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SELECTION OF THE MOST ACTIVE ENZYMES FROM A MIXTURE OF PHAGE-DISPLAYED β-LACTAMASE MUTANTS.

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Abstract: A mixture of phages displaying the wild type and four mutant Rtem ß-lactamases was submitted to three rounds of selection by incubation with a biotinylated suicide inhibitor followed by chromatography on streptavidin coated beads. The final mixture of selected phages was shown to be made up of two third of phages displaying the most active enzyme, present initially as a minor component and 25 percent of a mutant with a five fold lower activity. This technique allows to select for catalytic efficiency.

One of the goals of protein engineering is to produce enzymes with new properties and catalytic activities. The implementation of efficient random mutagenesis techniques allows to generate large libraries of mutants among which it is hoped that one or a few proteins will have the desired properties. The possibility of displaying enzymes on phage and of selecting rare phage-enzyme variants from huge libraries opens new avenues to find interesting enzymes. Phage display provides a very high sensitivity because selected phages can be replicated at will to give enough material for characterisation and recloning. However, the overall efficiency of the technique depends on the design and the application of suitable and effective selection protocols (for a recent review see ref. 1).

The first phage-enzymes displaying alkaline phosphatase [2] or trypsin [3] were shown to bind to immobilised inhibitors. Selection by binding to inhibitors can be useful to find enzymes with new specificities if inhibitors of new appropriate structures are used. Binding to phosphorothioate substrate analogs was used to select staphylococcal nuclease mutants obtained by error-prone PCR mutagenesis; an enrichment for binders was observed after each mutagenesis/selection cycle; however, most polymutants isolated and characterised had properties very similar to the wild type enzyme or a decreased activity, only one mutant featured a two-fold increase in k_{cat}/Km [4]. Binding to a relatively poor transition state analog was used to find mutants of phage displayed glutathione transferase with modified specificity; although no clear enrichment could be observed by counting the number of eluted phages *versus* input, more or less conserved sequence were found in the selected mutants but the catalytic activity of the isolated mutants was strongly reduced compared to the wild type enzyme [5]. As pointed out by Light and Lerner [4], there is a clear need for selection schemes for catalytic mechanism [6].

We have reported previously on a technique of selection by catalytic activity [7]. A mixture of phages displaying active ß-lactamase and an inactive mutant was incubated first with a biotinylated suicide inhibitor, then with streptavidin coated beads, the immobilised phages were recovered and analyzed; the mixture was shown to be enriched in active phage-enzymes. Here, we move one step further using the same technique by showing that we can select the most active phage-enzymes from a mixture.

Material and methods.

The construction of the phage displaying the wild type (wt) Rtem-ß-lactamase (fd-Bla$^+$) and the inactive S70A mutant has been described previously [7] ; the enzyme is connected to the minor coat protein g3p by a peptide linker containing the IEGR sequence recognized by the specific protease factor Xa. The other mutants were prepared by oligonucleotide extension mutagenesis according to the method of Kunkel [8] on the single stranded DNA of fd-Bla$^+$.

The ß-lactamase activity of the phage-enzymes was measured by following the decrease in absorbance at 232 nm of a 0.5 mM benzylpenicillin solution in 50 mM phosphate buffer at pH 7 [9]. The concentration of phage solutions was determined from their absorbance at 265 nm taking as molar extinction coefficient 1.12 x 10^8 M^{-1} cm^{-1}; this value was calculated by adjusting the value reported for phage fd [10] for the difference in size between fd of fd-Bla$^+$.

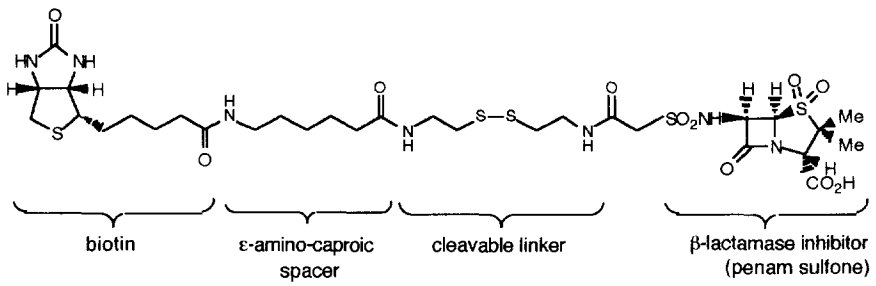
Selection of active phages. The mixture of phages ($\approx 4 \times 10^{12}$ ϕ/ml) was incubated at pH 5 in 1 ml of 50 mM acetate buffer containing 1% Bovine Serum Albumin (BSA) with the biotinylated inhibitor **1** ($\approx 2.10^{-5}$ M) after a time (tI), the labelling was stopped by addition of excess non biotinylated inhibitor **2** (2.10^{-4} M); the phages were precipitated twice (2% PEG 4000, 0.5 M NaCl) to remove the inhibitors and resuspended in 500 µl of 50 mM tris buffer at pH 7. On the other hand, streptavidin coated magnetic beads (from Dynal) were washed three times with 1 ml tris buffer and left in tris containing 2% skimmed milk for one hour. 50 µl of beads were then added to 450 µl of the phages solution; the mixture was incubated for one hour under mild stirring. After extensive washing (10 times) with 1 ml of Tris buffer containing 0.1% of Tween-20, the immobilized phages were recovered by proteolytic cleavage of the factor X_a site in the peptide connecting the enzyme to the minor protein g3p (10 units of factor X_a/ml for 4 hours in 50 mM Tris at pH 8, 100 mM NaCl, 1 mM $CaCl_2$). These phages were replicated by infection of an *E. coli* TG1 culture under exponential growth ($A_{570} \approx 0.5$).

Results and discussion.

Three mutants of the Rtem-ß-lactamase with reduced activity were prepared. In the first two mutants, Thr 71, in the vicinity of the essential serine 70, was converted to alanine or valine. These mutations have been shown to reduce the ampicillin resistance of Rtem-ß-lactamase producing bacteria [11]. In the third mutant, Val 103 and Tyr 105 were replaced respectively by isoleucine and phenylalanine. These two residues are located in a loop on the rim of the active site [12,13]; they are relatively well conserved [14]. The Y105F mutation has been shown to lead to a slight increase in k_{cat} and a two fold decrease in k_{cat}/Km [15]. The second mutation (V103I) is expected to lead to a further decrease in activity.

Phages displaying the wild type and the mutant enzymes were prepared and their lactamase activity was measured with benzyl-penicillin as substrate. The specific activities were obtained by dividing the experimental activities by the phage concentrations. These are, however, quite sensitive to the conditions in which the phages have been produced, among other reasons, because there is a temperature dependent proteolytic disconnection of the enzyme from the phage. The relative specific activities, are less sensitive to these conditions; the following values were obtained (mutant : specific activity *versus* wild type) : V103I-Y105F : 23%, T71V : 16%, T71A : 4%, S70A : 0% .

A mixture of phages made up of 24-25% of each of the four mutants above and ≈ 2% of wild type was incubated with the biotinylated ß-lactamase suicide inhibitor **1** [16] (scheme 1) to label the active ones; the labelling was stopped by addition of excess non biotinylated inhibitor **2** and the labelled phages were immobilized on streptavidin coated magnetic beads, then eluted by specific proteolytic cleavage of the peptide connecting the enzyme to the phage with factor X_a. The procedure was repeated twice identically except that the time of labelling was shortened from 15 to 8 and 5 minutes for rounds 2 and 3.



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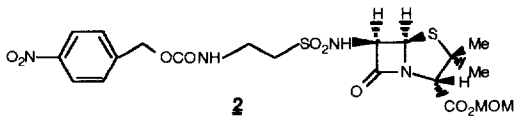

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Scheme 1

After each round of selection, the percentage of phages recovered was determined by titration on plates[17] of the tetracycline transducing units in the loaded and eluted solutions; this percentage did not increase significantly between rounds presumably because the labelling time was shortened so that the extent of labelling of the active phages was decreased. The specific activity of the selected mixture of phages was also measured after amplification. It increased regularly after each round from 43 s^{-1} for the starting mixture to 95 s^{-1}, 220 s^{-1} and finally 390 s^{-1}.

The analysis of the final mixture was performed by first painting the individual colonies growing on tetracycline plates with a solution of nitrocefin (10^{-3} M in pH 7 buffer / 5% DMSO). Colonies producing the wild type phage-enzyme and the V103I-Y105F mutant turn red relatively quickly, the first ones somewhat faster; the other ones remain white longer. 125 out of 140 colonies (90%) were found to turn red. Ten of these clones were picked for sequencing: 7 of them were found to contain the wild type gene and 3 the V103I-Y105F mutant. Overall, the wild type phages make up 63% and the double mutant 27% of the final mixture.

It is interesting to compare the enrichment observed with that calculated by assuming that the extent of labelling of the phages by the penicillin sulfone inhibitor under competitive conditions will be proportional to the activity versus the corresponding penicillin substrate. The contribution of each phage to the relative activity of the mixture (*versus* wt) is first calculated as the product of its relative activity by its mole fraction. The percentage of labelling of a phage is then given by the ratio of this contribution to the sum of the contributions (Table 1). The observed percentages after 3 rounds of selection are slightly better than the theoretical limit after 2 rounds for the wt and double mutant (in bold). Thus, this technique allows to select for catalytic efficiency.

Table 1.

Selection round	Phages	Wild type	V103I-Y105F	T71V	T71A	S70A	Σ(Relative activities)
0	Fraction in mixture	0.02	0.245	0.245	0.245	0.245	
	Contribution Rel. Act.	2	5.6	3.9	1.0	0.0	12.5
1	Fraction in mixture	0.16	0.448	0.312	0.08	0.0	
	Contribution Rel. Act.	16.0	10.3	5.0	0.32	0.0	31.6
2	Fraction in mixture	**0.506**	**0.326**	0.158	0.01	0.0	
	Contribution Rel. Act.	50.6	7.5	2.53	0.04	0.0	60.7
3	Fraction in mixture	0.834	0.124	0.042	0.0	0.0	
	Contribution Rel. Act.	83.4	2.85	0.64	0.0	0.0	86.9

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