Enzyme and Microbial Technology 113 (2018) 18–23

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

A simplified method for active-site titration of lipases immobilised on hydrophobic supports

Tim D. Nalder[a,b], Ivan Kurtovic[a,*], Colin J. Barrow[b], Susan N. Marshall[a]

[a] Seafood Unit, The New Zealand Institute for Plant & Food Research Limited, 293-297 Akersten Street, Nelson 7010, New Zealand
[b] Centre for Chemistry and Biotechnology, Deakin University, 75 Pigdons Road, Waurn Ponds, Victoria, Australia

ARTICLE INFO

Keywords:
Active-site titration
Candida rugosa lipase
Enzyme immobilisation
Fluorescence
Hydrophobic interaction
Lipase inhibition

ABSTRACT

The aim of this work was to develop a simple and accurate protocol to measure the functional active site concentration of lipases immobilised on highly hydrophobic supports. We used the potent lipase inhibitor methyl 4-methylumbelliferyl hexylphosphonate to titrate the active sites of *Candida rugosa* lipase (CrL) bound to three highly hydrophobic supports: octadecyl methacrylate (C18), divinylbenzene crosslinked methacrylate (DVB) and styrene. The method uses correction curves to take into account the binding of the fluorophore (4-methylumbelliferone, 4-MU) by the support materials. We showed that the uptake of the detection agent by the three supports is not linear relative to the weight of the resin, and that the uptake occurs in an equilibrium that is independent of the total fluorophore concentration. Furthermore, the percentage of bound fluorophore varied among the supports, with 50 mg of C18 and styrene resins binding approximately 64 and 94%, respectively. When the uptake of 4-MU was calculated and corrected for, the total 4-MU released via inhibition (i.e. the concentration of functional lipase active sites) could be determined via a linear relationship between immobilised lipase weight and total inhibition. It was found that the functional active site concentration of immobilised CrL varied greatly among different hydrophobic supports, with 56% for C18, compared with 14% for DVB. The described method is a simple and robust approach to measuring functional active site concentration in immobilised lipase samples.

1. Introduction

Lipases display a high degree of selectivity, making them useful biocatalysts which are used widely in industrial applications including textile and detergent manufacture, cosmetic and nutraceutical ingredient synthesis, and lipid processing in the oleo and food industries [1,2]. While lipases may be used in their free form for some processes, their use in industrial settings usually requires immobilisation onto solid supports. Immobilisation provides several advantages. For example, it enables the enzymes to be recovered post reaction so that the lipase does not remain as a potential contaminant in the final product. It also allows reuse of the enzymes in multiple reaction cycles, therefore reducing the cost of the process [3]. Understanding how immobilisation affects lipase properties is therefore critical for successful industrial applications.

Of key importance is knowing how much lipase has been bound to the immobilisation support, as well as how much of the bound lipase is present in an active form and able to react with the intended substrate. These factors can be difficult to monitor, because: 1. Lipase immobilisation is often carried out from crude or partially purified lipase-containing preparations, meaning that the actual concentration of lipase can be difficult to measure; 2. During the hydrophobic immobilisation process a number of factors can influence how the enzyme is bound to the solid support, some of which can lead to the lipases being bound in non-functional forms [4–7]. Successful immobilisation of different lipases has been found to be highly dependent on the properties of the support [8]. Therefore, methods are required that allow for the accurate determination of the concentration of functional lipase active-sites in immobilised samples, to enable comparisons between different immobilisation preparations.

Lipase inhibitors paired with a colorimetric or fluorogenic detection agent allow measurement of lipase active-site concentration. A range of compounds has been used to inhibit and study lipases [9–14]. Organophosphonate esters are a particularly potent group of inhibitors that act by irreversibly binding (covalently) to the hydroxyl group of the serine in the catalytic triad [15]. Upon inhibition of the lipase, the

Abbreviations: 4-MUHP, methyl 4-methylumbelliferyl hexylphosphonate; 4-MU, 4-methylumbelliferone; 4-MUB, 4-methylumbelliferyl butyrate; C18, octadecyl methacrylate resin; CaLB, lipase B from *Candida antarctica*; CrL, *Candida rugosa* lipase; DVB, divinylbenzene crosslinked methacrylate resin

* Corresponding author.
E-mail address: Ivan.Kurtovic@plantandfood.co.nz (I. Kurtovic).

https://doi.org/10.1016/j.enzmictec.2018.02.003
Received 8 December 2017; Received in revised form 8 February 2018; Accepted 9 February 2018
Available online 17 February 2018
0141-0229/

detection agent is released and subsequently measured spectrophotometrically to determine the rate of inhibition. These esters have been formed with common detection agents (leaving groups) such as para-nitrophenol (pNP) [10] or 4-methylumbelliferone (4-MU) [11], with the latter providing significantly improved sensitivity. Both pNP and 4-MU derivatives have been used to titrate lipase active-site concentrations [5,12,16–20]. While the use of these compounds to titrate the active-site concentration of free lipases is relatively straightforward, their application in studying immobilised lipases has been limited. Many of the macro-supports used in lipase immobilisation are hydrophobic in nature, so it is common for the released detection agent to be bound by the support, thereby altering the measurable concentration of the chromophore/fluorophore. Following inhibition progress via measurement of residual activity using short chain fatty acyl pNP or 4-MU ester substrates is also made more difficult with immobilised samples, with the accurate measurement of the released detection agent complicated by their adsorption to the supports. Previous work using organophosphonate esters for titrating active-site concentration of immobilised lipases has addressed the issue in different ways. The most common method accounts for 4-MU bound to the supports by adding an organic solvent, such as acetonitrile, to desorb it after the soluble concentration has been measured [5,15]. This requires additional sample handling and may result in incomplete fluorophore desorption. Furthermore, in a number of studies it is unclear whether binding of the respective detection agent to the support has been taken into account, and this may contribute to the detection of lower lipase concentrations than expected.

Here, we describe a simple protocol using the lipase inhibitor methyl 4-methylumbelliferyl hexylphosphonate (4-MUHP) for the sensitive titration of active-site concentration of lipases immobilised on highly hydrophobic supports. The method uses correction curves to take into account the binding of 4-MU by three different support materials: octadecyl methacrylate (C18), divinylbenzene crosslinked methacrylate (DVB) and styrene. We demonstrate the need to account for the uptake of the detection agent by highly hydrophobic materials in order to determine functional lipase active-site concentration. The method was accurate in detecting a range of concentrations of *Candida rugosa* lipase (CrL) immobilised on the different supports, giving linear relationships between lipase concentration and total inhibition.

2. Materials and methods

2.1. Chemicals and materials

All chemicals and reagents were purchased from Sigma-Aldrich® and were analytical grade or higher unless otherwise specified. CrL was sourced from Amano Enzyme Inc. (Japan), while lipase B from *C. antarctica* (CaLB) was purchased from Novozymes® (Denmark). The support resins C18, styrene and DVB were purchased from Purolite® Life Sciences (Purolite Corporation, Llantrisant, Wales).

2.2. Immobilisation of Candida rugosa lipase

CrL was immobilised on the three hydrophobic supports. Prior to immobilisation, each resin was washed with Milli-Q water, followed by 10 mM phosphate buffer, pH 6, before being collected on a sintered glass funnel under vacuum. The lipase load solution (0.1 g/mL in phosphate buffer) was mixed with the damp resin in a beaker such that the applied protein was 110 mg/g dry resin. Binding was carried out at 21 °C for 20–24 h, using a caged magnetic stirrer. The unbound material was recovered through a sintered glass funnel, the resin was washed with Milli-Q water twice (each wash consisting of 5–6 resin volumes of water), and the combined unbound fraction and washes were retained to assess protein concentration and activity. The resin was then washed with Milli-Q water 5 more times (each wash consisting of 10 resin volumes of water) and these washes were discarded. The fully drained, damp resin was recovered as the immobilised lipase preparation and then dried at 21 °C for 24 h in a desiccator under vacuum, for storage purposes.

2.3. Lipase activity assays

Activities of the lipase load solution, unbound material and the immobilised preparations were measured against tributyrin as described previously [21]. Reactions were carried out at 30 °C and pH 8.0.

After the titration of free lipase active site concentration, the residual lipase activity was measured using 4-MU butyrate (4-MUB) as substrate to determine the extent of inhibition that was achieved. A 10 mM stock of 4-MUB was made up in dimethyl sulfoxide (DMSO) and added to the assay reaction (50 mM Tris-HCl pH 8.0, 0.01% Triton X-100 v/v) to a final concentration of 30 μM, with the release of 4-MU detected continuously in a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies) using excitation and emission wavelengths of 363 and 445 nm, respectively. The rate of 4-MUB hydrolysis after inhibition of free lipase by 4-MUHP was then compared with the rate prior to addition of 4-MUHP, to determine the percentage of lipase inhibited (residual activity).

2.4. Measurement of protein concentration

2.4.1. Soluble protein

Protein concentrations were measured using the method of Lowry et al. [22] with some modifications to the concentration of reagents and incubation times. Bovine serum albumin (BSA) was used as the standard. A 2100 Bioanalyzer™ system (Agilent) run using the protein 230 kit and reagents (Agilent) were used to approximate the percentage of protein that was lipase. The peak area that correlated to the molecular weight of CrL from the chromatogram generated by electrophoretic separation was calculated as a percentage against all other peaks. It was approximated that 64% of protein in the soluble CrL preparation was lipase.

2.4.2. Bound protein

Using the Lowry method [22], the protein loading on each support (mg protein/g support) was calculated from the difference between the applied protein concentration in the lipase load and that in the combined unbound and wash fractions.

Elemental analysis (Carlo Erba Elemental Analyser EA 1108, University of Otago, New Zealand) was used as a direct method to determine the concentration of protein bound to the immobilisation supports. The protein concentration was calculated from the nitrogen content and molecular weight of CrL (662 mol N/mol enzyme and 57 149 g/mol, respectively).

2.5. Inhibition of free lipase by methyl 4-methylumbelliferyl hexylphosphonate

Inhibition of free lipase was measured in a continuous assay format. A 10 mM stock of 4-MUHP was made in DMSO. This was subsequently added to 50 mM Tris-HCl pH 8.0, 0.1% (v/v) Triton X-100 to a final concentration of 100 μM to make the inhibitor solution. The reaction was carried out using 2.80–2.98 mL of the inhibitor solution with an aliquot of lipase (20–200 μL) added to begin the reaction. A 'no enzyme' control was used to account for spontaneous hydrolysis of the 4-MUHP. Inhibition of the lipase was followed by measuring the release of 4-MU with the Cary Eclipse Fluorescence spectrophotometer (Ex. 363 nm; Em. 445 nm). Assays were run for 1–10 min until the increase in fluorescence levelled off. The concentration of 4-MU released was then calculated by calibration with a standard curve of 4-MU concentration under the same conditions.

The effect of 4-MUHP concentration on the rate of inhibition was investigated with 12.5, 25.0, 50.0 and 100.0 μM 4-MUHP in the