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

F. Taran[a], P. Y. Renard[a,b], C. Créminon[c], A. Valleix[a], Y. Frobert[c], P. Pradelles[c], J. Grassi[c*] and C. Mioskowski[a,d*].

[a] CEA, Service des Molécules Marquées, DBCM/DSV CEA Saclay F-91191 Gif sur Yvette Cedex. France.
[b] CEB, BP 3, F-91710 Vert le Petit. France.
[c] CEA, Service de Pharmacologie et d'Immunologie, DRM/DSV, CEA Saclay F-91191 Gif sur Yvette Cedex. France.
[d] Faculté de pharmacie, Université Louis Pasteur,Laboratoire de synthèse Bio-organique associé au CNRS 74, route du rhin. BP 24, F-67401, Illkirch France.

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Abstract.

A competitive immunoassay procedure for the screening of catalytic antibodies is reported. This screening approach (Cat-EIA) is a modification of the well-known Cat-ELISA technique avoiding the substrate modification step. It has been developed for a bimolecular reaction and has been tested on a high number of hybridoma clones. The current study explores for the first time the utility and feasibility of this method for the early detection of catalytic antibodies.

Keywords *: Antibodies; Catalysis; Phenol.*

The factor limiting the discovery of abzymes remains our capacity to identify from the huge repertoire of immunoglobulins those antibodies displaying catalytic activity for a given reaction. Among the analytical techniques allowing direct evaluation of the catalytic properties of monoclonal antibodies (mAbs) few days after fusion [1,2,3,4,5,6], immunoassays appear to be attractive because of their sensitivity and efficiency, as previously shown with Cat-ELISA [7,8,9]. However, the later procedure, like many others [10], involves a covalent link of the substrate to a solid phase which could generates some major drawbacks. First, it is limited to substrates possessing an activable chemical function allowing the covalent binding. Second, the hydrophobic environment surrounding the immobilized substrates could interfere with the reaction to be monitored [3]. Third, the modified substrate may not be correctly recognized by the catalytic antibody, particularly for poorly immunogenic or small substrate molecules[1]. Finally, using this strategy, the concentration of immunoreactive substrate cannot be precisely controlled, and is limited mainly for steric reasons. An interesting variant of the Cat-ELISA

[1] In the two previously desccribed Cat-ELISA, the authors had to introduce artificially strong antigenic determinants (similar to the linker used for immunogen preparation) in the substrate and the product in order to avoid this problem.

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procedure that avoids this substrate modification step has recently been described for the detection of enzymatic activity in solution [11]. Simultaneously, our work has also been focused on the development of such a strategy (named Cat-EIA) for the detection and measurement of abzyme activity involving low molecular weight substrates and products and bearing no particular structural features. In Cat-EIA (figure 1), a mixture containing the free substrate, the product and the catalyst is added with a product-enzyme conjugate[2] to a 96-well microtiter plate containing a specific anti-product antibody immobilized into the solid phase[3]. Catalytic activity results in the formation of product which competes with the enzyme-product conjugate for the occupation of the anti-product antibody binding sites. This leads to a decrease in the observed signal (solid-phase bound enzyme activity). Kinetic studies of the catalytic reaction can be easily carried out by stopping the reaction at different times prior to the addition of the reaction mixture to the plate.

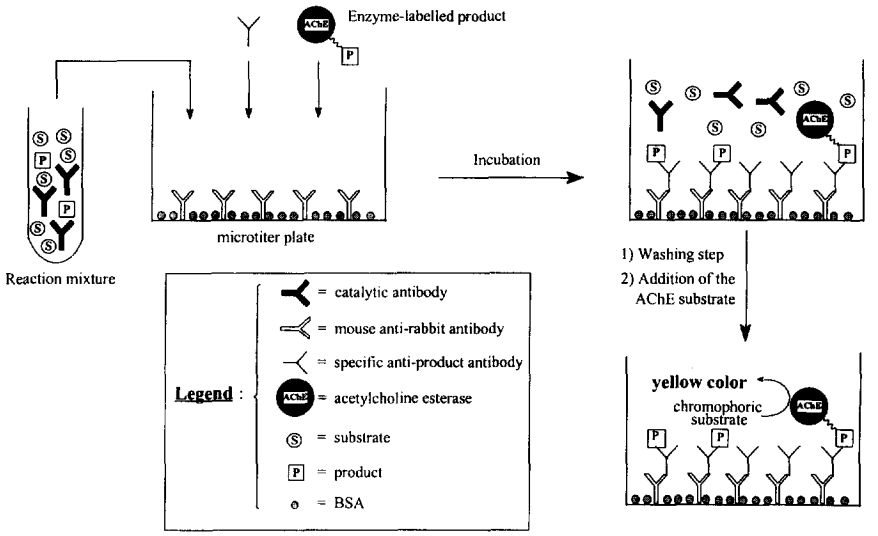
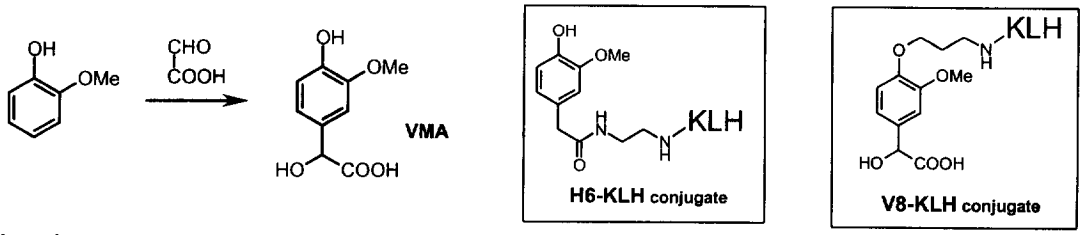


Figure 1.
Strategy of Cat-EIA. Catalytic activity results in a decrease in the absorbance due to a decrease in the amount of solid-phase bound AChE.

In this paper, we describe both positive features and potential drawbacks of this competitive immunoassay procedure for the detection of an antibody-catalyzed bimolecular condensation.

The electrophilic substitution of guaiacol by glyoxylic acid, a well-known reaction used for the synthesis of vanillin [12], produces **VMA** (scheme 1) as the major compound along with two regio-isomers. The yield of the byproducts (resulting from ortho or di-substitution of glyoxylic acid) is usually around 15%. Since this reaction involves poorly immunogenic and very small substrates (MW < 130 daltons), Cat-EIA seems suitable for rapid detection of catalytic antibodies. We decided to use hapten **H6** in order to elicit monoclonal antibodies with putative catalytic activity. Rabbit polyclonal anti-**VMA** antibodies raised against **V8**-KLH immunogen, were also produced to be used as anti-product antibodies in Cat-EIA (scheme 1).

[2] It is necessary to ensure that catalytic antibodies (present at higher concentration than the anti-product antibody) do not bind the product-enzyme conjugate, otherwise they must be eliminated (precipitation with EtOH, for example) before performing the assay.
[3] This specific antibody was currently bound to the plate by the intermediary of a second antibody (monoclonal mice anti rabbit antibody) directly coated on the plastic support



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

The anti-**V8** antibodies exhibited remarkable specificity toward **VMA**, cross-reactivity with substrates or byproducts was less than 0.001% [13]. The detection limit was found to be 5 nM for **VMA** (estimated precision, intra- and inter-assay repeatability < 10%). This allowed us to cover a large array of reaction conditions using up to 10 mM substrate without any interference. To validate our technique, we compared the kinetics of the uncatalysed **VMA** formation using Cat-EIA and HPLC detection at pH = 7.4. Measurements of second-order rate constants by the two methods proved to be identical (Cat-EIA: $k_{uncat} = 5.7 \pm 1.2 .10^{-9}$ $\mu M^{-1}.h^{-1}$; HPLC: $k_{uncat} = 5.7 \pm 0.3 .10^{-9}$ $\mu M^{-1}.h^{-1}$). An example of a kinetic study using Cat-EIA detection is shown in figure 2.

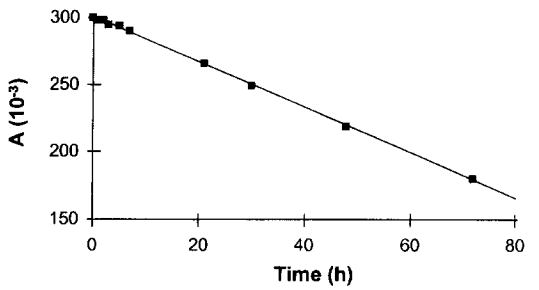


Figure 2.
Uncatalyzed reaction between guaiacol (100 µM) and glyoxylic acid (1 mM) in 0.1 M phosphate buffer (pH 7.4).**VMA** was detected by means of Cat-EIA. Initial rate $V_i = 0.54.10^{-3}$ µM/h.

Since the background reaction is extremely slow, product detection defines the lower limit of abzymatic activity that can be detected. Using this competitive immunoassay, we were able to monitor precisely very low levels of transformation of guaiacol into **VMA** (fig 2. time = 20 hours corresponds to only 0.01% yield). Under these conditions, the detection of **VMA** formed was not possible by HPLC analysis (Detection limit : 0.5 µM).

We were first faced with a complication due to the specific binding properties of the anti-**VMA** antiserum used in the Cat-EIA experiments. Although immunization was performed with hapten **V8** as a racemic mixture, we found that anti-**V8** antibodies recognized preferentially one of the optical isomers of **VMA** (affinity for S-**VMA** = 35 nM; RS-**VMA** = 48 nM; R-**VMA** = 1.17 µM) [14]. As a consequence, the rate of the catalyzed reaction could appear as low as 10% of the rate of the background reaction if the catalytic antibody catalyzes the formation of S or RS-VMA, but more than 200% if the reaction exclusively leads to the production of R-VMA[4]. Bearing this limitation in mind, spleen cells from a mouse immunized with **H6**-KLH conjugate were fused with myeloma cells. Among the resulting 2100 hybrid clones, 136 bound hapten **H6** and were assayed by Cat-EIA under different conditions (substrate concentrations, pH and temperature). A set of representative datas is given in figure 3.

[4] This phenomenon can be circumvented in our Cat-EIA strategy by using an appropriate optically active tracer.

Figure 3.
Cat-EIA results. Catalysis was performed with 100 µM guaiacol and 1 mM glyoxylic acid at pH 7.4 in 0.1 M phosphate buffer at 25°C. Similar results were obtained with 100 nM guaiacol and 10 mM glyoxylic acid at pH 9.5, 37°C; 10 µM guaiacol and 10 mM glyoxylic acid at pH 7.4, 4°C; 1 µM guaiacol and 10 mM glyoxylic acid at pH 8, 37°C. Controls were performed using 0.1 M phosphate buffer, BSA or irrelevant antibody (A54) instead of anti-**H6** antibodies.

Although 6 supernatants were clearly positive according to the Cat-EIA tests, further controls and kinetic experiments provided conclusive evidences that the Cat-EIA signal observed was not due to abzyme activity but to the contamination of culture supernatants with natural **VMA** (final metabolite of adrenaline and *Nor*-adrenaline) present in the fetal calf serum used in cell culture. This was confirmed by HPLC analysis of the corresponding supernatants (5 to 50 nM of VMA was detected). It is worth noting that, among the 136 hapten-binding antibodies, mAbs **H6**-4, 9, 32, 78, 93, 127 displayed the greatest affinity for natural VMA. It seems very likely that VMA in the supernatant was concentrated or protected from degradation by binding to the mAbs. Bound VMA was therefore removed from mAbs binding sites by dialyzing the corresponding supernatants against a dissociating medium (0.1 M citrate buffer, pH 5). After this treatment, none of these antibodies still exhibited catalytic properties.

In this paper we analyzed the advantages and the drawbacks of Cat-EIA detection of catalytic activities in hybridoma supernatants. This study demonstrated the feasability and utility of this screening approach (thousands of catalytic tests were performed under several conditions within few hours) but revealed also two important limitations of this procedure. In our opinion, these problems deserve to be seriously considered for any immunoassay (including Cat-ELISA) detecting products with chiral centers and/or natural compounds present in biological media. This was successfully achieved for hydrolytic catalyses (part II).

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