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# Activation of *Mucor circinelloides* lipase in organic medium

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**Abstract**

An intracellular *Mucor circinelloides* lipase either in the form of mycelium-bound enzyme, or in the homogeneous and soluble form, was subjected to activation experiments. It was found that some compounds, such as pyridine, diethanolamine (DEtA), triethanolamine (TEtA), and cetylpyridinium bromide, either increase or decrease the synthetic lipase activity in organic solvents, dependently on their concentration. Differential spectrophotometry of the homogeneous lipase dissolved in toluene, indicate that the variation in the enzyme activity results from an interaction of these substances with the indole group(s) of the tryptophan residue(s), situated on the surface of this enzyme. Our results prove that *M. circinelloides* lipase is activated not only in the aqueous milieu, but also in organic systems. The molecular background of this phenomenon seems to be similar to the interfacial activation of lipases in the aqueous system (namely the 'lid'-helix translocation), thought the reason is different.


*Keywords:* *Mucor*; Mycelium-bound lipase; Homogeneous lipase; Differential spectrophotometry; Activation in organic solvent

## 1. Introduction

An activity against water-insoluble hydrophobic substrates is the characteristic property of lipases. This feature has a crucial impact on the mechanism of reaction, which occurs on the interface between the water-insoluble substrate-containing phase, and the water phase, which contains the enzyme. Lipases are activated by their apolar substrates. This phenomenon is called the interfacial activation of lipases [1].

The molecular background of the activation has been discussed for years. Brockerhoff and Jansen [2] has proposed the so-called substrate theory, which has emphasized the role of factors such as the form and concentration of a substrate in the lipid–water interface. Desnuelle et al. [3] have postulated that lipase molecules undergo some conformational changes after their adsorption on the interface. Determination of the first crystallographic structure of lipases has confirmed the earlier theories, since it was proved that the enzyme's active site was inaccessible for the solvent, if the enzyme was dissolved in water. Further studies on the structure of lipases revealed that except the cutinase [4,5] and the acetylcholinesterase [6], all known lipases contained an amphipathic polypeptide domain, which had a helical structure and was localized above the catalytic site. This domain was called a lid or a flap. Grochulski et al. [7] found that *C. rugosa* lipase was crystallized in the open-lid conformation from the solvent with low ionic strength. Lipases display poor activity against water-soluble substrates, since the lid covers their catalytic site. Putting the water-insoluble substrate in touch with the lipase molecule generates some conformational changes in the protein, due to

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which the lid uncovers the active site and the enzyme activity is increased. The newly formed lipase conformation is probably stabilized by some structural elements, present in the enzyme molecule [6,8–14]. The interfacial activation mechanism is believed to be more complicated, since lipases from *Pseudomonas aeruginosa* (CVL and CAL-B) do not give in the interfacial activation though they posses the lid-helix. Furthermore, the *Staphylococcus hyicus* lipase yields to the activation but only in the presence of some of its substrates. Similarly, the novel, so called *coypu* lipase, which was found between the pancreatic lipases, has the lid composed of 23 amino acid residues and homologous to the 'classical' pancreatic lipase, but does not give in the interfacial activation [1,8].

Derewenda et al. [14,15] found that the lids of *Rhizomucor miehei* and *Humicola langinosa* (now re-named as *Thermomyces lanuginosus*) lipases showed a high degree of motility, and that the tryptophan residue was situated on their surface, that enabled its detection using spectroscopic methods. For instance, Peters and co-workers [16,17] applied fluorescence spectroscopy and molecular dynamics simulations, confirmed high motility of Trp residues in molecules of *R. miehei* and *H. langinosa* lipases. Also Jutila et al. [18] used the method of fluorescence spectroscopy to study the changes in conformation of *H. lanuginosa* lipase induced by the detergent (pentaoxyethylene octyl ether). Investigation into molecular dynamics of lipases done by Graupner et al. [19] were focused on changes in exposition of Trp residues in lipases of *Chromobacterium viscosum* and *Pseudomonas* sp., dissolved in water, in micelles and in water–alcohol solution. Stobiecka et al. [20,21] carried out fluorescence studies on *H. lanuginosa* lipase found that distinct configuration of this fungal lipase could be monitored using the fluorescence of the Trp89 residue located in the lid-helix, and involved in the interfacial activation of the enzyme. del-Val and Otero [22] detected the simultaneous changes of the enzyme activity and fluorescence emission of the izolipase B from *Candida rugosa*, which occurred both in water and a microgell matrix.

It is known that Trp residue shows an affinity towards carbonyl groups of the lipid [23]. This observation casts light on the mechanism of the contact between the water-insoluble substrate and the lipase, since the specific interaction between Trp of the lid with the lipid, may be responsible for the uncovering of the lipase active site.

The authors of this publication have been involved for several years in research of intracellular *M. circinelloides* and *M. racemosus* lipases, which have been poorly recognized before. The results of our studies have been published elsewhere [24–27]. In the recent studies, we have applied differential spectrophotometry for detection of changes in conformation of *M. circinelloides* lipase dissolved in toluene in the presence of selected substances, including pyridine, DEtA and TEtA. Our earlier studies revealed that these substances, added at certain concentrations, activated this enzyme in the synthesis of esters in organic solvents [24–26].

## 2. Materials and methods

### 2.1. Materials

*M. circinelloides* strain was obtained from the microbial culture collection of the Institute of Technical Biochemistry of Technical University of Lodz. All reagents were analytical grade and were purchased from Sigma, Baker, Fluka and ICN.

### 2.2. Crude preparations of lipases

Strain of *M. circinelloides* was cultured in 14 l Chemap C-2000 fermentors in a medium containing 3% (v/v) corn steep liquor and 2% (v/v) sunflower oil. The mycelium was washed thrice with acetone and dried at room temperature, thus giving the crude preparation of in situ immobilized lipases.

### 2.3. Lipase purification

The lipase was extracted from the crude preparation (see Section 2.2) with 0.8% sodium cholate in 0.06 M Tris–HCl buffer pH 7.2, at 37 °C. The procedure was repeated several times in order to release all the lipase activity from the mycelium. The supernatants were collected, dialyzed and lyophilized. The enzyme was purified on CNBr–Sepharose 4B coupled with bacitracin [28]. Because the *Mucor* mycelium contained no proteases, this method was applied for the lipase purification. The protein solution was applied

to a column (1 cm × 15 cm), eluted with 0.05 M Tris–HCl buffer pH 7.5 containing 25% isopropanol and 1 M NaCl, and concentrated on ultrafiltration membrane (10 kDa) and lyophilized. Molecular mass of the lipase was determined by SDS–PAGE [29].

### 2.4. Spectrophotometric studies

Substrates of lipases were dissolved in toluene (separately). Solution (no. 1) of 16-hydroxyhexadecanoic acid (0.33 mg ml$^{-1}$), and oleic acid (0.5 mg ml$^{-1}$), contained a perturbing substance (concentrations are presented under the figures). Concentrations of solution of the homogeneous lipase in toluene were 0.5 or 1 mg ml$^{-1}$ (solution no. 2). Solution no. 3 contained the lipase (the same concentrations), one of the substrates and a perturbing compound.

Differential spectrophotometry studies were executed using a spectrophotometer UV–VIS Beckman DU75000, at 30 °C, in quartz cuvettes with light path equal to 10 mm. The differential spectrum was obtained by subtraction of both spectra of solution no. 1 and 2 from the spectrum of the solution no. 3.

### 2.5. Activity assays

For determination of the synthetic lipase activity ($A_s$), 1 mmol of acid, 1 mmol of alcohol, 0.05 g of lipase preparation and 5 ml of organic solvent were incubated for 10–20 min at 37–50 °C on a shaker at 220 rpm. The residual free fatty acids were titrated with 0.05 M NaOH up to pH 10.0. One unit (U) of lipase synthetic activity was defined as the amount of enzyme that synthesized 1 mol of ester equivalents per second under the conditions described above. The specific activity was expressed as a number of activity units per 1 g of mycelium. The synthesis efficiency (mol%) was calculated from the amount of acid consumed in the reaction. The yield of synthesis of 16-hydroxyhexadecanoic acid lactone was estimated as reported earlier [24]. Details are presented in Tables 1–4.

Table 1
The effect of activators on the lipase activity of in synthesis of butyl oleate

| Substance | Concentration (mM) | Activity $A_s$ (μkat g$^{-1}$) | Relative activity (%) |
|---|---|---|---|
| Reference sample | – | 4.5 | 100 |
| Diethanolamine (DEtA) | 13.8 | 8.3 | 185 |
| Triethanolamine (TEtA) | 11.2 | 8.3 | 184 |
| Piperazine | 12.0 | 6.3 | 140 |
| Pyridine | 39.0 | 6.1 | 136 |
| DMF | 15.0 | 6.3 | 141 |

Reaction conditions: 1 mmol of 1-butanol, 1 mmol of oleic acid, 5 ml of petroleum ether, 50 mg of mycelium-bound lipase, 20 min, 37 °C.

## 3. Results and discussion

Lipase activation in the presence of low concentrations of DMF (di-methyl formamide) in organic milieu was for the first time reported by Zaks and Russell [30]. The authors believed that molecules of this compound replace some water molecules in the enzyme essential water layer, thus modifying this layer. Our initial studies [24] proved that the *M. circinelloides*

Table 2
The effect of activators on activity of the lipase in synthesis of various sugars esters

| Ester | Reference sample[a] $A_s$ (μkat g$^{-1}$) | Cetylpyridinium bromide[b] | | Astaxanthin[c] | |
|---|---|---|---|---|---|
| | | $A_s$ (μkat g$^{-1}$) | Relative activity (%) | $A_s$ (μkat g$^{-1}$) | Relative activity (%) |
| Sucrose caprylate | 6.1 | 7.7 | 125 | 11.3 | 185 |
| Sucrose oleate | 5.9 | 8.3 | 140 | 11.4 | 192 |
| Glucose caprylate | 6.6 | 9.1 | 138 | 13.4 | 204 |
| Glucose oleate | 6.1 | 9.5 | 156 | 13.8 | 225 |

Reaction conditions: 1 mmol of sugar, 1 mmol of acid, 5 ml of di-*n*-penthyl ether, activators, 50 mg of mycelium-bound lipase, 10 min, 50 °C.
[a] Samples without additives.
[b] Samples with 0.042 mmol (16.5 mg) of cetylpyridinium bromide per sample.
[c] Samples with 14 mg of astaxanthin (from shrimps) per sample.

Table 3
The efficiencies of various esters synthesis catalysed by the homogeneous lipase soluble in toluene

| Esterification (%) | | | |
|---|---|---|---|
| Alcohol/acid | Caprylic acid | Palmitic acid | Oleic acid |
| 1-Propanol[a] | 38.2 | 43.2 | 45.3 |
| 1-Butanol[a] | 46.5 | 46.0 | 47.4 |
| Oleic alcohol[a] | 34.3 | 33.5 | 30.9 |
| Glycerol[a] | 28.5 | 16.1 | 15.9 |
| Sucrose[b] | 21.1 | 17.3 | 18.5 |
| Glucose[b] | 28.7 | 24.7 | 24.1 |
| Lactone[b,c] | 5.5 | | |

Reaction conditions: 0.1 mg of lipase dissolved in 5 ml of toluene, 1 mmol of acid, 1 mmol of alcohol or 1 mmol of 16-hydroxyhexadecanoic acid.
[a] At 30 °C.
[b] At 50 °C.
[c] One gram of 4-Å molecular sieve, 18 h, 180 rpm.

lipase (mycelium-bound enzyme) activity in the lactone (hexadecanolide) synthesis was approximately five times higher in the presence of DMF or pyridine. The similar effect of pyridine was also noticed when this lactone synthesis was catalyzed by the pancreatic lipase (unpublished data). Further investigation showed that in the presence of some compounds (Tables 1 and 2) the preparation of mycelium-bound lipase from *Mucor* displayed higher catalytic activity also in other esters synthesis [25,26,31]. These substances (identified empirically) probably modify the enzyme's essential water layer, like DMF, and furthermore, dependently on concentration in reaction mixture, either activate or inhibit the lipase (Fig. 1). Strongly activating influence of DEtA has been even utilized for preparative synthesis of higher fatty acids [32].

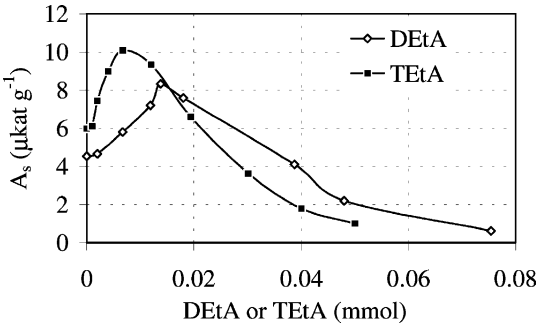


Fig. 1. The effect of DEtA and TEtA concentration on synthetic activity of the mycelium-bound lipase in butyl oleate synthesis. Reaction conditions are presented in Table 1.

The *M. circinelloides* lipase was isolated from the mycelium and purified as described in Section 2.3. Molecular mass of this lipase is 42 kDa. The homogenous lipase formed aggregates with molecular mass of 80–160 kDa in diluted Triton X-100 solution (below 0.05%). They precipitated from water in the absence of the detergent, similarly to other lipases [33]. Important properties of the homogeneous, lyophilized lipase from *M. circinelloides* are its solubility and catalytic activity in certain organic solvents, e.g. toluene (Table 3). These features were exploited in studies on molecular dynamics of this protein using differential spectrophotometry.

### 3.1. Spectral studies of homogeneous lipases dissolved in toluene

It was assumed that regulation of lipases activity in non-aqueous milieu with chemical compounds

Table 4
The effect of selected compounds on the activity of the homogenous *Mucor* lipase

| Synthesis of | Activity (μkat g$^{-1}$) | | Relative activity (%) |
|---|---|---|---|
| | Control | Sample | |
| Lactone of 16-hydroxyhexadecanoic acid | 8.5 | 66.6[a] | 790 |
| Lactone of 16-hydroxyhexadecanoic acid | 8.5 | 46.6[b] | 549 |
| Sucrose oleate | 28.6 | 68.0[c] | 238 |
| Butyl oleate | 73.2 | 137.5[d] | 188 |

Activity of the enzyme was determined in the conditions of the reactions are described in Table 3.
[a] Additives of 5 μl ml$^{-1}$ (49.5 mM) of pyridine.
[b] Additives of 2 mg ml$^{-1}$ (5.1 mM) of cetylpyridinium bromide.
[c] Additives of 5 mg ml$^{-1}$ (12.7 mM) of cetylpyridinium bromide.
[d] Additives of 5 mg ml$^{-1}$ (50.2 mM) of DEtA to the samples were applied.

modifying their essential water layer resulted from variation in spatial location of their lid. These variations are believed to result from perturbation with substances, which modify the indole group of the tryptophan residue located outside the lid.

Spectral studies carried out as described in Section 2.4, showed that the lipase's Trp residues could not be spectrophotometrically detected when the enzyme was dissolved in a medium of organic solvent. Also, the presence of substrates (16-hydroxyhexadecanoic acid or oleic acid) in the lipase solution in organic solvent did not change the enzyme spectrum. The observed differential spectra were linear, that meant that these conditions did not generate any changes in spatial location of Trp residues in the enzyme molecules. An absence of light absorption peaks in differential spectra of samples, which contained the enzyme dissolved in toluene and the substrate (16-hydroxyhexadecanoic acid) was in agreement with results of [14,34] indicating that in the non-aqueous milieu, even in the presence of hydrophobic substrates, the lipase's active site remained closed with the lid, that in consequence brought about the lower enzymatic activity.

On the other hand, compounds such as pyridine, cetylpyridinium bromide and diethanolamine, present in a solution of the homogeneous lipase in toluene, generate the characteristic for Trp maximum of UV-absorption at wavelengths from $\lambda = 287$ to 291 nm. At the same time, an increase in the enzyme synthetic activity was observed. In presence of 4 μl of pyridine in the solution, the lipase displayed the highest activity, and in its differential spectrum the maximum of UV-light absorption was observed at 288 nm. This shift of absorption maximum in comparison to peaks corresponding to 2 and 10 μl of pyridine (in both cases, the enzyme had lower catalytic activity than in the case of 5.6 μl of pyridine) is a result of an increase in polarity of Trp residues microenvironment (bathochromic shift, Fig. 2).

Differential spectra of the lipase, determined in the presence of various compounds, when added to reaction mixtures at various concentrations, confirmed the correlation between the height of the maximum of UV-light absorption ($\Delta A$) and the enzyme catalytic activity. It was found that concentrations of these substances, corresponding to the highest lipase activity, coincided with the maximum of absorption (Figs. 2–5; Table 4). Thus, pointing to changes in spatial location of Trp indole groups, that in consequence, probably gave rise to variation in the lid position above the lipase catalytic center. Hydrogen bonds between the indole group of the Trp residue localized on the lid surface, and the activator molecules, are supposed to change the lid conformation into the catalytically favorable position.

The mycelium-bound *Mucor* lipase shows high thermal stability in organic solvents at 100 °C [25,35]. This observation prompted our research into

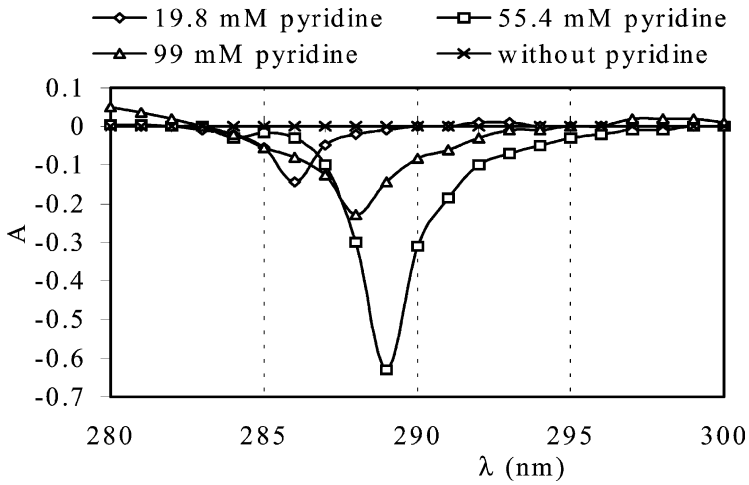


Fig. 2. The differential spectrum of the homogeneous lipase dissolved in toluene in the presence of 16-hydroxyhexadecanoic acid and various volumes of pyridine. Lipase concentration: 0.5 mg ml$^{-1}$.

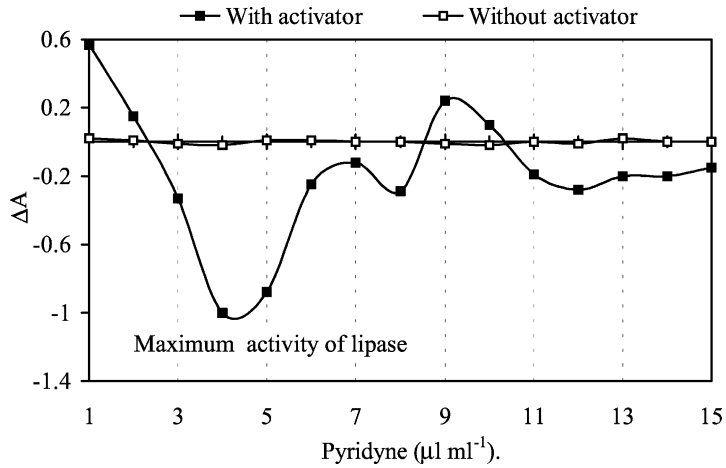


Fig. 3. Variation in absorbance ($\Delta A$) at maximum activity of the homogeneous lipase dissolved in toluene, subjected to perturbation with pyridine in the presence of 16-hydroxyhexadecanoic acid. Lipase concentration: 0.5 mg ml$^{-1}$.

the stability of the homogeneous *Mucor* lipase at 100 °C in toluene. Conformational changes of its molecules under extreme conditions were observed by means of differential spectrophotometry (Fig. 6). The lipase dissolved in toluene and perturbed with pyridine (5.6 μl ml$^{-1}$) in the presence of 16-hydroxyhexadecanoic acid displayed high negative spectrum with maximum at $\lambda = 288$ nm ($\Delta A = -1.277$). After its incubation for 10 min at 100 °C its differential spectrum had only minor peak of absorbance ($\Delta A = -0.087$) shifted towards longer wavelengths ($\lambda = 293$ nm, bathochromic shift). We suppose that this almost flat spectrum is not a proof of the lipase denaturation. The character of spectral variations rather confirms that the secondary and tertiary enzyme structure were not damaged by incubation at 100 °C, because Trp residues localized inside the enzyme molecule would be more accessible after thermal denaturation that would be visualized by an increase in UV-light absorbance. Probably, the

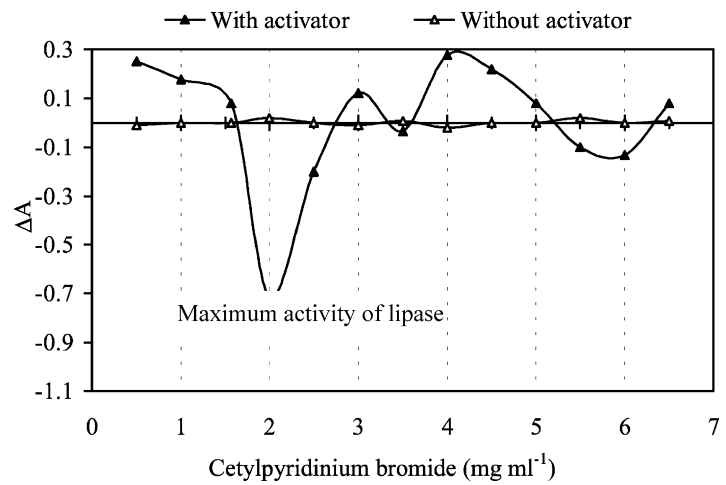


Fig. 4. Variation in absorbance ($\Delta A$) at maximum activity of the homogeneous lipase dissolved in toluene, subjected to perturbation with cetylpyridinium bromide in the presence of 16-hydroxyhexadecanoic acid. Lipase concentration: 0.5 mg ml$^{-1}$.

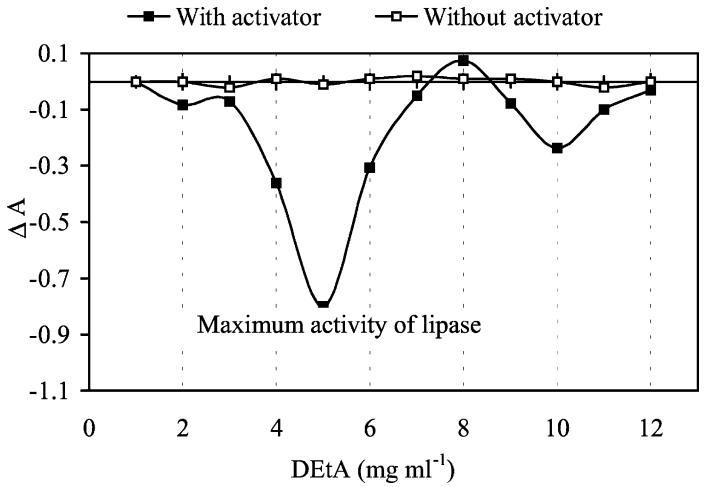


Fig. 5. Variation in absorbance ($\Delta A$) at maximum activity of the homogeneous lipase dissolved in toluene, subjected to perturbation with diethanolamine in the presence of oleic acid. Concentration of lipase: 0.5 mg ml$^{-1}$.

enzyme incubation at 100 °C brought about 'burying' of residues of tryptophans. In this way, the possibility of the lipase indole groups perturbation was almost omitted. Incubation at 30 °C of the lipase preparation that was previously incubated at 100 °C, caused partial enzyme renaturation, and generated the peak of UV-light absorption at $\lambda = 290$ nm ($\Delta A = -0.6$) in the presence of a perturbing substance (Fig. 6). The

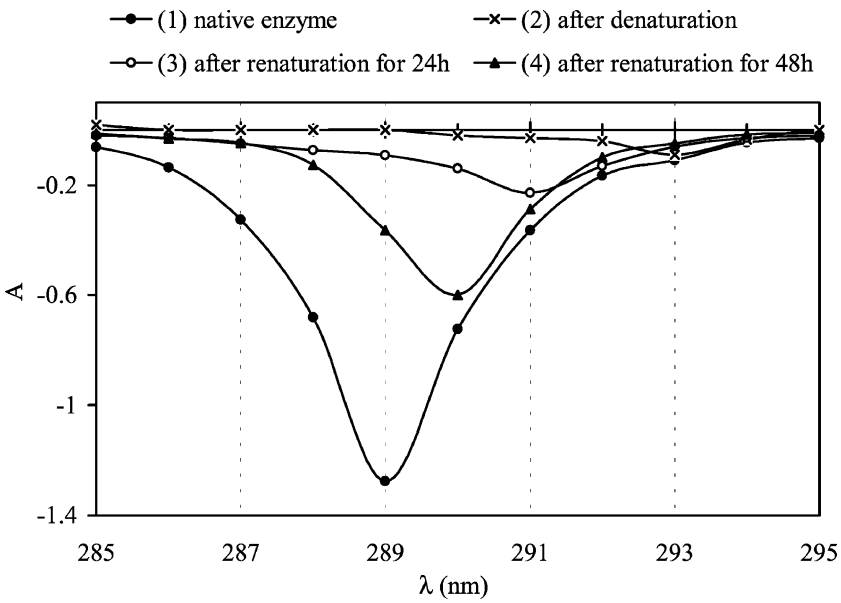


Fig. 6. Differential spectrum of the homogeneous lipase from *M. circinelloides* dissolved in toluene and subjected to thermal denaturation and renaturation in the presence of 16-hydroxyhexadecanoic acid and 10 μl of pyridine. Annotation: the relative activities of the lipase in synthesis of 16-hydroxyhexadecanoic acid lactone were: (1) 100%; (2) 1%; (3) 18.6%; and (4) 30.5%, respectively. Reaction conditions are specified in Table 3.

reason of this observations is probably connected with the partial rearrangement of spatial position of the Trp residue located in the lid, resulting in 30% recovery of the enzyme catalytic activity (see annotation under Fig. 6).

## 4. Conclusions

The *M. circinelloides* lipase is activated in organic solvents by some hydrogen bond-forming substances such as diethanolamine, triethanolamine, pyridine, piperazine, dimethyl formamide and cetylpyridinium bromide. These compounds generate changes in conformation of the *Mucor* lipase molecules, detectable by means of differential spectrophotometry. The possible activation mechanism involves translocation of the lid with respect to the lipase active site. Probably, the molecular background of this phenomenon is identical as in the case of the interfacial activation of lipases in aqueous systems though the reason is different.

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