to improve lipase stability and activity. Thus porcine pancreatic lipase immobilized on mesoporous silica supports retained over 70% of its activity after incubation at 60 °C for 2 h, while the free enzyme lost all activity [3]. After modification with pNPCF-PEG, *Candida rugosa* lipase was more stable iso-octane than its native form at 50 °C [6]. Noel and Combes [12] found that the half-life of *Rhizomucor miehei* lipase was prolonged by a factor of 500 at 50 °C in the presence of 4 M sorbitol. The use of protective agents is a simple, fast, and economic means to improve/maintain the stability and activity of lipases. Various types of substances have been used as additives, including surfactants [10,13], polyhydric alcohols [9,11], methyl esters [14], metal ions [15] and other chemical reagents [8,16].

Yarrowia lipolytica (*Y. lipolytica*) lipase has been produced in our lab and confirmed to be an efficient catalyst for several industrially relevant reactions [17–19]. The applications of the enzyme for industrial purposes depend on its stability under harsh conditions such as high temperature or the presence of organic solvents.

In this study, the relationship between thermal inactivation and conformational change of the enzyme has been investigated using fluorescence, circular dichroism (CD) and ultraviolet (UV) spectroscopy, and dynamic light-scattering (DLS). The effect of additives on lipase stability was also studied in order to be applied to commercial preparations and industrial applications.

2. Materials and methods

2.1. Materials

The organism *Y. lipolytica* (formerly named *Candida* sp. 99-125) was isolated and preserved in our laboratory. Olive oil, span 80, span 85, Tween 80, Triton X-100, polyvinyl alcohol (PVA), trehalose, acacia gum and glycerol were from Sanbo Biotech (Beijing, China). Bovine serum albumin (BSA), dithiothreitol (DTT), phenylmethyl sulfonylfluoride (PMSF), cysteine and racemic naproxen were purchased from Sigma (St Louis, USA).

2.2. Purification procedure of the lipase

Lipase-containing yeast culture was obtained as described previously [20] produced in 30 l bioreactor. The broth was clarified by centrifugation (2000 × *g*, 15 min, 4 °C). The proteins in the supernatant were precipitated at 40% ammonium sulfate saturation followed by dehydration using cold acetone. The dry acetone powder was used as starting material for the lipase purification pro-

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cedure. The chromatographic purification of the crude lipase was carried out as previously described [21]. The purified lipase had a specific activity of 5000 U mg^{-1}.

2.3. Lipase assay

Lipase activity was assayed using the olive oil emulsion method [22]. Olive oil (5%, v/v) was emulsified in distilled water containing 2% (w/v) of PVA (as an emulsifier) for 6 min using a knife homogenizer. To 5 ml emulsion was added 4 ml 100 mM potassium phosphate buffer, pH 8.0, and 1 ml enzyme solution. After incubation for 10 min at 37 °C the reaction was stopped by adding 15 ml ethanol. Enzyme activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit (U) of lipase was defined as the amount of enzyme which released 1 μmol fatty acid per minute under the assay conditions.

2.4. Fluorescence measurements

Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorimeter over the 325–380 nm range, with excitation at 300 nm to minimize the emission arising from tyrosine residues. A 700-μl sample was analyzed in the chamber quartz cuvette with a scan rate of 2 °C min^{-1}. The protein concentration of the lipase was, in all cases, 0.2 mg ml^{-1}. All measurements were repeated three times.

2.5. CD and UV spectra

CD and UV spectra were obtained using a JASCO J-810 spectropolarimeter (JASCO, Japan). The working buffer was 10 mM Tris–HCl buffer (pH 8.0). Wavelength spectra were recorded at 25 °C using a 0.1-cm path-length cuvette. The sample concentration was 0.2 mg ml^{-1}. Thermodynamic stability was measured at 208 and 222 nm by recording the CD signals and 280 nm by recording the UV signals with a scan rate of 2 °C min^{-1}. All measurements were repeated three times.

2.6. DLS analysis

The concentration of the protein was 1.0 mg ml^{-1}. A 50-μl sample was analyzed in the chamber quartz cuvette. DLS measurements were performed on a DynaPro^{TM} Titan molecular sizing system (Wyatt, USA) with a scan rate of 2 °C min^{-1}. The data were analyzed using Dynamics V6 software. All measurements were repeated three times.

2.7. DSC analysis

DSC analysis was carried out using a 200F3 DSC instrument (NETZSCH, Germany). A 20 μl sample (5 mg ml^{-1}) was put into the calorimeter's measurement cell, and the whole mass was recorded. A reference cell was filled with the blank solution without lipase. Both cells were hermetically sealed and measured with a scan rate of 2 °C min^{-1}. Each differential scanning calorimetry measurement was repeated three times.

2.8. Preparation of lipase solution with different additives

Additives were mixed with 50 mM phosphate buffer (pH 8.0), and dispersed by sonication for 15 min at 50 °C. The lipase was dissolved in 50 mM phosphate buffer (pH 8.0) with or without additives. The enzyme solution had an initial activity of 5000 U ml^{-1}. All measurements were repeated three times.

Table 1
Kinetics equation parameter estimates for the one-step model (1) of thermal inactivation of *Y. lipolytica* lipase.

Temperature, °C	k, $\times 10^{-2}$ h^{-1}	$t_{1/2}$, h	R^2
4.0	0.279	231	0.996
10.0	0.358	173	0.998
15.0	0.598	116	0.997
20.0	0.694	99.0	0.998
25.0	0.780	86.6	0.998
30.0	2.40	28.9	0.996
35.0	6.14	11.4	0.997
37.5	11.0	6.30	0.986
40.0	9.67	7.15	0.997
42.5	21.0	3.30	0.997
45.0	45.8	1.51	0.997
47.5	72.6	0.955	0.991
50.0	213	0.325	0.984

2.9. Thermal inactivation

Thermal inactivation was carried out at temperature ranging from 4 to 50 °C. The lipase samples (5000 U ml^{-1}) were incubated at a selected temperature, withdrawn at predetermined intervals of time, immediately chilled to 4 °C and assayed for residual lipase activity as previously described, with unheated samples as reference. Thermodynamic stability was measured at temperature ranging from 25 to 60 °C with a scan rate of 2 °C min^{-1}. All measurements were repeated three times.

2.10. Thermal stability

For the determination of thermal stability and the effect of additives, the samples (5000 U ml^{-1}) were incubated at 50 °C for 3 h. Samples were withdrawn and assayed for residual relative activity as described above. All measurements were repeated three times.

3. Results and discussion

3.1. Inactivation kinetics

The dependences of the relative activity (U_t/U_0) (U_0 and U_t are the values of the initial and residual activity, respectively) on time (t) are linear in the coordinates {ln(U_t/U_0): t}.

The inactivation rate (k) is calculated by the first-order expression:

$$\ln\left[\frac{U_t}{U_0}\right] = -kt \quad (1)$$

The model parameters at different temperatures are shown in Table 1. Above 40 °C, the lipase quickly loses its activity and its half-life is reduced to 0.3 h at 50 °C. The parameter k is temperature dependent and can be expressed by the Arrhenius equation:

$$k = A\exp\left(-\frac{E_A}{RT}\right) \quad (2)$$

where E_A is the energy of activation for the inactivation process, and A is the pre-exponential factor. The E_A value can be deduced from the Arrhenius plot of ln k vs. $1/T$, represented in Fig. 1. The enzyme on heating involves three stages. From the slope of the Arrhenius plot, three values of 41.2, 160 and 247 kJ mol^{-1} were obtained for the temperature ranges of 4–20, 25–37.5 and 40–50 °C, respectively.

3.2. Relationship between the conformational change and the thermal inactivation

Spectroscopic analysis can provide more data of thermal protein unfolding.

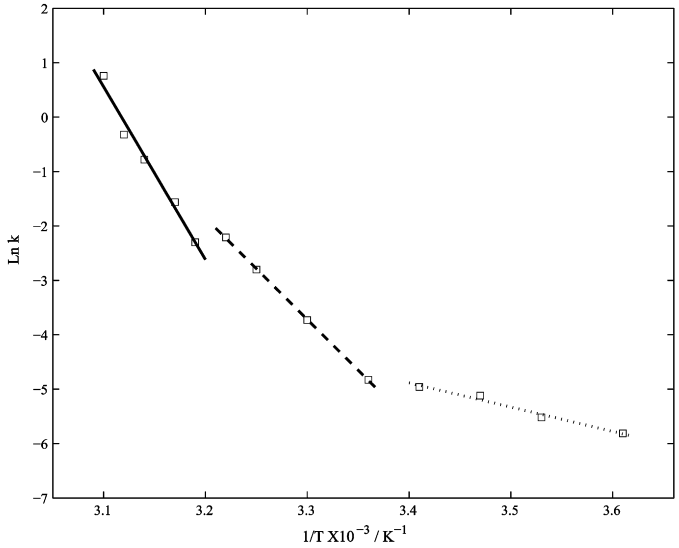


Fig. 1. Arrhenius plot for the thermal inactivation of *Y. lipolytica* lipase—solid line: $y = -29.7x + 92.7$, $R^2 = 0.987$; dash line: $y = -19.3x + 59.8$, $R^2 = 0.999$; dot line: $y = -4.96x + 12.0$, $R^2 = 0.956$.

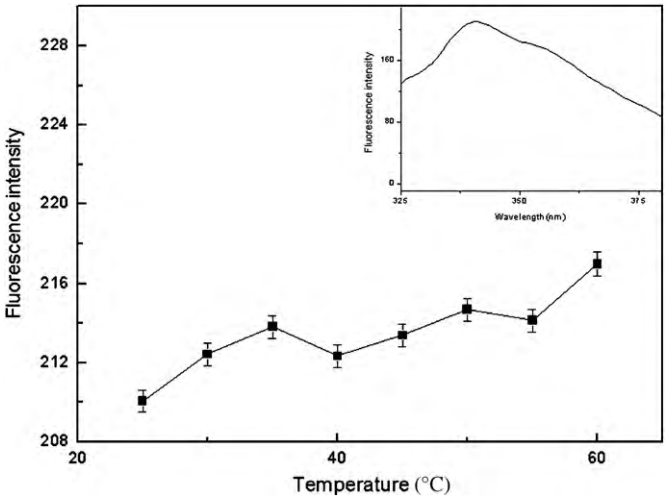


Fig. 3. Fluorescence emission of *Y. lipolytica* lipase as a function of temperature. The thermal unfolding was recorded at 340 nm by spectrofluorimeter at a constant rate of 2 °C min^{-1}. The inset shows fluorescence emission spectra of the lipase at initial 25 °C.

DLS allows the measurement of the size of the particles formed during protein aggregation. In Fig. 2 is shown the process of aggregation and inactivation of the lipase as a function of the temperature. The hydrodynamic radius R_h of the protein increases dramatically when the temperature reaches the range 50–60°C, indicating the formation of protein aggregates. According to the inactivation plot, the aggregation is correlated to the activity loss. At 60 °C, the enzyme is completely inactive.

The fluorescence emission of the lipase shows a maximum at 340 nm (Fig. 3), caused by the protein's three Trp residues [21]. By observing the shift in fluorescence emission at an excitation wavelength of 300 nm, where the absorbance of Tyr residues is negligible, the Trp residues can be studied as a function of temperature [23]. As is well known, Trp emission is extremely sensitive to the polarity of the environment and a red-shift is observed with an increase in polarity [24]. Thermally induced lipase emits maximally at 340 nm with minor increase of fluorescence emission and no obvious red-shift. During thermal unfolding, although Trp residues are still buried within the interior of the protein, the polarity of its microenvironment changes, indicating changes of the enzyme tertiary structure. Fig. 4 shows the effect of heat on the UV spectra of the lipase. The protein exhibits a maximal UV absorption at about 280 nm which is due to the presence of 3 Trp and 13 Tyr residues in the molecule. The absorption intensity increases with increasing temperature in the range 25–60 °C. The result of the UV spectroscopy further demonstrates changes of the tertiary structure of the lipase.

Fig. 5 shows the degree of ellipticity as a function of temperature in the range 25–90 °C, obtained from CD spectroscopy. Two minima at around 220 and 208 nm, which are typical of α-helix secondary structure, can be distinguished. The enzyme unfolding was investigated through monitoring the characteristic CD peaks for α-helix at 208 and 222 nm. The CD bands decrease from 50 °C at 222 nm and from 70 °C at 208 nm, indicating that heat promotes helix unfolding which is often correlated with activity loss [25]. The minimum at 208 nm is the Moffitt excitation, which is attributed to parallel (helical axis) polarized $\pi \rightarrow \pi^*$ transitions; and the minimum at 222 nm has a $n \rightarrow \pi^*$ transition [26]. The change of CD signal at

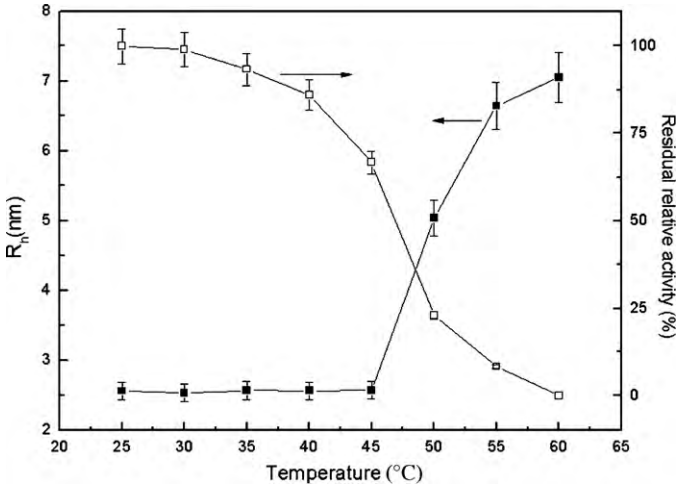


Fig. 2. Evolutions of aggregation and inactivation of *Y. lipolytica* lipase with temperature at a constant rate of 2 °C min^{-1}. The samples were incubated to reach different temperatures, immediately chilled to 4 °C, then subjected to the measurements of residual relative activity (□) with the native lipase activity (5000 U ml^{-1}) being 100% and hydrodynamic radius (R_h) (○) using DLS.

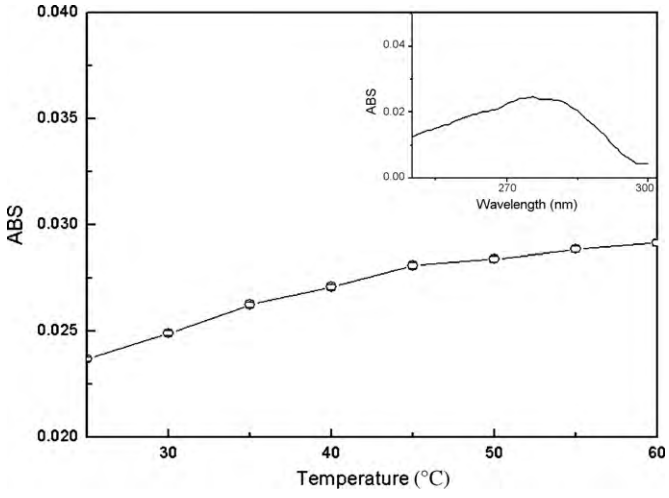


Fig. 4. UV absorption of *Y. lipolytica* lipase as a function of temperature. The thermal unfolding was recorded at 280 nm by CD spectropolarimeter at a constant rate of 2 °C min^{-1}. The inset shows UV spectra of the lipase at initial 25°C.

Table 2
Effect of additives on the residual activity of *Y. lipolytica* lipase after incubation at 50 °C for 3 h.

Additive		Residual activity %	Additive		Residual activity %
Type	Concentration (w/v,%)		Type	Concentration	
None	–	0	Olive oil	0.05	38.4 ± 1.1
Trehalose	10.0	9.0 ± 0.3		0.25	88.3 ± 2.6
	20.0	79.1 ± 2.4		0.50	100.0 ± 3.0
	30.0	69.2 ± 2.4	Span 80	0.05	8.1 ± 0.2
Acacia gum	10.0	41.8 ± 1.3		0.25	63.2 ± 1.9
	20.0	69.4 ± 2.1		0.50	97.2 ± 2.9
	30.0	66.8 ± 2.0	Span 85	0.05	16.3 ± 0.5
PMSF	0.1*	0		0.25	77.4 ± 2.3
DTT	0.1*	59.4 ± 1.8		0.50	100.0 ± 0.5
	0.5*	50.6 ± 1.5	Tween 80	0.05	0
	1.0*	0		0.25	9.0 ± 0.3
Cysteine	0.1	3.0 ± 0.1		0.50	23.4 ± 0.7
	0.5	78.4 ± 2.3	Triton X-100	0.05	0
	1.0	53.7 ± 1.6		0.25	0
				0.50	0

* Expressed in molar concentration (M).

208 nm probably results from the flexibility of the structure with a pronounced effect on the parallel polarized $\pi \rightarrow \pi^*$ band [27].

Developments in spectroscopy have made it possible to study the mechanism of protein denaturation in more detail. Li et al. [28] reported that rubisco undergoes a three-stage thermal unfolding of its tertiary and/or quaternary structure, secondary structure and tertiary and/or quaternary structure. Golub et al. [29] studying mitochondrial aspartate aminotransferase, found that loss of enzyme activity preceded denaturation of the enzyme, which was observed as aggregate formation. The results point to that the lipase undergoes a three-stage conformational change during thermal unfolding. In the first stage, the tertiary structure changes, leading to minor activity loss. The excited state as well as the ground state of the lipase is sensitive to heat and subtle conformational rearrangements can be detected by fluorescence and UV spectra, respectively (Figs. 3 and 4). The CD data show that the secondary structure is still stable and native (Fig. 5). It means that the tertiary structure is more fragile and more easily destroyed than the secondary structure [28,29]. In the second stage, changes in the tertiary and secondary structures lead to protein aggregation and most of the activity loss. It is generally accepted that the first step in the protein aggregation is unfolding (or partial unfolding) [30], which may be relevant to the inactivation kinetics of the lipase at high temperature. In the final stage, the inactivated lipase is further unfolded and finally totally denatured. The major cause is the breakdown of the secondary conformation. The data also show that thermal inactivation of the lipase proceeds faster than thermal unfolding.

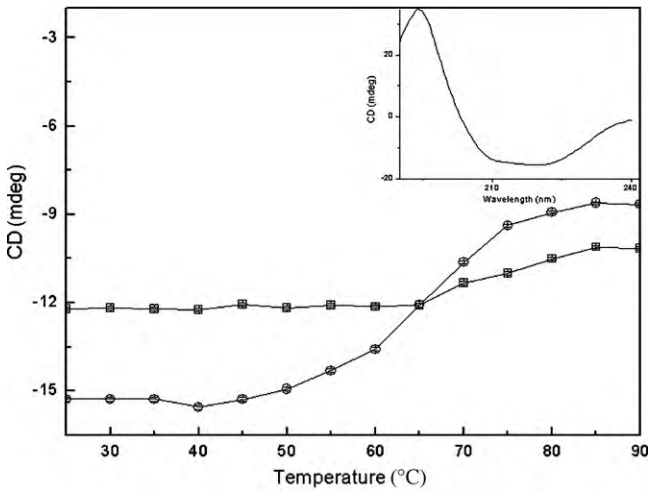


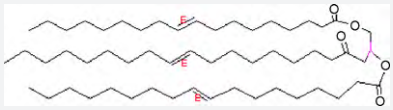
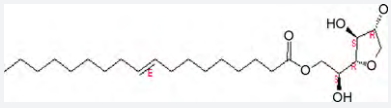
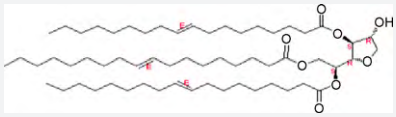
Fig. 5. CD plots of *Y. lipolytica* lipase as a function of temperature. The thermal stability was recorded at 208 nm (□) and 222 nm (○) by CD spectropolarimeter at a constant rate of 2 °C min^{-1}. The inset shows CD spectra of the lipase at initial 25 °C.

3.3. Effect of additives on the thermal stability

In Table 2 are listed a number of reagents tested for their effect on lipase thermal stability. Without additive the lipase activity was completely lost after incubation for 3 h at 50 °C, Trehalose, Acacia gum, DTT, cysteine, olive oil, span 80 and span 85 promote the thermal stability to different degree depending on concentration. It is well known that polyols such as sugar preferentially dissolve in the bulk water, reducing the thickness of the hydration layer of proteins [31]. A decrease in the hydration layer of the lipase reduces its flexibility and enhances its stability. For DTT and cysteine, the protective effect may be explained such that they may protect protein from oxidation. However, at high concentrations they destroyed the native structure and reduced the enzyme activity. It is noticeable that the best protective effect was obtained in the presence of olive oil, span 80 and span 85. These additives maintained the original lipase activity at a concentration as low as 0.5% (w/v) after incubation at 50 °C for 3 h.

The protective effect of olive oil, span 80 and span 85 on lipase thermal stability was studied at 50 °C (Fig. 6). Polynomial regression was used to fit the relation between relative activity and incubation time. The half-life of the lipase in the presence of additives was calculated using the fitting equation. The protective effect parameter (PE) was calculated from the half-life values obtained, and was defined as the ratio of enzyme half-life in the presence of additive to the half-life in the absence of additive. In Table 3 is shown the variation in PE among the three additives tested. The half-lives are significantly increased at 50 °C by a factor of 622, 768, and 850, respectively. The increase in enzyme stability might be attributed to interactions with the active site or other epitopes on the protein surface thus maintaining its active conformation [32]. Previous reports have shown similar effects of additives on enzyme stability. For example, 4 M sorbitol could prolong the half-life of *Rhizomucor miehei* lipase at 50 °C by a factor 500 [12], and 1–2 M sucrose increased pea lipoxygenases stability at 70 °C by 400–600% [33]. Successful additives are mostly hydrophilic and soluble, while hydrophobic and less soluble ones are rarely studied. It has been reported that the addition of methyl stearate to a lipase solution during the freeze-drying increased its organic solvent stability [14].

Table 3
Protective effect of additives on the thermal stability of *Y. lipolytica* lipase at 50 °C.

Additive	Main composition	$t_{1/2}$, h	Protective effect
Olive oil		8.70 ± 0.45	622 ± 32
Span 80		10.8 ± 0.3	768 ± 22
Span 85		11.9 ± 0.6	850 ± 43

Olive oil, span 80 and span 85 are all hydrophobic/amphipathic and have oleate side chains. This leads us to think that the long-chain fatty acids can stabilize active conformation of the enzyme. Unfortunately, the spectroscopy investigations cannot be carried out in the presence of additives, because of spectrophotometrical transparency. Besides spectroscopic techniques, DSC which provides the thermodynamics of protein unfolding was used to measure the lipase stability of the protein. The denaturation temperature (T_m) measured by DSC is directly related to protein stability with a higher T_m indicating greater stability. The DSC transitions of the lipase were calorimetrically irreversible since no thermal effect was observed in a second heating of the enzyme solution. Thermograms obtained by DSC for the lipase showed one main endothermic peak (Fig. 7) at a temperature of 82.8 °C without any additives and at 86.4 °C with span 85, the most effective additive. Span 85 causes a higher T_m, which indicates that it possesses a higher stabilizing effect thus maintaining the ordered structure to some degree during thermal unfolding of the enzyme. The DSC data correlate with those obtained from CD spectra. Together with data from DLS, fluorescence and UV measurements it supports the notion that the secondary structure, because of its higher stability, plays the role of molecular skeleton of the protein and can be completely unfolded only at higher temperatures.

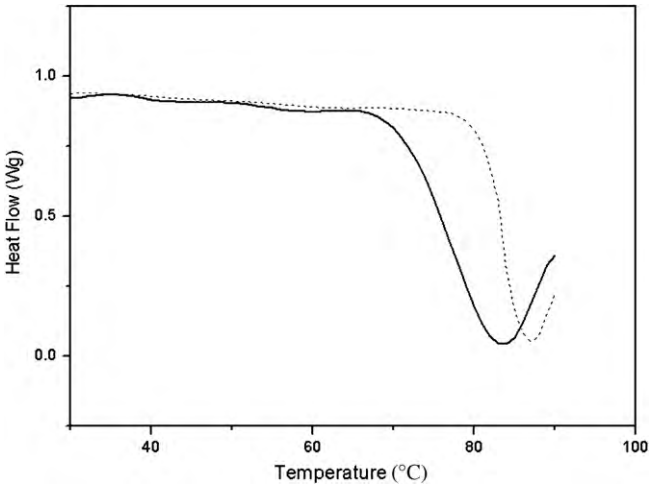


Fig. 7. Heat absorption of *Y. lipolytica* lipase as a function of temperature at a constant of 2 °C min^{-1} obtained by DSC. The curves show the thermal unfolding transition of the lipase without additive (thick solid line) or in the presence of span 85 (thin dash line).

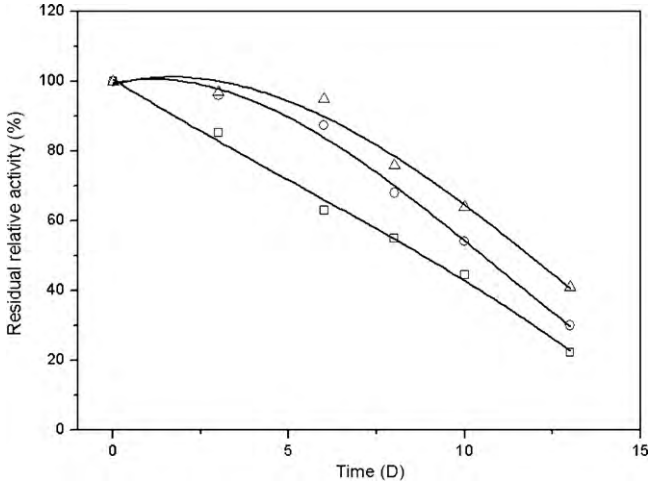


Fig. 6. Time course of residual relative activities of *Y. lipolytica* lipase in presence of olive oil (□), span 80 (○) and span 85 (△) at 50 °C. The samples were incubated and taken at suitable time intervals. The activity was then measured, with the native lipase activity (5000 U ml^{-1}) being 100%. The fitting curves are: (□) $y = -3.99 \times 10^{-4} x^2 - 5.41 \times 10^{-2} x + 1.00$, $R^2 = 0.995$; (○) $y = -3.74 \times 10^{-3} x^2 - 7.20 \times 10^{-3} x + 1.01$, $R^2 = 0.995$; (△) $y = -4.09 \times 10^{-3} x^2 - 6.64 \times 10^{-3} x + 1.00$, $R^2 = 0.982$.

4. Conclusions

Lipases have potential for industrial applications. This is why a study of their thermal stability and activity is of interest. In this report the results of studies of the inactivation kinetics and conformational change of *Y. lipolytica* lipase in the process of thermal unfolding are presented. The inactivation model can be expressed by first-order kinetics. Insight of the structural changes has been obtained by DLS, fluorescence, UV, CD measurements as a function of temperature. The results reveal that inactivation precedes denaturation and is accompanied by changes of tertiary/secondary structure as well as aggregation. Moreover, the thermal stability of the enzyme is significantly increased by the addition of olive oil, span 80 and span 85. It is confirmed by DSC data that the enzyme is more stable in the presence of span 85 than in the absence of additive.

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