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Enzyme Microarrays: On-Chip Determination of Inhibition Constants Based on Affinity-Label Detection of Enzymatic Activity

Jörg Eppinger*, Daniel P. Funeriu*, Masato Miyake, Lucile Denizot, Jun Miyake

Dr. J. Eppinger: ForschungsDozentur Molekulare Katalyse Lehrstuhl für Anorganische Chemie, Technische Universität München, Lichtenbergstr. 4, 85748, Garching. Germany, Tel. +49 (89) 289 13097, E-mail: joerg.eppinger@ch.tum.de

Dr. D.-P. Funeriu, Dr. M. Miyake, L. Denizot, Dr. J. Miyake: Tissue Engineering Research Center, National Institute of Advanced Industrial Science and Technology, 3-11-46 Nakouji, Amagasaki, Hyogo, 661-0974, Japan. Tel. +81 (6) 6494 7809, E-mail: danielpetru-funeriu@aist.go.jp

*These authors contributed equaly to this work. Correspondence to joerg.eppinger@ch.tum.de or danielpetru-funeriu@aist.go.jp

I) ***Synthesis of 2***

1 mg of epoxy-peptide **1** was dissolved in 1 mL of dry DMF containing 5 % DIPEA and added into a vial of 1mg NHS-CY3 (Amersham). After 1 h at RT the reaction mixture was purified by HPLC on a C18 column, with a linear gradient of acetonitrile/water mixtures.

II) ***Microarray preparation***

papain (Merck) was microarrayed on Hydrogel-NHS functionalized glass slides (NoAb Diagnostics, CA) at a 0.5mg/mL in PBS at 86% humidity and incubated for 1 h. The surface was blocked with 2% BSA in PBS pH 6.48 (PBSA) for 10 minutes and the microarray was dip-washed with PBSA and ddH_2O and dried by vacuum suction.

III) ***Post-printing processing***

MA1: Each sub-array was pre-incubated with 8µL RB (2mM DTT in PBSA) for 40 min. Optimization experiments have shown that the results obtained are independent of the pre-incubation time, provided that it is longer than 15 minutes and shorter than 1 hour. After the preincubation, 4µL of a solution of FAL **2** in RB is added, at 4 different stock concentrations (3X, X, 0.3X, 0.1X), with X=5µM. The final concentrations of FAL **2** are 5, 1.66, 0.5, 0.166 µM. After the appropriate time (see figure 2) the reaction in each sub-array is stopped by flow washing with 200µL of SDS buffer, pH 9. In a control experiment, it was proven that no reaction proceeds between the microarrayed enzyme and the FAL 2 in SDS buffer. After all the sub-arrays are treated, the entire slide is washed with SDS, 70°C for 3 min under vigorous shaking and sonicated for 2 min, then abundantly washed with ddH_2O and dried by centrifugation at 2000rpm. Repeated washings under these conditions resulted in no significant differences in fluorescence. The obtained microarray was scanned using an ArrayWorks microarray and the fluorescence, quantified using Imagene software (supplementary material IV). Further data analysis was performed as described (supplementary material V).

MA2: Each sub-array was pre-incubated with 8µL RB (2mM DTT in PBSA) containing the appropriate concentration of leupeptin (56µM, 5.6µM, 0.56µM, 56nM) for 1hour. After the preincubation, 4µL of a solution of **2** in RB at 1.5µM is added. Final concentrations are: leupeptin 37.3µM, 3.73µM, 373nM, 37.3nM; FAL **2** 0.5µM. After the appropriate time (see figure 3) the reaction in each sub-array is stopped by flow washing with 200µL of SDS buffer pH 9. After all the sub-arrays are treated, the entire slide is washed with SDS, 70°C for 3 min under vigorous shaking and sonicated for 2 min, then abundantly washed with ddH2O and dried by centrifugation at 2000rpm. The obtained microarray was scanned using an ArrayWorks microarray and the fluorescence quantified using Imagene software (supplementary material IV). Further data analysis was performed as described (supplementary material V).

MA3: Each sub-array was pre-incubated with 8µL RB (2mM DTT in PBSA) containing the appropriate concentration of E-64 (560nM, 56nM, 5.6nM, 0.56nM) for 1hour. After the preincubation, 4µL of a solution of FAL **2** in RB at 1.5µM is added. Final concentrations are: E-64 373nM, 37.3nM, 3.73nM, 0.373nM; FAL **2** 0.5µM. After the appropriate time (see figure 3) the reaction in each sub-array is stopped by flow washing with 200µL of SDS buffer pH9. After all the sub-arrays are treated, the entire slide is washed with SDS, 70°C for 3 min under vigorous shaking and sonicated for 2 min, then abundantly washed with ddH2O and dried by centrifugation at 2000rpm. The obtained microarray was scanned using an ArrayWorks microarray and the fluorescence quantified using Imagene software (supplementary material IV). Further data analysis was performed as described (supplementary material V).

MA4:

Procedure 1: alternatively to procedure 1, the preincubation was realized with 8µL RB containing E-64 or leupeptin at a concentration of C for 1 hour. Then 4µL of a solution of **2** in RB at 1.5µM is added (see figure 4). Final concentrations are identical to procedure 1. After 2 minutes the reaction in each sub-array is stopped by flow washing with 200µL of SDS buffer pH9. After all the sub-arrays are treated, the entire slide is washed with SDS, 70°C for 3 min under vigorous shaking and sonicated for 2 min, then abundantly washed with ddH2O and dried by centrifugation at 2000rpm. The obtained microarray was scanned using an ArrayWorks microarray and the fluorescence quantified using Imagene software (supplementary material IV). Further data analysis was performed as described (supplementary material V).

Procedure 2: Each sub-array was pre-incubated with 8µL RB (2mM DTT in PBSA) for 1h. Then 4µL of a solution of FAL **2** in RB at 1.5µM with E-64 or leupeptin at a concentration 2C is added

(see figure 4). Final concentrations are: E-64 or leupeptin: 373µM, 124µM, 41.4µM, 13.8µM, 4.6µM, 1.53µM, 512nM, 170nM, 57nM, 19nM, 6.32nM; FAL **2** 0.5µM. After 2 minutes the reaction in each sub-array is stopped by flow washing with 200µL of SDS buffer pH 9. The obtained microarray was scanned using an ArrayWorks microarray and the fluorescence quantified using Imagene software (supplementary material IV). Further data analysis was performed as described (supplementary material V).

IV) ***Scanning and Imagene analysis***

The microarrays were scanned immediately after processing. Storage for 1 day at room temperature in the dark did not significantly affect the obtained data. The main scanner settings were the following: exposure time: 0.8 seconds, High Dynamic Range mode, observation wavelength: CY3-optimized. Tiff files were generated and further analyzed by Imagene software. The images presented in the text are jpeg files derived from the tiff files automatically generated after the scanning. Although visually informative from a qualitative point of view and for representation purposes, only the tiff files ought to be considered as quantitatively relevant. They are available upon written request.

The tiff files obtained above were imported in the Imagene software. The spots were detected using the automated procedure. The spots unsuitable for measurement (typically less than 1% of the spots) were rejected. Then the background and signal intensities were measured at various rejection limits. The rejection limits (the highest x% of the signal and the lowest y% of the signal rejected, only considered for analysis the X-Y interval) were chosen as a function of the data quality. Their choice does not significantly modify the quantification of the raw data; however, according to the choice operated, it provides standard deviations that are more or less relevant. Standard deviations reported were obtained with settings that take into account almost entire spot and background regions.

V) ***determination of kinetic constants***

Quantification of the fluorescence data was achieved via a standard curve. This standard curve was derived by scanning a standard slide, on which different concentrations of the FAL **2** were spotted (figure SM1). Application of the equation (standard curve) $y = 4.122 * x^{1.064}$ (y: mean fluorescence signal of a spot minus the average background around this spot, x: molar amount of fluorescent label) allowed the direct conversion of fluorescence intensities into absolute concentrations.

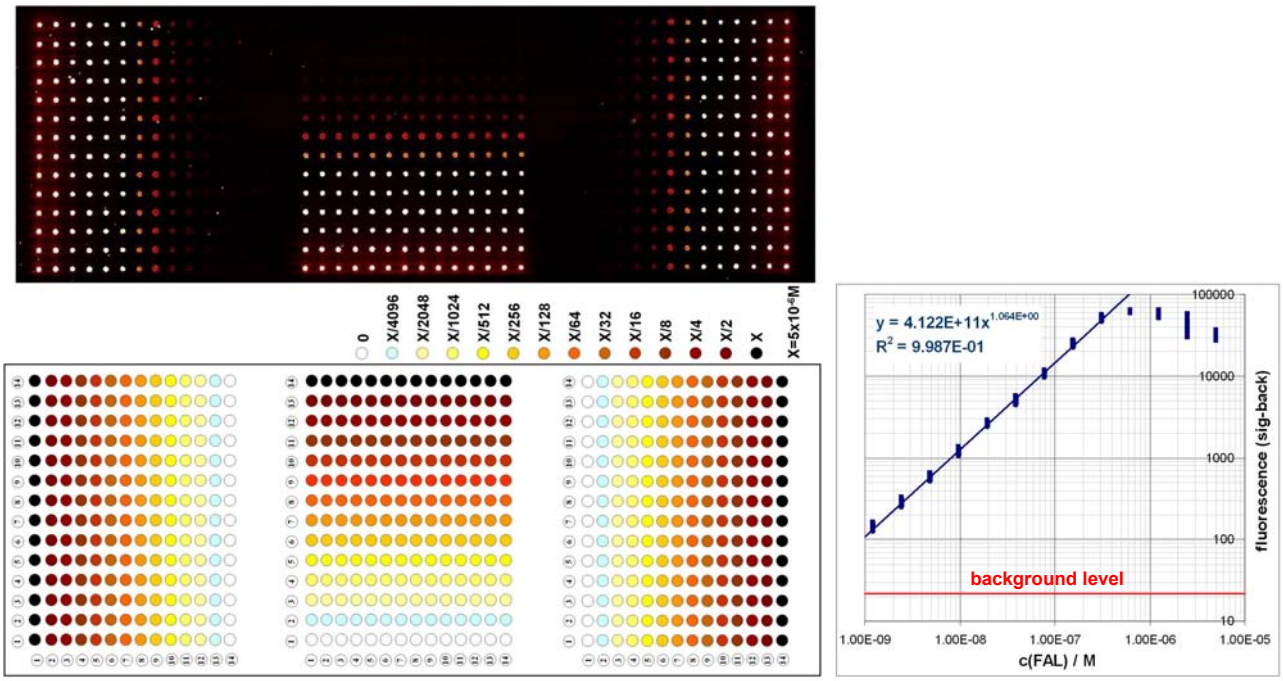

Figure SM1: Quantification of fluorescence data via a standard curve derived from a calibration slide.

Data analysis was performed by fitting of a theoretical progress curve to the measured concentrations using the program DYNAFIT (see: Refernce [9] in the article). The kinetic constants derived are listed in table SM1. For the side reaction, E_{total} was used, as the reactive groups on the enzyme surface are not consumed by the catalytic reaction (E_{total} (t = 0) = E (t = 0)).

Table SM1: kinetic constants

	k(+0) [$\mu M^{-1}s^{-1}$]	k(-0) [s^{-1}]	k(+1) [$\mu M^{-1}s^{-1}$]	k(-1) [s^{-1}]	k(cat) [s^{-1}]	k(side) [$\mu M^{-1}s^{-1}$]	k(+i) [$\mu M^{-1}s^{-1}$]	k(-i) [s^{-1}]	k(irrev) [s^{-1}]	c(E) [nM]
solution	---	---	3.2	1.7	0.086	5.3E-5	0.0046	0.00073	---	72
MA1	11	92	5.6	2.3	0.059 [a)]	5.3E-5 [a)]	---	---	---	2.8
MA2	11 [a)]	92 [a)]	5.6 [a)]	2.3 [a)]	0.059 [a)]	5.3E-5 [a)]	0.0019	0.00022	---	2.8
MA3	11 [a)]	92 [a)]	5.6 [a)]	2.3 [a)]	0.059 [a)]	5.3E-5 [a)]	2.1	0.79	0.12	2.9
MA4 [b)]	11 [a)]	92 [a)]	5.6 [a)]	2.3 [a)]	0.059 [a)]	5.3E-5 [a)]	0.0061	0.00061	---	2.6
MA4 [c)]	11 [a)]	92 [a)]	5.6 [a)]	2.3 [a)]	0.059 [a)]	5.3E-5 [a)]	0.94	0.33	0.043	2.6

[a)] Not refined in this experiment. [b)] Constants for leupeptin inhibition derived from the co-incubation experiments. [c)] Constants for E-64 inhibition derived from the co-incubation experiments.

The rate constants are attributed to the single processes in the mechanism as follows:

(I)	I	$\rightleftharpoons$	I_{in}	k(+0), k(-0)
(II)	S	$\rightleftharpoons$	S_{in}	k(+0), k(-0)
(III)	$E + S_{in}$	$\rightleftharpoons$	ES	k(+1), k(-1)
(IV)	ES	$\longrightarrow$	P	k(cat)
(V)	$E_{tot} + S_{in}$	$\longrightarrow$	P	k(side)
(VI)	$E + I_{in}$	$\rightleftharpoons$	EI	k(+i), k(-i)
(VII)	EI	$\longrightarrow$	X	k(irrev)

Equilibrium (I) accounts for the diffusion limitation of the reaction of the free-floating **FAL** with the immobilized enzyme. Thus, I_{in} and S_{in} stand for the apparent concentration of inhibitor and substrate (**FAL**) inside the enzyme spot. Therefore k(+0) and k(-0) can thus be rationalized as the constants characterizing the diffusion of small molecules into the microarrayed spot of densely packed enzymes. It is a rough estimation, that for substrate and inhibitor the same diffusion constants can be applied. A model with different constants for substrate and inhibitor did not yield in an improved the goodness of fit and was thus rejected. For the reversible inhibitor including process (VII) expectedly results in a decreased goodness of fit. A simplification of the model resulted in a much worse goodness of fit parameter in all cases tested, as there are:

1) first order kinetics for S (E + S → P)
2) first order kinetics for S including side reaction (V)
3) Michaelis-Menten derived kinetics without diffusion limitation for S and I without side reaction (omitting processes (I), (II) and (V))
4) Michaelis-Menten derived kinetics without diffusion limitation for S without side reaction (omitting processes (II) and (V))
5) Michaelis-Menten derived kinetics without diffusion limitation for I without side reaction (omitting processes (I) and (V))
6) Michaelis-Menten derived kinetics without diffusion limitation for S and I (omitting processes (I) and (II))
7) Michaelis-Menten derived kinetics without diffusion limitation for S (omitting process (II))
8) Michaelis-Menten derived kinetics without diffusion limitation for I (omitting process (I))
9) First order reaction of the Inhibitor (omitting process (VI))

Technically, the terms S (Substrate) k_{cat} and V_{max} are defined for a catalytic reaction. However, the FAL reacts irreversibly with the enzyme. Thus, the k_{cat} is the kinetic constant of the irreversible formation of fluorescent product out of the enzyme-FAL complex. V_{max} is the maximal speed of the inactivation that can be reached Nevertheless, as the model applied is Michaelis Menten derived, the values of these constants are similar to those, which result from an truly catalytic assay. Only k_{cat} should display a small deviation, as the dissociation of the product is not included.

characteristic inhibitor constants were calculated as follows:

leupeptin: $Ki = k(-i)/k(+i)$
E-64: $k_{2nd} = k(cat)* k(-i)/k(+i)$
FAL: $k_{2nd} = k(cat)* k(-0)/k(+0)$

For the case of the analysis of the pre-incubation experiment, Ki can be derived from the initial velocities only, because the following conditions are fulfilled:

1) the FAL (c = 0.5 μM) is used at a concentration below its reversible binding constant (K_B(FAL) = 3.5 μM) , and
2) the percentage inhibition is measured at a time point prior to substantial inactivation of the enzyme by the FAL probe (maximal deactivation of 35 % of the enzyme within the 2 minutes time period of the reaction).

The side reaction that has to be introduced in order to account for the observed data is likely the result of a nucleophilic attack on the epoxyde moiety of FAL. Since the NH2 terminated compound **1** is chemically stable, we conclude that the attack is not by the NH2 group of lysines on the surface of the enzyme. According to our estimations, and consistent with E-64 active site titrations performed in solution (Supplementary Material VI) about 10% of the surface bound papain is active. Even though papain does not have other SH groups, the "inactive" SH groups from inactive chip-bound papain account for 90% of the total SH groups. Therefore, we consider as justified our hypothesis that the side-reaction can be attributed to attack on the FAL by a thiol group. In fact, the residual reactivity of heat denatured papain, which was spotted in a control experiment corresponds well with the observed background reactivity of the active enzyme.
More generally, when non-cystein protease enzymes are microarrayed and treated with cysteine-protease specific FAL, after a long incubation time (> 60min), very weak fluorescence can be observed at the addresses on which these enzymes were microarrayed. The level of this very weak fluorescence is enzyme-dependent but the reaction responsible for this signal does not appear to be of enzymatic type. This clearly shows the importance of high signal/background ratios as well as one of the possible confusions that could be made with this method: mere appearance of (weak) signal does not necessarily mean enzymatic activity. This must be confirmed by careful kinetic analysis.

VI) ***quantification of the reaction of FAL 2 with papain and leupeptin inhibition in solution***

400μL of solution of 0.005 mg/mL papain was incubated for 1 hour with either RB, RB with leupeptin 56nM, RB with leupeptin 560nM or RB with leupeptin 5600nM. Then 200 μL of a solution of FAL **2** at 150nM in PBSA was added. Final concentrations: active papain (determined by E-64 titration) 25nM, FAL 2: 50nM, Leupeptin: 3733nM, 373.3nM, 37.3nM. 15μL Aliquots of each reaction mixture were quenched at 5, 15, 30, 60, 120, 180, 300, 420, 840, 1380 seconds by rapid mixing with 5μL of Invitrogen 4X NuPAGE LDS sample buffer at 98 °C and heated at 98 °C for 10 min. Each sample was loaded on 4-12% Bis-Tris gel. The fluorescent bands on the gel (figure SM1) were quantified using a fluorescence scanner. The obtained data was fitted as explained in the supplementary material V.

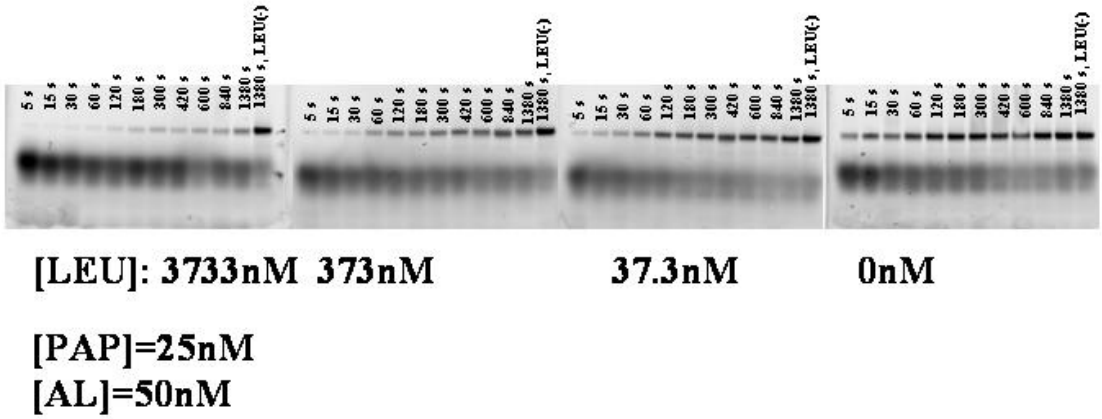

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Figure SM1: Fluorescence images of the gels obtained after solution reactions with different concentrations of leupeptin and FAL **2**.