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Competitive immunoassay (Cat-EIA), a helpful technique for catalytic antibody detection. Part II.

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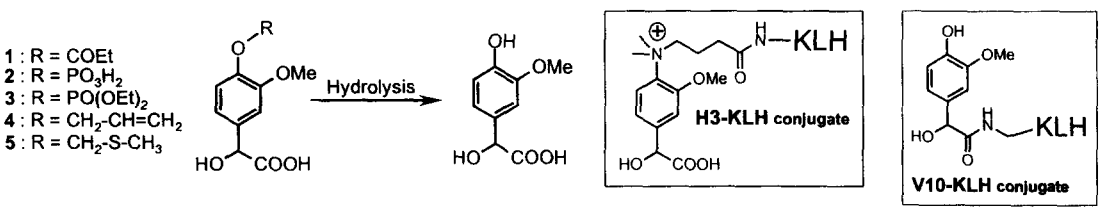
Abstract.

The Cat-EIA procedure, described in part I, was successfully applied to the screening of 5 different catalytic activities on a given set of 11 mAbs. The precautions required to devoid false-positive identification (preceding paper), were taken into account. Two catalylic activities were thus detected, including a newly thioacetal hydrolysis.

Keywords *: Antibodies; Catalysis; Hydrolysis; Thioacetal.*

Taking into account the parameters (part I) which can give rise to false-positive identification if no additional controls are performed, we applied the Cat-EIA procedure to the screening of a given set of monoclonal antibodies. MAbs raised against hapten **H3** (scheme 1) have been previously shown to catalyze an oxidative decarboxylation of VMA in the presence of $NaIO_4$ [1]. We decided to screen these mAbs for various hydrolytic activities (scheme 1) using the Cat-EIA procedure. This allowed us to scan a large panel of reaction conditions (substrate concentration, pH, temperature) and to perform rapidly a large number of catalytic tests.



Scheme 1.
Target reaction generating **VMA** as product and hapten structures.

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The potential interest of the Cat-EIA approach depends on its ability to measure selectively the product but not the substrate, to detect low amounts of product, and to provide results allowing more accurate calculation of kinetic data. These factors are related to the intrinsic properties of the anti-product antibody as seen in part I. It was not possible to carry out the Cat-EIA using the previously described anti-**V8** antibodies because of their high cross-reactivity with the substrates **1-5** (scheme 1). We therefore produced new polyclonal antibodies by immunizing rabbits with hapten **V10** (scheme 1). The selected antiserum was suitable to detect VMA by Cat-EIA (cross-reactivity for substrates **1-5** < 0.1%; detection limit = 400 nM; precision CV, intra- and inter-assay repeatability < 10%). Moreover, no enantioselectivity toward the VMA enantiomers was observed, thus avoiding the detection problem previously mentioned (part I). All the anti-**H3** mAbs were purified using the prot.A technique [2,3] and dialyzed against the required dissociating medium (0.1 M citrate buffer, pH 5) before use in order to remove potential VMA contamination (see part I).

We first focused our attention on the hydrolysis of ester **1**. Kinetic studies of the non-catalyzed reaction, at pH 8.0, gave identical first-order rate constants for the Cat-EIA and HPLC detection (Cat-EIA : $k_{uncat} = 9.3 \pm 0.7 .10^{-5}$ min^{-1}; HPLC : $k_{uncat} = 9.2 \pm 0.5 .10^{-5}$ min^{-1}), thus validating the Cat-EIA measurements. Catalytic tests were performed using 50 µM of **1** and 2 µM of anti-**H3** mAb at pH 8, 25°C. Under these standard conditions and after one hour reaction, the Cat-EIA experiments allowed us to detect a rise of 20% of the background reaction. According to the Cat-EIA detection, two anti-**H3** mAbs (**H3**-12 and 32; figure 1) were clearly positive while mAbs **H3**-6 and 15 could also be considered as good candidates. To assess the Cat-EIA screening, these results were compared with those obtained using HPLC detection. The results (figure 1) roughly confirmed the selection made by Cat-EIA[1].

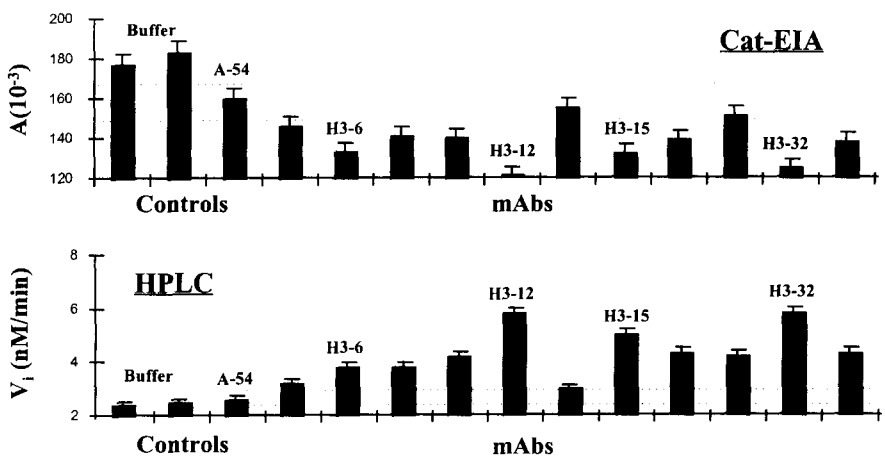

Figure 1.
Screening of anti-H3 mAbs for esterolytic activity. Comparison between Cat-EIA and HPLC. Catalysis was performed with 50 µM of **1**, 2 µM of anti-H3 mAb at pH 8.0 in 0.05 M phosphate buffer, 0.05 M borate buffer at 25°C. V_i is the initial rate of the reaction. Buffer and A-54 are control experiments. Dotted lines represent the confidence interval as estimated from reproductibily experiments.

[1] Note that the apparently low activity detected by Cat-EIA with irrelevant monoclonal antibody A54 is due to the known cross-reactivity of the mouse monoclonal anti-rabbit IgG coated on the plate with the mouse mAbs tested. This leads to competition with rabbit antibodies, and removal from the solid phase of anti-**V10** antibodies leading to a lowering of the signal. Absorbance observed with irrelevant mAbs, used at the same concentration as anti-**H3** antibodies, should therefore be taken as the uncatalyzed reference rather than the buffer experiments.

Careful controls are required to ensure that the observed rate enhancement of esterolytic activity is due to the abzyme and not to enzyme contamination. The first control experiment was to inhibit the catalytic activity with hapten **H3**. This was easily achieved by Cat-EIA (figure 2). Results obtained with mAbs **H3**-12, 15 and 32 demonstrated that catalytic activities were clearly inhibited by hapten **H3**[2]. This is a good evidence that the rate enhancement was indeed due to the catalysis of the hydrolysis of ester **1** by the specific binding antibodies. On the other hand, apparent activity of antibody **H3**-6 was not inhibited by hapten **H3**, and this mAb was therefore not retained for further studies. All of these results were confirmed by HPLC analysis. The calculated inhibition constants K_i (**H3**-12: K_i = 7.3 μM; **H3**-15: K_i = 3.3 μM; **H3**-32: K_i = 0.3 μM) were very close to the estimated hapten affinity.

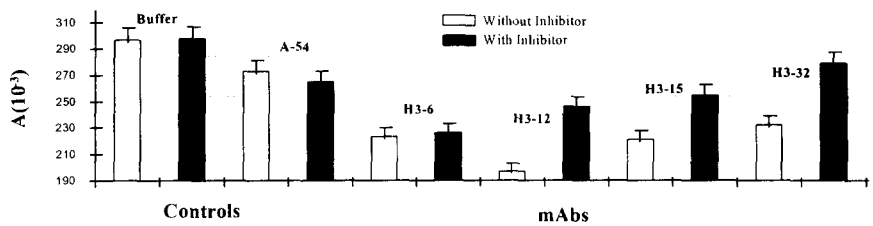

Figure 2.
Inhibition controls. Detection of **VMA** by Cat-EIA. Catalysis was performed with 50 μM of **1**, 2 μM of anti-H3 mAb at pH 8.0 in 0.05 M phosphate buffer, 0.05 M borate buffer at 25°C in the presence or absence of 25 μM hapten H3. Buffer and A-54 are control experiments. Dotted lines represent the confidence interval as estimated from reproductibily experiments.

The abzymatic character of the observed catalysis was also supported by the following datas : The catalytic properties of the three selected mAbs were proportional to the antibody concentration. Purity of the monoclonal antibodies was attested by SDS-PAGE analysis under reducing conditions. Successive purifications by the prot.A procedure did not decrease the observed catalytic activities, and monoclonal antibodies from various batches of ascitic fluids showed the same catalytic activity. These three catalysts were characterized by HPLC in order to determine the kinetic parameters of the catalysis using Lineweaver-Burk plots (**H3**-12: k_{cat} = 0.053 min^{-1}, k_{cat}/k_{uncat} = 570; K_m = 480 μM; **H3**-15: k_{cat} = 0.015 min^{-1}, k_{cat}/k_{uncat} = 160; K_m = 250 μM; **H3**-32: k_{cat} = 0.045 min^{-1}, k_{cat}/k_{uncat} = 480; K_m = 710 μM). The calculated K_m and substrate affinities were found to be similar. The low rate accelerations underline the capacity of the Cat-EIA approach to detect abzymes with low catalytic activities (figure 1 rate of the catalyzed reaction is 70 to 130% of the background reaction).

Monoclonal anti-**H3** antibodies were then assayed for their ability to hydrolyze substrates **2**-**5**. None of them increased the rate of cleavage of substrates **2**, **3** and **4**, but mAb **H3**-32 showed significant catalytic activity for the hydrolysis of thioacetal **5** in the presence of $MgCl_2$. Once again Cat-EIA and HPLC identified the same antibody as a potential catalyst (figure 3). The same controls as those undertaken for esterase catalytic activities (inhibition and purity controls) proved that the observed rate enhancement was due to the mAb **H3**-32 binding site. To the best of our knowledge such an antibody-catalyzed reaction has not been described in the literature so far. The catalytic parameters and mechanism of this new antibody-catalyzed reaction are currently investigated in the laboratory.

[2] Control competitive immunoassays were used to check that anti-V10 antibodies did not bind H3 and that hapten H3 did not interfere with the detection of VMA by Cat-EIA.

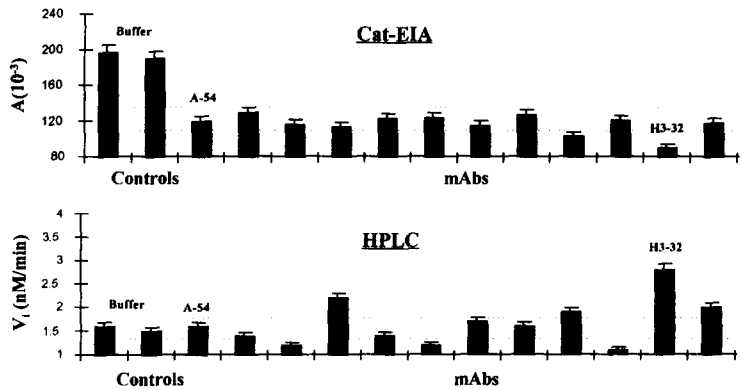

Figure 3.
Screening of anti-H3 mAbs for thioacetal hydrolysis activity. Comparison between Cat-EIA and HPLC results. Catalysis was performed with 1 mM of **5**, 3 μM of anti-H3 mAbs, at pH 5.5 in 0.05 M phosphate buffer and 0.05 M acetate buffer containing 75 mM $MgCl_2$ at 25°C. V_i is the initial rate of the reaction. Dotted lines represent the confidence as estimated from reproductibily experiments.

In this paper we analyzed the ability of the Cat-EIA technique to screen rapidly and efficiently a given set of mAbs for numerous catalytic activities. The selection of catalytic anti-**H3** mAbs toward the five studied hydrolysis reactions was completed within few days using the Cat-EIA procedure (for each catalysis, more than 4 conditions of pH or substrate concentration were assayed). By HPLC, the same screening would have taken weeks. Moreover, the accuracy of this method has been demonstrated here since rather low catalytic activities were identified. Cat-EIA is a method easy to use and does not require the use of modified substrates. It is adapted to the detection of abzymatic activity in homogenous conditions and has many advantages from a practical and fundamental point of view. In conclusion, Cat-EIA appears to be a very attractive technique, especially as it involves simple, time-saving experimental procedures. It is very helpful for a primary catalytic selection.

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Typical Cat-EIA procedure.
Anti-VMA antibodies and enzymatic tracers were diluted in EIA buffer [4] containing 0.1 M phosphate buffer (pH 7.4), 0.15 M NaCl, 10^{-3} M EDTA, 0.1% BSA (bovine serum albumin) and 0.01% sodium azide. Microtiter plates (Nunc, Maxisorb) were coated with 10μg/ml of purified mouse monoclonal anti-rabbit IgGs and then saturated with BSA (1 mg/ml in phosphate buffer). The reagents were dispensed in the following order: 50 μL of diluted ($1/10^6$) anti-VMA antiserum, 50 μL of the crude (diluted or not) catalyzed reaction mixture (including substrate, product and catalyst) and 50 μL of the **V8**-AChE conjugates (enzymatic tracers used at 2 Ellman Units/ml). After 12-h immunoreaction at 4°C, plates were washed, and 200 μL of Ellman's reagent [5] were added to each well. After 2 h of enzymatic reaction, the absorbance at 414 nm was measured in each well.

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