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Detection Of Enzyme Activity At Trace Levels: A New Perspective For The Direct Screening Of Active Catalytic Antibodies.

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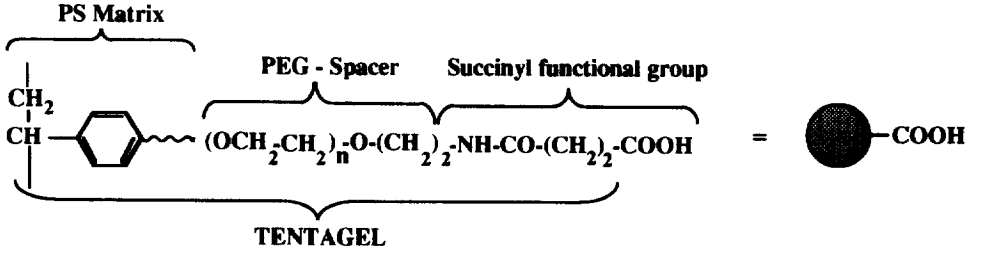
Abstract : A very low level of enzyme activity (10^{-11} mol) has been detected using a PCR method following a vector hybridisation of a supported oligonucleotide. This methodology could be very useful for direct active catalytic antibody screening from a combinatorial library.

Over the last few years, considerable effort in many laboratories has been directed towards the development of catalytic antibodies. Usually this involves the selection of antibodies against an analogue of the transition state of the reaction of interest. However, many antibodies specific to the analogue of the proposed transition state are devoid of catalytic activity. For an easy and rapid detection of activity, new highly sensitive methodologies for screening are required. Although some sensitive systems of detection have been published over the last few years, they are unfortunately unsuitable for general use. For example the cat Elisa system [1] requires antibodies specific for each desired detected product. In a similar manner, the methodology developed by Lerner and co-workers[2] requires a specific peptide-oligonucleotide for each reaction. Futhermore, the elegant screening developed by Benkovic and co-workers is applicable to only few biological reactions [3]. In our system using a cleavable disulphide bridge, the same thiopyridine-oligonucleotide can be used for screening any kind of bimolecular reaction.

We tested our screening system in a model reaction, i.e. the formation of a peptide bond between phenylalanine and leucine catalysed by α -chymotrypsin.

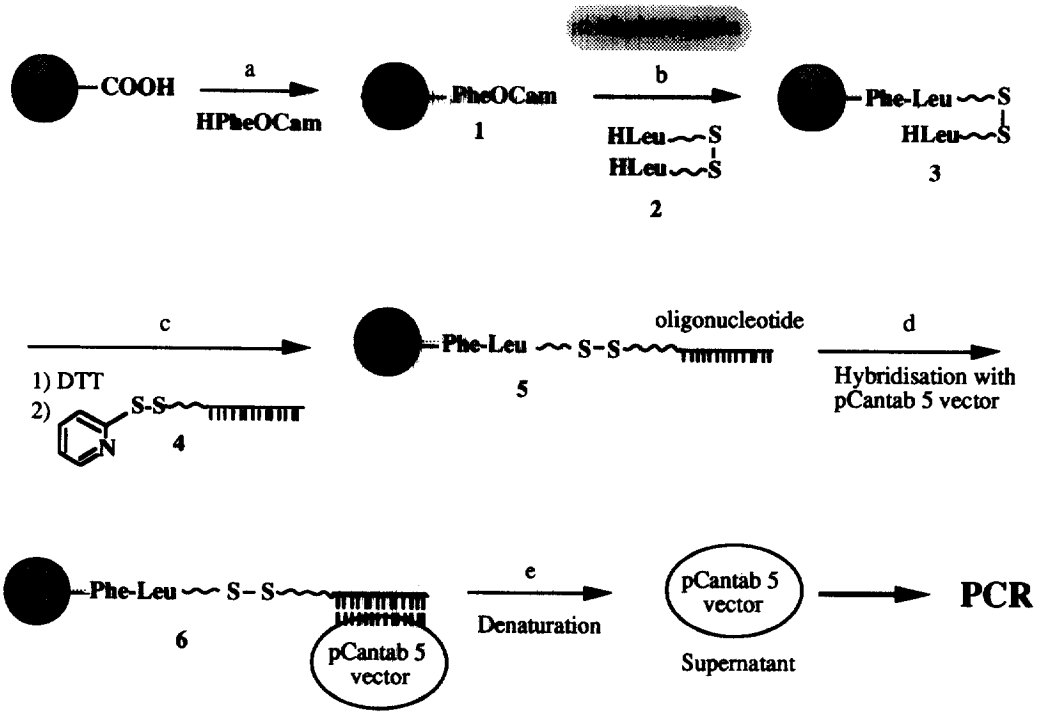
Because the use of solid phase would allow make the washing steps easier, the starting ester (HPheOCam) was chemically linked by a succinyl bridge to tentagel resin (Rapp Polymere)(Figure1) leading to the compound **1** (Figure2). This gel bears a polyethyleneglycol (PEG) spacer which allows a better substrate accessibility.

Figure 1 : Tentagel solid support



In step 2, the aminonucleophile **2** bearing a disulphide bridge (HLeu-cystamine-LeuH) [4] was linked to the resin **1** by α-chymotrypsin under kinetic control (figure 2) [5,6].

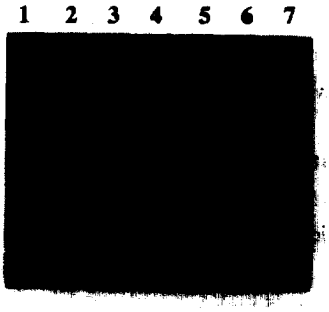
Figure 2 : General methodology to detect catalytic activity by PCR



a) Bop, DMF, DIEA, 5 hours, 20°C. b) α -chymotrypsin, carbonate buffer, pH 10, 15 min, 20°C. c) 1) DTT, Phosphate buffer, pH 7.5, 3 h, 37°C. 2) Dithiopyridine-oligonucleotide, water, 1 h, 37°C. d) pCantab 5 vector, 1x SSC buffer, 15min, 85°C, then 1 h at room temperature. e) water, 3 min 94°C, then immediately 4°C, centrifugation 2 min 12000g.

The resulting product **3** bearing the peptide bond was then subjected to reduction of the disulphide bridge by DTT yielding a free terminal thiol group [7]. An activated thiol derivative 30-mer oligonucleotide **4** (Appligen), able to link specifically via a disulphide bridge to the supported thio-peptide, was then added [8]. The reaction leads to the formation of the supported product **5** presenting the accessible oligonucleotide. We then hybridised **5** with pCantab 5 vector **6** (Pharmacia) [9]. After repeated washings to remove unhybridised vector, the product **6** was dissociated from the gel by heating (denaturation step [10]) and amplified by PCR method (figure 3) [11].

Figure 3 : PCR product analysis



lane 1 : 100 base-pair ladder (Pharmacia).
lane 2 : negative control, all the reaction is performed without α-CH
lane 3 : positive control, PCR on 1 pmol of pCantab 5 vector.
lane 4 : PCR product of coupling reaction with 10^{-13} mol of α-CH
lane 5 : PCR product of coupling reaction with 10^{-12} mol of α-CH
lane 6 : PCR product of coupling reaction with 10^{-11} mol of α-CH
lane 7 : PCR product of coupling reaction with 10^{-10} mol of α-CH

Our results (lane 6 figure 3) show an easy detection of the activity of 10^{-11} mole of α- chymotrypsin. Our methodology is very simple, rapid and inexpensive. The detection sensitivity should be increased by using more efficient DNA staining procedures. We are developing a system which will allow to detect catalytic antibodies among several hundred clones from a combinatorial library.

Abbreviations : Aca = ε-aminocaproic acid, Cam = carboxamidomethyl ester, PEG = Polyethyleneglycol, PS = Polystyrene, α-CH = α-Chymotrypsin, SSC buffer = sodium chloride/ sodium citrate buffer.

References and Notes:

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4. HLeu-cystamine-LeuH preparation: Cystamine dichloride (12mmol) was dissolved in 100 ml of CH2Cl2. After cooling to 0°C, triethylamine (34 mmol) was added followed by the addition of Boc-Leu-Aca-OH and BOP reagent (22mmol). The reaction was stirred for 5 hours at room temperature and monitored by TLC. The mixture was concentrated under vacuum. This residue was extracted with a mixture of ethyl acetate and 10% citric acid. The aqueous solution was washed with 5% $NaHCO_3$. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. (Boc-Leu-Aca-CH2-CH2-S-)$_2$ was obtained cleaved with dry HCl gas. The reaction was monitored by TLC. Leucine dimer HCl (HLeu-Aca-CH2-CH2-S-)$_2$ crystals were obtained with a quantitative yield after solvent removal. 250 mHz ^{1}H N.M.R.(D_2O) ; δ ppm 1 (d, 6H, 2x $\underline{CH_3}$ (Leu)) ; 1.35-1.58 (m, 2H, C-$\underline{CH_2}$ (Aca)) 1.65-1.169 (m,4H, 2x $\underline{CH_2}$ (Aca)) ; 1.7 (m, 1H, $\underline{CH}$-(CH_3)$_2$ (Leu)) ; 1.75 (m, 2H, CH-$\underline{CH_2}$ (Leu)) ; 2.3 (t, 2H, CO-$\underline{CH_2}$ (Aca)) ; 2.9 (t, 2H, $\underline{CH_2}$-S(cystamine)) ; 3.2-3.38 (m, 2H, N-$\underline{CH_2}$ (Aca)) ; 3.6 (t, 2H, $\underline{CH_2}$-NH (cystamine)) ; 4. (t, 1H, CH α (Leu)) ; FAB: $[M+H]^+$ = 605
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6. Enzymatic coupling under kinetic control

In order to detect a very low enzymatic activity, dilutions of α-chymotrypsin ranging from 10^{-10} (2.5µg) to 10^{-16} mol (2.5pg) were used to perform the coupling reaction (figure 2, b) between HPheOCam **1** and Leucine dimer **2** (only the amount of enzyme varies, substrates and buffer stay unchanged). α–chymotrypsin was added to a suspension of (pH 8). The reaction proceeded for 15 min at room temperature and was then quenched by acidification to pH 3.0 with 1M HCl. The gel was washed several times with water .

7. The disulphur reduction was performed on the gel in 100 µl of phosphate buffer at pH 8 over 3 hours using 200 µmol of DTT per 10 µmol of disulphide bridge.
8. Peptide-oligonucleotide synthesis: 10 µl of dithiopyridine-oligonucleotide (1 nmol) solution were added to the supported thio-peptide. Then the mixture was heated to 65°C for 15 min and one hour at 37°C, unreacted thio-oligonucleotide was removed by washing the gel with water (5 times).
9. Hybridisation : the pCantab 5 vector (10^{-12} mol) was added to 20ml of 1xSSC buffer containing the peptide-oligonucleotide gel. After heating the reaction mixture at 85°C for 4 min, the sample was leaved at room temperature for 1 hour and was washed carefully 3 times with 1x SSC (sodium clhoride/sodium citrate solution) and once with 0.5x of SSC.
10. The denaturation step was carried out in 15 µl of water at 94°C for 4 min.
11. PCR (polymerase chain reaction): the amplification of the pCantab 5 vector DNA was done by polymerase chain reaction (PCR) using a thermostable DNA polymerase. 15µl reaction mixture was prepared with : 5µl supernatant obtained after the denaturation step, 1.5 µl of each dNTP (2.5mM), 0.75 µl of Taq polymerase buffer (20x), 0.3 µl of RS primer (RPAS Pharmacia), 0.2 µl of ReplithermTaq polymerase (Epicentre), and 7.25 µl of water. Each 15 µl of reaction mixture was overlaid with paraffin oil and subjected to 35 rounds of temperature cycling with a Minicycler MJ Research programmable heating block. A typical cycle was 1 min at 94°C (denaturation), 2 min at 55°C (annealing), and 2 min at 72°C (extension). After 35 cycles the results were analyzed on an 1.5% agarose gel. The negative control (lane 2, figure 3) corresponds to a PCR performed on the supernatant obtained without α-chymotrypsin catalysis, all other steps being strickly similar to those described in figure 2. A positive control was performed by PCR on 1pmol of pCantab vector (lane 3, figure 3).

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