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Short communication

In situ evaluation of esterase stereoselectivity in two-dimensional electropherograms and tissue sections

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Abstract

Staining with both enantiomers of an α-naphthyl ester plus a diazonium salt and comparing the color intensities given by the two enantiomers is a convenient method to evaluate the esterase stereoselectivity for that ester in two-dimensional electropherograms and tissue sections. Application of this method for rat liver has shown that (1) several esterases, e.g., one of p*I* 6.4 and M_r 118 kDa, are moderately stereoselective against α-naphthyl (*R*)-*N*-acetylalaninate and (*R*)-*N*-methoxycarbonylalaninate but strictly stereoselective against α-naphthyl (*S*)-*N*-methoxycarbonylvalinate, implying that esterase stereoselectivity may be inverted by changing the ester structure; and (2) these esterases are mainly contained in the hepatocytes around central veins. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Stereoselectivity; Esterase; Enzymes

1. Introduction

Esterase is an important enzyme in the conversion of ester-type prodrugs to the parent active drug in the patient body [1]. Its usefulness is due to its broad substrate specificity, but the stereoselectivity is high for some chiral esters. Esterases in different tissues (e.g., tumor and normal tissue) sometimes show opposing stereoselectivities to each other depending on the ester structure [2]. This property may be used to activate a chiral prodrug in specific cells which contain esterases of the corresponding stereoselectivity while keeping it inactive in other cells whose esterases are counter-stereoselective. A simple analytical method is currently required to observe stereoselectivity of esterases in the tissue to be targeted with the chiral prodrug. Recently we have reported that staining of electrophoretic gels with a pair of enantiomers of a chiral α-naphthyl ester plus a diazonium salt indicates stereoselectivity of esterase in each band upon comparing the color intensities given by the two enantiomeric substrates [3]. The previous experiment was conducted only with density gradient electrophoresis so there remained some ambiguity that the apparently low stereoselectivity might have been caused by presence of more than two enzymes with opposite stereoselectivities in the same band. In this communication we describe applications of the above chiral staining method to

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2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and histochemical examination.

2. Experimental

2.1. Reagents

The chiral naphthyl esters, α-naphthyl (*R*)- and (*S*)-*N*-acetylalaninate [AcAla(ONap)], (*R*)- and (*S*)-*N*-methoxycarbonylalaninate [MocAla(ONap)] and (*R*)- and (*S*)-*N*-methoxycarbonylvalinate [MocVal(ONap)] (Fig. 1), were prepared in the previous work [3]. Fast Blue RR salt, Fast Garnet GBC salt and methyl green were purchased from Sigma–Aldrich Japan (Tokyo, Japan), diisopropyl fluorophosphate from Wako Pure Chemical Industries (Osaka, Japan), acrylamide, *N,N′*-methylenebisacrylamide and Bio-Lyte (3/9) from Bio-Rad Labs. (Hercules, CA, USA), isoelectric point (p*I*) marker proteins from Oriental Yeast (Osaka, Japan), density gradient precast gels for 2D-PAGE from Daiichi Kagaku Kogyo (Tokyo, Japan) and O.C.T. Compound from Miles (Elkhart, IN, USA).

2.2. Biological samples

Small pieces of liver were taken from a male Wistar rat (four weeks old). Some pieces (about 0.05 cm^3) were ground by a Potter-type homogenizer with 10 μl digitonin solution (2% in 0.1 *M* phosphate buffer, pH 7.5) for each piece. After treatment with a sonicator (Tomy UR-20P) for 2 min, the homogenates were centrifuged at 15 000 rpm for 3 min to give supernatants, which were used as enzyme extracts. Other pieces (10×10×3 mm) were embedded in O.C.T. Compound blocks by freezing.

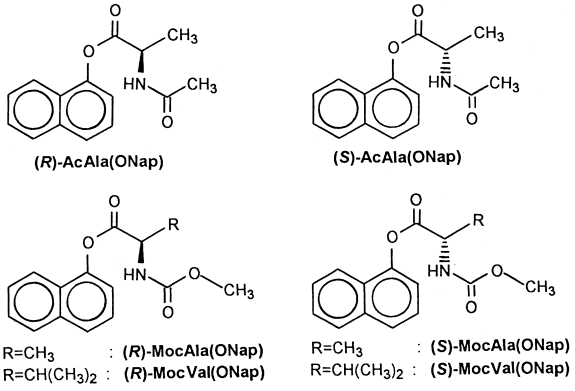

Fig. 1. Structures of α-naphthyl esters.

2.3. 2D-PAGE

Gels for isoelectric focusing (IEF) were prepared on supporting films with a casting stand (Model 111 Mini IEF Cell, Bio-Rad) with 2% Bio-Lyte (3/9) according to the supplier's manual. The enzyme extracts were applied to the gel as soaked in filter chips (5×2 mm). After 10-min contact, the chips were taken off and electric power was applied at 100 V for 15 min, at 200 V for 15 min, and then at 450 V for 60 min. Parts of the gel containing electrophoresed proteins (i.e., lanes) were cut, as they were attached on the supporting film, to give strips of 35×5 mm. Two of the strips were placed in a line in the slit between two glass plates at the top of one density gradient gel (10×10 cm). Electrophoresis was done with 0.3% Tris–1.44% glycine as the electrode solution at a constant current of 20 mA for 4.5 h in a cold room. The gel was vertically divided at the center in two parts corresponding to each of the two IEF strips. One part was immersed in a mixture of 10 ml 0.1 *M* phosphate buffer (pH 6.25) containing 4 mg Fast Blue RR salt with 0.1 ml ethanol containing 2.5 mg (*R*)-AcAla(ONap). Another part was immersed in the similar mixture containing (*S*)-AcAla(ONap) instead of (*R*)-AcAla(ONap). After standing at room temperature for 5 min, the gels were thoroughly washed with water and then recorded by photography. The same procedure was carried out with (*R*)- and (*S*)-MocAla(ONap) and (*R*)- and (*S*)-MocVal(ONap) for staining. In the case of MocVal(ONap), the gels were allowed to stand for 30 min before washing.

2.4. Staining of tissue sections

The rat liver embedded in the polymer blocks was sliced to 10 μm-thick sections at −16°C with a cryostat (MICROM HM500-OM). The sections were attached on slide glass plates coated with polylysine. Each section was overlaid with a mixture made of 3 μl ethanol containing (*R*)- or (*S*)-MocVal(ONap) (50 mg/ml) and 0.2 ml phosphate buffer (0.1 *M*, pH

6.25) containing Fast Garnet GBC salt (0.3 mg/ml) and left at room temperature for 20 min. After washing with water, the sections were stained with an aqueous solution of methyl green (0.1 mg/ml) for 3 min, washed again with water, and finally covered with a thin glass plate using 90% glycerol for microscopic examination.

3. Results and discussion

3.1. Staining for zymograms

Fig. 2 shows the gels of 2D-PAGE for rat liver esterases stained by the present method.

The spots for an isoenzyme of p*I* 6.4 and M_r 118 kDa (indicated by arrow) were stained heavier with the (*R*)-enantiomers of AcAla(ONap) and MocAla-(ONap) than with the corresponding (*S*)-enantiomers, showing that this isoenzyme hydrolyzed the (*R*)-enantiomer of the two esters more rapidly than the counter enantiomer. Therefore, the stereoselectivity was an (*R*)-enantiomer preference for AcAla-(ONap) and MocAla(ONap). This isoenzyme was, however, hardly stained with (*R*)-MocVal(ONap), while its spot still appeared by staining with (*S*)-MocVal(ONap). The stereoselectivity for Moc-Val(ONap) was strictly toward the (*S*)-enantiomer. Thus the stereoselectivity of this isoenzyme was reversed by changing the substrate from the alanine esters to the valine ester. On the other hand, another isoenzyme of p*I* 4.9 and M_r 92 kDa (indicated by arrowhead) showed the spot that was stained with every (*S*)-enantiomer slightly heavier than with the corresponding (*R*)-enantiomer. The stereoselectivity of the latter isoenzyme seems to be independent of the substrate structure. It can be concluded that there are two kinds of relationships between enzyme stereoselectivity and substrate structure, i.e., independence and dependent alteration leading even to inversion.

This conclusion was also suggested by the previous experiment with the one-dimensional electrophoresis [3], but there was a possibility that more than two enzymes with different substrate specificity and different stereoselectivity might have come in the same electrophoretic band to give the apparent phenomena regarding stereoselectivity. The present data with 2D-PAGE support the above conclusion more firmly because protein singularity for every spot is more reliable in 2D-PAGE.

3.2. Histochemical application

The present chiral staining is also applicable for the location of stereoselective esterases in tissue

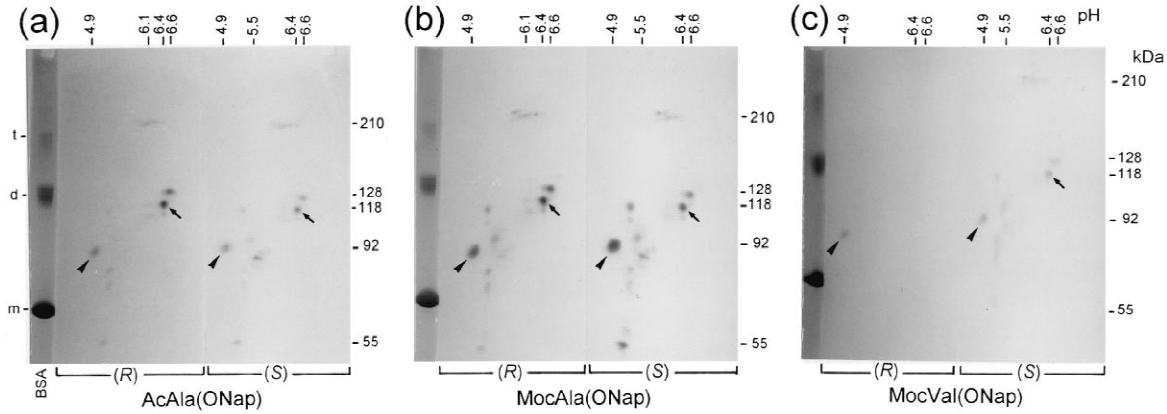

Fig. 2. Two-dimensional PAGE for rat liver esterases detected by activity staining with (*R*)- and (*S*)-AcAla(ONap) (a), (*R*)- and (*S*)-MocAla(ONap) (b), and (*R*)- and (*S*)-MocVal(ONap) (c) in the presence of Fast Blue RR salt. Left lane of each panel shows BSA oligomers as molecular-mass-markers (t=201, d=134 and m=67 kDa) which were concomitantly electrophoresed and stained with Coomassie Brilliant Blue G. Numbers on the top and on the right side indicate pH of the IEF gel (determined with p*I* marker proteins) and M_r values, respectively.

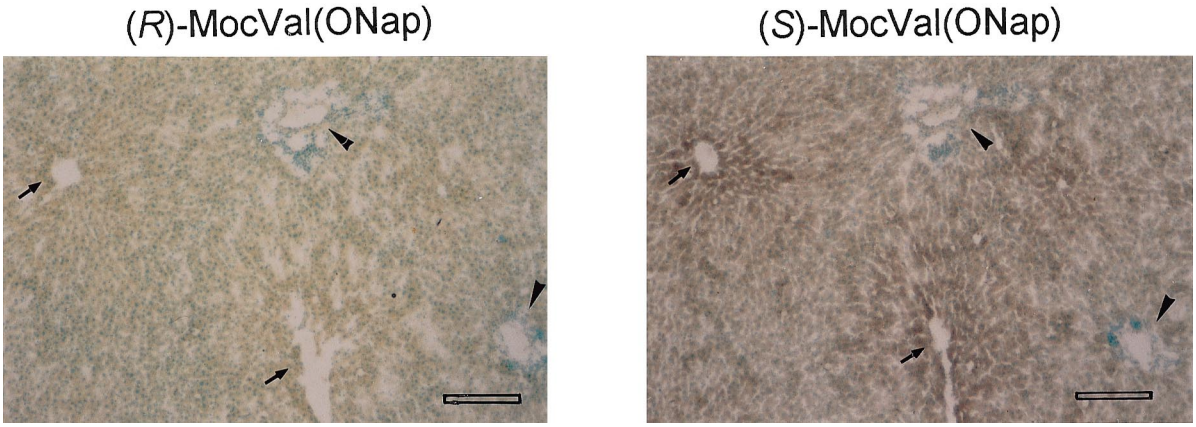

Fig. 3. Staining of rat liver cryostat sections with (*R*)- and (*S*)-MocVal(ONap) and Fast Garnet GBC salt. After the esterase activity staining, the sections were stained with methyl green. Scale bar, 250 μm.

sections. Fig. 3 shows rat liver tissue sections stained with (*R*)- and (*S*)-MocVal(ONap) plus Fast Garnet dye and then with methyl green. The red coloration indicates presence of esterase activity and the blue–green dots represent cell nuclei. Red color did not appear when the staining solution lacked the α-naphthyl ester or was supplemented with 1 m*M* diisopropyl fluorophosphate, an esterase inhibitor (data not shown). A comparison between the left and right parts of Fig. 3 shows that most of the esterases in this tissue are stereoselective toward (*S*)-MocVal(ONap), consistent with the electrophoresis result (Fig. 2c). The comparison also suggests that these esterases are abundant in the hepatocytes around central veins [4] (indicated by arrow), whereas there is less esterase activity in cells in portal area (arrowhead). The faint stain with (*R*)-MocVal(ONap) is probably due to the isoenzyme of p*I* 4.9 and M_r 92 kDa which is the major enzyme active with this (*R*)-enantiomer (Fig. 2c).

3.3. Concluding remarks

Stereoselectivity inversion of esterases with different substrates has been already reported from studies on the enantiomeric products by chiral-phase chromatography or nuclear magnetic resonance with chiral shift reagents or counter-ions [5,6]. These methods are applicable to any substrate in principle, but have the disadvantage that the enzyme should be purified prior to the experiment. Our strategy (i.e., electrophoresis and activity staining) is limited to special esters having a prochromophore such as an α-naphthyl group, but it can bypass the time-consuming step of enzyme purification. Although the structure of alcohol moiety possibly affects the esterase reaction of two enantiomeric esters differently, investigation with chiral α-naphthyl esters may be useful as a preliminary survey for esterases contained in the targeted tissue. In conclusion, information concerning stereoselectivity of esterases directly in electropherograms and tissue sections is easily obtained by the present method. Application of this method to cancer esterases is in progress [7].

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