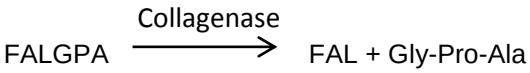
Protocols for Internal reaction

Enzymatic assay of Collagenase

Principle



Abbreviations used:

FALGPA = N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala
FAL = N-(3-[2-Furyl]Acryloyl)-Leu

Reagents:

1. 50 mM Tricine Buffer with 10 mM Calcium Chloride and 400 mM Sodium Chloride. The pH is adjusted to 7.5 at 25 °C with 1 M NaOH.
2. 1.0 mM N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala Solution (FALGPA). The needed volume of substrate is prepared in Reagent 1. Approximately 30 minutes of stirring is required for this product to dissolve completely.The pH is adjusted to 7.5 at 25 °C with either 1 M NaOH or 1 M HCl.
3. Collagenase Enzyme Solution

Procedure:

The following reagents are pipetted into suitable cuvettes:

	Test	Negative control
Reagent 2 (FALGPA)	2.90 ml	2.90 ml
Deionized Water	------	0.10 ml

The solution is mixed by inversion and equilibrated to 25 °C. A_{345nm} is monitored until constant, using a suitably thermostatted spectrophotometer. In the following step it is added:

	Test	Negative control
Reagent 3 (Enzyme Solution)	0.10	------

The reaction is immediately mixed by inversion and the decrease in A345nm is recorded for approximately 5 minutes. Using the maximum linear rate for both the Test and the Negative control the ∆A345nm/minute is obtained.

Calculation:

Units/ml enzyme = $\frac{(\Delta A345nm/min\ Test - \Delta A345nm/min\ Negative\ control)(3)(df)}{(0.53)\ (0.1)}$

3 = Total volume (in milliliters) of assay

df = Dilution factor

0.53 = Millimolar extinction coefficient of FALGPA at 345 nm2

0.1 = Volume (in milliliters) of enzyme used

Units/mg protein = $\frac{units/ml\ enzyme}{mg\ protein/ml\ enzyme}$

Unit definition:

One unit hydrolyzes 1.0 μmole of furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) per minute at 25°C at pH 7.5 in the presence of calcium ions.

Final assay concentration:

In a 3.00 ml reaction mix, the final concentrations are 48 mM tricine, 9.7 mM calcium chloride,
387 mM sodium chloride, 0.97 mM N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala.

Reference:

Van Wart, H.E., and Steinbrink, D. R. (1981) Analytical Biochemistry 113, 356-365

Conjugation of Collagenase to TAMRA amine-reactive fluorescent dye

Reagents:

1. 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE), Invitrogen
2. Collagenase from *Clostridium hystoliticum* , Sigma, Type V

Labeling protocol:

1. 10 mg of enzyme are dissolved in 1 ml of 0.1M sodium bicarbonate buffer, pH 8.3.
2. 5 mg of dye are dissolved in 500 μl of DMSO. The dye should be dissolved immediately before the reaction. The solution is protected from light.
3. While vortexing the protein solution, 100 μl of the TAMRA solution is added slowly. This corresponds to 1 mg of dye into 10 mg of protein. TAMRA NHS ester is amine-reactive dye and reacts with non-protonated aliphatic amine groups, including the amine terminus of proteins and the ϵ-amino group of lysines.
4. The reaction is incubated for 1h at room temperature with continuous stirring and protected from light.
5. Separating the conjugate from the unreacted dye is performed using Sephadex G-25 size exclusion column. The first excluded fraction which corresponds to the first fluorescent band to elute is the conjugate.
6. The protein concentration is determined after collecting the fractions. The protein-dye conjugate is diluted 100 times. The absorbance of the diluted sample is measure at 280 nm and at 555 (the excitation maximum) .
7. The protein concentration is determined using the following formula:
   $A_{protein} = A_{280} – A_{555}(CF)$

   CF is the correction factor used to correct for the contribution of the dye to the absorbance at 280nm.

   The protein concentration is calculated assuming that 1.7 $A_{protein}$ units = 1mg/ml. This value is different for each protein and it was experimentally determined for collagenase.

8. The degree of labeling is calculated using this formula:

$$D.O.L = \frac{A\max \times MW}{[protein] \times \varepsilon dye}$$

MW = molecular weight of the protein, ε_{dye} = the excitation coefficient of the dye at its absorbance maximum, [protein] is the protein concentration in mg/ml

UPLC measurements

The detection of reaction products were performed with ACQUITY Ultra Performance Liquid Chromatography system, using BEH C18 columns. The detection limit of this system is 10 μM. The retention time for the substrate and the reaction products were obtained, after elution with water.For the cases when UPLC measuremnts were performed with nanoreactors, filtration of the samples were performed with 0,22 μm filters in order to remove the polymersomes from the solution.

Confocal imaging

The confocal images were taken using Zeiss LSM 780/FCS confocal microscope. The samples were pipetted into a PCR tube cap and then placed with inversion on a coverslip. The coverslips were mounted on the microscope and images were taken, keeping the pinhole equal to 1 AU. The laser power and the gain were adjusted in a way that best images could be obtained.

Enzymatic assay with nanoreactors

The final reaction assay with nanoreactors were performed using the tri-block and di-block polymersomes without channels as a negative control and the polymersomes with channels with different hydrophobic modifications. The reaction assay was performed for lower and

higher concntration of the polymersomes - 22 μg/ml and 100 μg/ml respectively. The reaction mixtures were incubated for the same time and samples were taken for 3h, 6h, 8h 30 min, 24 h and 48h. These samples were checked with UPLC for the presence of reaction products.