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Journal of Molecular Catalysis B: Enzymatic 3 (1997) 127–130

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

New fluorescent glycerolipids for a dual wavelength assay of lipase activity and stereoselectivity

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Abstract

A new type of fluorogenic alkyldiacyl glycerols was synthesized and used as fluorogenic substrates for the analysis of lipase activities and stereoselectivities. These compounds contain pyrene or perylene as a fluorophore and the trinitrophenylamino- (TNP) residue as a quencher. Both substituents are covalently bound to the ω-ends of the *sn*-2 and *sn*-1(3) acyl chains, respectively. Upon glycerolipid hydrolysis, the residues are separated from each other thus allowing determination of lipase activity from the continuous increase in fluorescence intensity which is caused by dequenching. Mixtures of enantiomeric fluorogenic alkyldiacyl glycerols, selectively labeled with pyrene or perylene as fluorophores, can be used for a dual-wavelength 'stereoassay' of lipases. Since absorption and emission maxima of both labels are clearly separated, hydrolysis of the respective enantiomeric substrates can be distinguished simultaneously, and the difference in their rates of hydrolysis can be taken as a parameter for the stereopreference of a lipase.

Keywords: Alkyldiacylglycerols; Pyrene glycerolipids; Perylene glycerolipids; Resonance energy transfer

1. Introduction

Lipases are hydrolytic enzymes, that are important for intracellular lipid metabolism and extracellular lipid degradation. In synthetic chemistry they are used as biocatalysts [1] for stereoselective hydrolysis and esterification of artificial substrates, leading e.g., to optically pure pharmaceutical agents. Other applications of lipases in industry include food processing, industrial reagents and detergent additives [2]. In all these cases, the activities of the employed lipases have to be determined in order to assess enzyme purity, stability, and activity. In medicine, lipase activities are determined for the diagnosis of impaired lipid metabolism [3].

Fluorogenic alkyldiacyl glycerols are useful substrates for the analysis of lipase activities and stereoselectivities. So far lipids have been available, containing pyrene as a fluorophore and the trinitrophenylamino- (TNP) residue as a quencher [4]. Both substituents were covalently bound to the ω-ends of the *sn*-2 and *sn*-1(3) acyl chains, respectively. Upon glycerolipid hydrolysis both residues were separated from each other and lipase activity could be determined from the continuous increase in fluorescence intensity due to dequenching. For reasons of sensitivity and suppressing background effects

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PII S1381-1177(96)00047-1

of biological samples, it was desirable to have a longer-wavelength probe. This could be achieved by the preparation of alkyldiacyl glycerols containing perylene as a fluorescent label bound to the *sn*-2 acyl chain. A mixture of *sn*-1 (or *sn*-3) perylene and *sn*-3 (or *sn*-1) pyrene-labeled alkyldiacyl glycerols can be used in a dual wavelength assay for the characterization of lipases by their stereoselectivity toward the *sn*-1 and *sn*-3 positions of the respective glycerolipid substrates as described in this paper.

2. Results and discussion

A new type of enantiomeric alkyldiacyl–glycerols (Fig. 1) was synthesized. These compounds are useful as fluorogenic substrates for the continuous determination of lipase activities. They contain a hexadecyl chain linked by an ether bond to the *sn*-1 or *sn*-3 position of the glycerol backbone. This alkyl-substituent can neither be released by alkali nor lipolytic enzymes. Fluorescent pyrene decanoic acid (Fig. 1, in substrate I) or perylene dodecanoic acid (Fig. 1, in substrate II) is esterified to the *sn*-2 position of glycerol. Positions *sn*-3 or *sn*-1 are substituted with the trinitrophenylaminododecanoyl residue, which is a quencher of pyrene and perylene fluorescence.

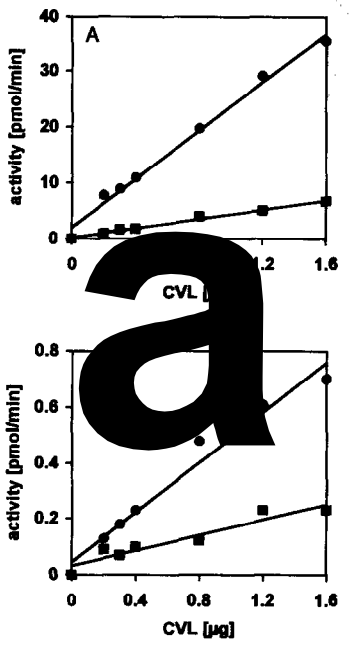

Fig. 2. Activity of lipase from *Chromobacterium viscosum* (CVL) towards *sn*-1 and *sn*-3 acyl enantiomers of fluorescently labeled alkyldiacyl glycerol (● *sn*-1; ■ *sn*-3). Lipolysis was carried out at 30°C in aqueous buffer (0.1 M Tris–HCl, pH 7.4), containing BSA (0.13 mg/ml), and (A) pyrene-labeled lipid analogs (2 nmol/ml), and (B) perylene–lipid analogs (2 nmol/ml). The increase of pyrene fluorescence at 378 nm and the increase of perylene fluorescence at 451 nm was recorded continuously for 5 min.

Due to the presence of a quencher, label fluorescence in the intact substrate molecules I and II is low. However, if one of the labeled

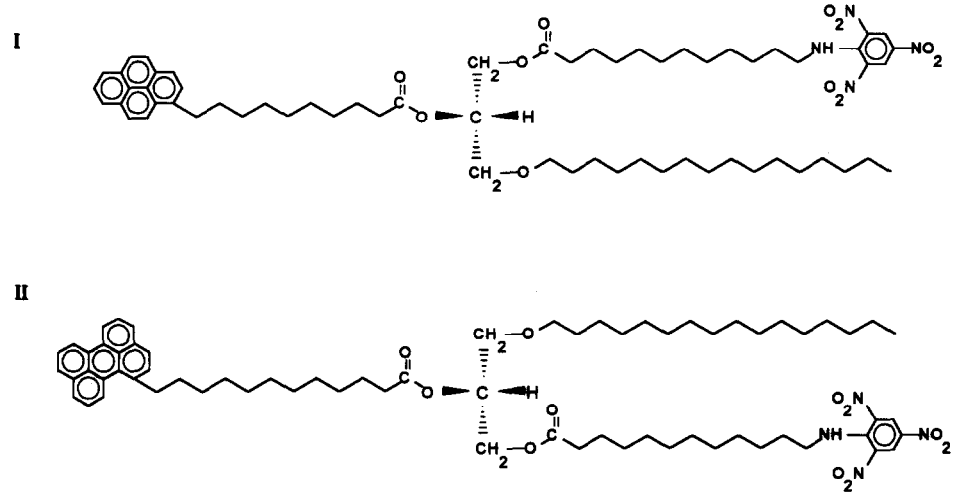

Fig. 1. Chemical structures of fluorogenic alkyl–diacylglycerols. I: (*S*)-1-trinitrophenylaminolauroyl-2-pyrenedecanoyl-3-*O*-hexadecyl-*sn*-glycerol, II: (*R*)-3-trinitrophenylaminolauroyl-2-perylenedodecanoyl-1-*O*-hexadecyl-*sn*-glycerol.

acyl chains is released from the glycerolipid by lipase-catalyzed hydrolytic cleavage, label fluorescence increases. Thus, from the continuous increase in fluorescence intensity, lipase activity can be easily determined.

Enzyme activity observed with pyrene- and perylene-labeled substrates depended on the enzyme used, the configuration of the glycerolipid, and the form of substrate solubilization. If the substrates were solubilized in the presence of albumin, all enzymes under investigation preferred the *sn*-1 enantiomer of pyrene- (Fig. 2A) or perylene-labeled (Fig. 2B) compounds.

A mixture of enantiomeric alkyldiacyl glycerol analogs, one containing pyrene (Fig. 1, substrate I) and the other one perylene (Fig. 1, substrate II) as a fluorophore, is suited for a dual wavelength 'stereoassay' of lipases. Since absorption and emission maxima of both labels are well separated (Fig. 3), hydrolysis of both labeled substrates can be determined simultaneously (Fig. 4), and the ratio of their rates of hydrolysis can be taken as a parameter for the apparent stereopreference of a lipase.

Hydrolysis rates measured with perylene lipids are lower than those obtained with the pyrene analogs (Fig. 2). Thus, we observe a higher difference in apparent stereoselectivity of lipases (which is in fact a combination of stereo-

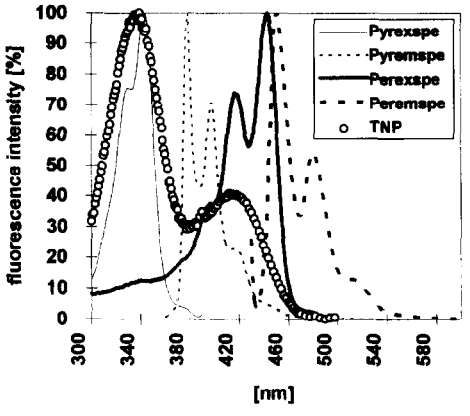

Fig. 3. Excitation- and emission spectra of chromophores in labeled alkyldiacylglycerols (see Fig. 1). Pyrexspe, pyrene excitation spectrum. Pyremspe, pyrene emission spectrum. Perexspe, perylene excitation spectrum. Peremspe, perylene emission spectrum. TNP, trinitrophenylamine absorption spectrum.

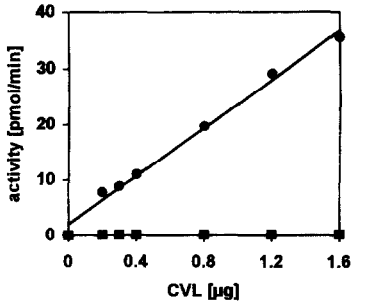

Fig. 4. Activity of lipase from *Chromobacterium viscosum* (CVL) in a mixture of perylene- and pyrene-labeled alkyldiacyl glycerols. Samples contained *sn*-1-acyl (pyrene-labeled ●) and *sn*-3-acyl (perylene-labeled ■) lipid (1 nmol/ml each; total lipid concentration: 2 nmol/ml) in aqueous 0.1 M Tris–HCl, pH 7.4, containing fatty acid-free albumin (0.13 mg/ml). Lipase activities at various enzyme concentrations were determined from the increase in fluorescence intensity at 378 nm (pyrene) and 451 nm (perylene) during a 5 min incubation.

and substrate selectivity) if a mixture of both labels is used as a substrate, particularly in the presence of albumin. Since all microbial lipases tested so far exhibit stereopreference for the *sn*-1 glycerol position in the presence of albumin [4], the apparent stereoselectivities should be highest if the pyrene and perylene substrates contain the label at the *sn*-1 and *sn*-3 position, respectively.

The dual-wavelength method as proposed here can be suitably carried out using existing and cheap instrumentation for the fluorimetric analysis of Ca^{2+} concentrations. The same technique applies to our lipase stereoselectivity assay requiring the alternating measurement of two different fluorescence intensities (of perylene and pyrene) in a single cuvette.

Apparent stereoselectivities obtained by the dual-wavelength assay, could provide a very effective means for the characterization of lipases in biotechnology and medicine. It could be used for the fast determination of enzyme purity during purification, enzyme identification in complex biological sources or commercial enzyme products, and for the analysis of lipases in mixtures of lipolytic enzymes (lipases and esterases). It is not likely that two different lipases exhibit the same apparent stereoselectivity. Therefore, during lipase purification or

screening of biological material, measurement of apparent stereoselectivity rather than activity could serve as a parameter for lipase identity. Related studies on the applicability of this new method are under way in our laboratory.

Acknowledgements

Financial support by the Fonds zur Förderung der wissenschaftlichen Forschung (project F 0107 to A.H.) is gratefully acknowledged.

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