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Conformational dissection of *Thermomyces lanuginosus* lipase in solution

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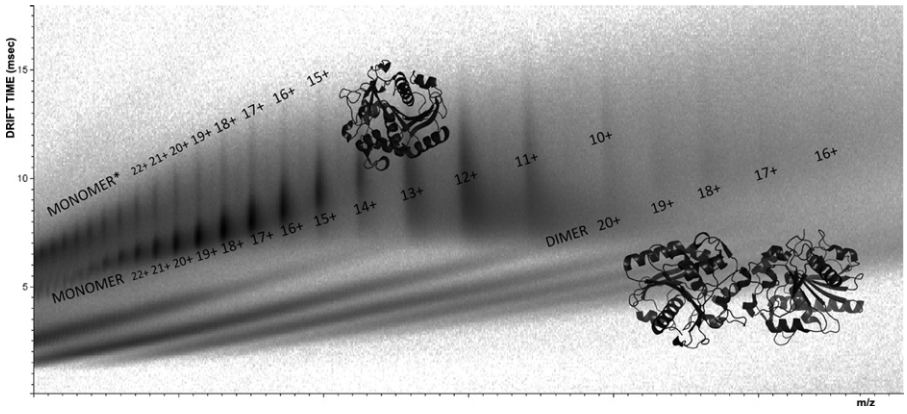
HIGHLIGHTS

- The conformation and oligomeric state of a commercial lipase (TLL) were evaluated.
- SAXS data evidenced the presence of monomers and dimers of TLL in solution.
- Mass spectrometry analysis confirmed that TLL is present in both forms.
- The presence of dimeric species might compromise overall enzyme activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Lipases are triacyl glycerol acyl hydrolases, which catalyze hydrolysis of esters, esterification and transesterification reactions, among others. Some of these enzymes have a large hydrophobic pocket covered by an alpha-helical mobile surface loop (the lid). Protein–protein interactions can occur through adsorption of two open lids of individual lipases. We investigated the conformation and oligomeric state of *Thermomyces lanuginosus* lipase (TLL) in solution by spectroscopic and mass spectrometry techniques. Information about oligomerization of this important industrial enzyme is only available for TLL crystals; therefore, we have done a throughout investigation of the conformation of this lipase in solution. SDS-PAGE and mass spectrometry analysis of size-exclusion chromatography eluted fractions indicated the presence of both monomeric and dimeric populations of TLL. The stability of the enzyme upon thermal and guanidine hydrochloride treatment was examined by circular dichroism and fluorescence emission spectroscopy. Small angle x-ray scattering and ion mobility mass spectrometry analysis revealed that TLL is found as a mixture of monomers and dimers at the assayed concentrations. Although previous x-ray diffraction data showed TLL as a dimer in the crystal (PDB: 1DT3), to our knowledge our report is the first evidencing that TLL co-exists as stable dimeric and monomeric forms in solution.

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1. Introduction

Lipases are carboxyl-esterases that catalyze the hydrolysis of long-chain acylglycerols [1]. Despite these enzymes are diverse in their amino acid sequences, previous crystallographic analysis revealed their typical α/β hydrolase scaffold, with catalytic residues constituted by a highly conserved trypsin-like triad of Ser–His–Asp(Glu) residues [2].

The lipase from *Thermomyces lanuginosus* (TLL) (previously *Humicola lanuginosa*) is obtained as a commercial soluble lipase preparation supplied by Novozymes® and is produced by a genetically modified strain of *Aspergillus oryzae*. The molar mass of this lipase is ~30 kDa, and it is mono-glycosylated at Asn33, which adds approximately 2 kDa to the final mass of the native enzyme [3]. The TLL has 269 amino acid residues in its primary sequence, four of which are tryptophan (89, 117, 221 and 260). Thus, an efficient method to follow the protein unfolding is to monitor Trp fluorescence upon application of physical or chemical variables [4]. Besides, as a consequence of the proximity of Trp89 to the active site, changes in its fluorescence emission can be related to conformational changes in the active site, specifically in the lid. Moreover, Trp89 seems to be important for the catalysis, while Trp residues 117, 221 and 260 have been reported to participate in the structural stability of *T. lanuginosus* lipase, as seen by steady-state and time resolved fluorescence spectroscopy of wild-type and TLL mutants [5].

X-ray diffraction studies showed that the TLL presents a central eight-stranded predominantly parallel β-sheet structure, with five interconnecting α-helices [2]. The lid is an α-helical mobile surface loop that covers the active site [2], and the catalytic triad of Ser146-Asp201-His258 is similar to those seen in serine proteases [5].

Despite that TLL crystallographic studies evidenced three distinct conformational states, an unstable intermediate form and two stable forms (closed and open lid conformations) [2], it is suggested that the enzyme conformation is closed in aqueous environments; thus, the access to the catalytic triad would be blocked by the lid. It was also shown that when TLL is bound to substrate analogs, the helix forming the lid is displaced and the active site becomes exposed [4].

In contrast, it was reported that lipases might crystallize in their open conformation without the presence of substrates or inhibitors, suggesting that exposition of hydrophobic areas surrounding the active center occurs in the unbound enzyme. The exposed large hydrophobic pocket can promote the association between two open lipases, hence enabling oligomer formation [6].

Dimerization of the lipase of *Pseudomonas fluorescens* was proposed previously [7]. Reduced activity was observed at higher enzyme concentrations, indicating that dimers are less active than monomers. Besides, when detergents such as Triton X were added in the solution, there was no difference in activities of preparations with different lipase concentration, indicating dissociation of dimers into monomers. The same behavior was observed for the lipase from *Alcaligenes* sp. [6]. Additionally, it was shown that *Alcaligenes* sp. lipase dimers were more stable to thermal denaturation than the monomers [6].

Some authors suggest that the *T. lanuginosus* and *Mucor miehei* (MML, Novozym® 388) lipases have a strong tendency to dimerize, even at very low protein concentrations [8,9]. However, the existence of TLL dimers in solution is still controversial, and the structure and conformation of this species were not evaluated in aqueous solution. Dimeric forms of these lipases were only investigated for immobilized enzymes, in the presence or absence of detergents, or by gel exclusion chromatography, mainly with enzyme concentrations higher than 300 μg/mL. Only a small percentage of dimers was found at lower concentrations (below 50 μg/mL) [8].

For the TLL, another indication of its tendency to form dimers (i.e., less active species) is the fact that solubilizing the enzyme in the presence of detergents leads to an increase in the activity by more than one order of magnitude [10]. This fact is not related to the interfacial activation, but rather to the dissociation of intermolecular interactions that keep the enzyme in the dimeric conformation.

Here we investigated the conformation and oligomeric state of the native *T. lanuginosus* lipase through size-exclusion chromatography (SEC), small angle x-ray scattering (SAXS) and Electrospray Ionization–Ion Mobility Spectrometry–Mass Spectrometry (ESI–IMS–MS) in aqueous phase. SAXS and ESI–IMS–MS techniques were employed to evaluate the conformation and oligomerization state of TLL in solution, since they are powerful tools to study biological systems in conditions close to physiological [11–14]. Moreover, the stability of the enzyme upon thermal and guanidine hydrochloride (GdnHCl) denaturation was investigated by circular dichroism (CD) and intrinsic fluorescence spectroscopy, to provide structure–stability relationships. Using such methodologies, the present study shows that TLL is present as a mixture of dimeric and monomeric states in solution.

2. Materials and methods

2.1. Materials

T. lanuginosus lipase (TLL) was obtained as crude extract from Novozymes® (Bagsvaerd, Denmark) and its concentration was determined by the Bradford method [15] or, for the purified enzyme, by its extinction coefficient at 280 nm (36,900 M^{-1} cm^{-1}), calculated from the TLL primary sequence in http://web.expasy.org/protparam/. GdnHCl (99.9% pure) and the purified enzymes for SEC analysis were acquired from Sigma-Aldrich (St. Louis, MO, USA). Buffers used in the experiments were sodium phosphate and tris(hydroxymethyl) aminomethane from VETEC (Duque de Caxias, RJ, Brazil). The SDS-PAGE standard used was Precision Plus Protein™ Dual Color (Bio-Rad, CA, USA), containing a mixture of 10 recombinant proteins (from 10 to 250 kDa).

2.2. Size-exclusion chromatography (SEC)

The enzyme was purified by SEC using TSK gel 3000 (7.5 mm ID × 30 cm × 10 μm) (TosoH Corp., Tokyo, Japan) or Superdex 75 10/300 GL (GE Healthcare, USA) columns in a Jasco PU 2089 Plus chromatograph (Jasco Corp., Japan). Elution was done in 50 mM phosphate buffer, pH 7.0, at flow rates of 1.0 mL/min or 0.5 mL/min for TSK or Superdex columns, respectively. The collected aliquots were analyzed by SDS-PAGE (12.5%) and further lyophilized. The calibration curve was made using the purified proteins from Sigma-Aldrich: lysozyme (14.3 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa) and β-amylase (200 kDa). The chromatographic run was monitored with a UV-absorption detector at 280 nm.

2.3. Enzyme activity

The lipase activity was determined as described by Invernizzi et al. [16]. The measurements were made by following the increase in absorbance at 410 nm generated by the release of *p*-nitrophenol produced by the hydrolysis of 5 mM *p*-nitrophenyl palmitate (dissolved in isopropanol) in 100 mM Tris–HCl buffer, pH 7.5, supplemented with 0.005% Triton X-100 at room temperature. The enzyme and substrate solution were heated separately at different temperatures and the reaction was started with the lipase addiction, as described [16].

2.4. Intrinsic and extrinsic fluorescence measurements

Fluorescence measurements were carried out in a Jasco FP 6300 spectrofluorimeter (Jasco Corp., Japan) with excitation set at 280 nm and emission was monitored from 300 to 420 nm. The center of spectral mass values ‹λ› of TLL fluorescence emission spectra were calculated