Biochimie 128-129 (2016) 127–132

Contents lists available at ScienceDirect

Biochimie

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

Research paper

4-Hydroxy-*N*-propyl-1,8-naphthalimide esters: New fluorescence-based assay for analysing lipase and esterase activity☆

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ARTICLE INFO

Article history:
Received 8 June 2016
Accepted 27 July 2016
Available online 29 July 2016

Keywords:
Carboxylester hydrolase
Enzyme assay
Fluorescence
Lipase
1,8-Naphthalimide

ABSTRACT

Research using 1,8-naphthalimide derivatives has expanded rapidly in recent years owing to their cell-permeable nature, ability to target certain cellular locations and fluorescent properties. Here we describe the synthesis of three new esters of 4-hydroxy-*N*-propyl-1,8-naphthalimide (NAP) and the development of a simple and sensitive assay protocol to measure the activity of carboxylester hydrolases. The NAP fluorophore was esterified with short (butyrate), medium (octanoate) and long (palmitate) chain fatty acids. The esters were spectroscopically characterised and their properties investigated for their suitability as assay substrates. The esters were found to be relatively stable under the conditions of the assay and levels of spontaneous hydrolysis were negligible. Non-specific hydrolysis by proteins such as bovine serum albumin was also minimal. A simple and rapid assay methodology was developed and used to analyse a range of commercially available enzymes that included enzymes defined as lipases, esterases and phospholipases. Clear differences were observed between the enzyme classes with respect to the hydrolysis of the various chain length esters, with lipases preferentially hydrolysing the medium chain ester, whereas esterases reacted more favourably with the short ester. The assay was found to be highly sensitive with the fluorophore detectable to the low nM range. These esters provide alternate substrates from established coumarin-based fluorophores, possessing distinctly different excitation (447 nm) and emission (555 nm) optima. Absorbing at 440–450 nm also offers the flexibility of analysis by UV–visible spectrophotometry. This represents the first instance of a naphthalimide-derived compound being used to analyse these enzymes.

1. Introduction

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are amongst the most widely used enzymes in industry and are utilised as biocatalysts in many applications, including the manufacture of pharmaceuticals, cosmetics and detergents, as well as in oil and biodiesel processing [1]. These enzymes belong to the ester hydrolase family and are sub-classes of carboxylic ester hydrolases (EC 3.1.1.-). Of the two enzyme sub-classes lipases react preferentially with long chain water-insoluble carboxylic ester substrates, whereas esterases act upon more soluble compounds. The ability of lipases to act on insoluble substrates at a lipid-aqueous interface has traditionally been defined as distinct from other carboxylic ester hydrolases [2]. In reality, lipases are specialised esterases and the divide between lipases and other carboxylesterases is not clear-cut. A recent book chapter [3] discusses this issue and highlights

Abbreviations: BSA, bovine serum albumin; CalA, *Candida antarctica* lipase A; CalB, *Candida antarctica* lipase B; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NAP, 4-hydroxy-*N*-propyl-1,8-naphthalimide; NAP-B, 4-hydroxy-*N*-propyl-1,8-naphthalimide-butyrate; NAP-O, 4-hydroxy-*N*-propyl-1,8-naphthalimide-octanoate; NAP-P, 4-hydroxy-*N*-propyl-1,8-naphthalimide-palmitate; PfE, *Pseudomonas fluorescens* esterase; TlL, *Thermomyces lanuginosa* lipase.

☆ The NAP esters have been made available through Advanced Molecular Technologies and AK Scientific. Catalogue # AMTGC207-NAB16, AMTGC208-NAO16, AMTGC209-NAP16.

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http://dx.doi.org/10.1016/j.biochi.2016.07.016
0300-9084/

problems that currently cause confusion in the field. The potential variety of reactions these enzymes can perform means that attempting to categorise them in a meaningful manner by any single criterion, i.e. *in silico* analyses, kinetic parameters or structural properties, is not currently possible. As such, biochemical characterisation methods are still of importance, particularly for understanding the application of these enzymes in particular reactions.

Numerous enzyme activity assays for the detection and characterisation of hydrolytic enzymes have been applied over the years. While the use of natural glyceride substrates is required for full characterisation of so-called 'true lipase' activity, these methods are generally slow and laborious. Amongst the most widely used methods are those that apply synthetic substrates that are specifically designed for targeted enzymatic reactions. Mono-ester substrates offer simple and rapid methodologies for investigating activity. Despite some limitations, they are highly useful substrates that allow for the investigation of different facets of activity and selectivity in a high-throughput manner. Chromogenic substrates such as *p*-nitrophenyl esters have been used extensively [4,5] and are still amongst the most commonly reported [6,7]. Fluorogenic substrates have also been in use for some time [8]. However, despite a number of compounds being commercially available, such as the coumarin-derivatives 4-methylumbelliferone [9] and umbelliferone [10], colorimetric-based assays remain more widely used. This is surprising considering the greatly improved sensitivity offered by fluorogenic substrates. Whether this is due to instrumental requirements, or simply because the colorimetric substrates are more accessible is unclear.

Fluorophores based on a 1,8-naphthalimide scaffold are known to possess excellent photostability, high quantum yields and large stokes shifts. These properties make them suitable for a range of applications in analyte sensing and cell imaging [11,12]. We have recently published a library of *N*-substituted 4-hydroxy-1,8-naphthalimide derivatives that can be functionalised to produce a variety of compounds with analytical utility [13]. The compounds possess useful photophysical characteristics, with excitation and emission optima in aqueous solutions of ~447 nm and ~555 nm, respectively. In addition to fluorescent detection, they can also be analysed using a standard UV/visible spectrophotometer, albeit at reduced sensitivity.

One of these derivatives (4-hydroxy-*N*-propyl-1,8-naphthalimide (NAP)) was selected as a fluorophore for enzyme assay development. Herein we describe the synthesis of three novel NAP esters of different chain length and their use in an activity assay. A sensitive, simple and rapid methodology was developed and then applied in the analysis of a range of carboxylester hydrolases, allowing for comparisons of activity between substrates.

2. Experimental

2.1. Chemicals and enzymes

All chemicals were purchased from Sigma-Aldrich and were of analytical grade or higher, unless specified. Lipozyme CalB L (lipase B from *Candida antarctica*), Lipozyme TL-100 L (lipase from *Thermomyces lanuginosa*), Novocor AD L (lipase A from *Candida antarctica*) and Lecitase® Ultra (phospholipase A1, hybrid of lipase from *Thermomyces lanuginosa* and phospholipase from *Fusarium oxysporum*) were obtained from Novozymes, while esterase from *Pseudomonas fluorescens* (recombinant from *E. coli*) and bovine serum albumin (BSA) was purchased from Sigma-Aldrich.

2.2. Fluorophore analysis

The molar absorptivity of the previously characterised 4-hydroxy-*N*-propyl-1,8-naphthalimide derivative [13] was investigated at different pH (4.0–10.0). The absorption and fluorescence of the compound was measured over a range of concentrations (0–50 μM) at 447 nm (absorbance maximum). The linear dynamic range of the fluorophore was also defined using substrate concentrations ranging from 0.01 to 20 μM using excitation and emission wavelengths of 447 and 555 nm, respectively.

2.3. Synthesis of the 4-hydroxy-1,8-naphthalimide esters

The 4-hydroxy-*N*-propyl-1,8-naphthalimide (NAP) derivative synthesised previously [13], was used to synthesise esters of butyric, octanoic and palmitic acids. This was achieved using a Steglich esterification [14], similar to that performed in Ref. [6] with modifications. A stirring solution of 4-hydroxy-*N*-propyl-1,8-naphthalimide (1.0 equiv) in dichloromethane (CH_2Cl_2) (10 mL per mM NAP) was treated with the appropriate carboxylic acid (1.3 equiv), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (1.5 equiv) and 4-dimethylaminopyridine (DMAP) (0.01 equiv) at ambient temperature under and atmosphere of N_2 for 1 h. NAP is insoluble in CH_2Cl_2, while the esters are soluble, allowing for reaction progress to be monitored visually (yellow opaque solution to a clear brown). Thin layer chromatography was performed to monitor reaction progress and chromatography fractions using a mobile phase of CH_2Cl_2, methanol and ammonium hydroxide (94:5:1 v,v,v) and visualised under UV-light. After completion of the reaction CH_2Cl_2 was removed under reduced pressure giving a crude residue that was subsequently purified by silica gel column chromatography, eluting with the mobile phase described above. Fractions containing the ester product were pooled and the solvent removed under reduced pressure. In some circumstances the palmitate ester was re-crystallised (ethanol/H_2O) to obtain sufficient purity. The dry NAP esters were then stored at −20 °C prior to use. Fig. 1.

2.4. Compound characterisation

The synthesised esters were spectroscopically characterised using ^1H and ^{13}C NMR, high resolution mass spectroscopy, as well as UV–vis and fluorescence scanning spectrophotometry. High resolution mass spectra were collected using an Agilent Technologies 6210 MSD TOF time-of-flight mass spectrometer. NMR spectra were recorded with an AVANCE III 500 MHz NMR spectrometer. The absorbance maxima (λ_{ab}) of each ester was determined from a spectrum scan (200–600 nm) performed using a Cary 300 Bio UV–visible spectrophotometer (Agilent Technologies), while the emission maxima (λ_{em}) were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). Photophysical data were collected in 50 mM Tris-HCl pH 8.0.

NAP-B; 72% as a waxy orange/yellow solid. λ_{ab} = 340 nm, λ_{em} = 490 nm. ^1H NMR ($CDCl_3$, 500 MHz) δ 8.63 (d, J = 7.3, 1H), 8.61 (d, J = 8.0, 1H), 8.24 (dd, J = 8.4, 1.0, 1H), 7.77 (dd, J = 8.4, 7.3, 1H), 7.55 (d, J = 8.0, 1H), 4.16–4.13 (m, 2H), 2.77 (t, J = 7.4, 2H), 1.91 (app. sext. J_{app} = 7.4, 2H), 1.76 (app. sext. J_{app} = 7.5, 2H), 1.13 (t, J = 7.4, 3H), 1.01 (t, J = 7.4, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.3, 164.1, 163.5, 151.5, 131.7, 131.6, 129.3, 127.6, 127.2, 125.2, 123.0, 120.3, 119.4, 41.9, 36.2, 21.4, 18.5, 13.7, 11.5. Found; $[M+H]^+$ 326.1396 $C_{19}H_{19}NO_4$ requires 326.1387.

NAP-O; 81% as a yellow solid. λ_{ab} = 340 nm, λ_{em} = 490 nm. ^1H NMR ($CDCl_3$, 500 MHz) δ 8.63 (dd, J = 7.3, 1.1, 1H), 8.61 (d, J = 8.1, 1H), 8.23 (dd, J = 8.4, 1.1, 1H), 7.77 (dd, J = 8.4, 7.3, 1H), 7.55 (d,