A Novel Assay for Sequence-Specific Cleavage of an Immobilized Oligopeptide

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Abstract

We have developed a novel assay for protease activity using a new assay platform developed by Meso Scale Discovery™ (MSD™). This platform combines array technologies and electrochemiluminescence detection to achieve ultra-fast, highly sensitive assays. This poster presents an assay for matrix metalloproteinases (MMPs) activity. Oligopeptides containing the cleavage sequence for a subset of MMPs and bearing an electrochemiluminescent complex were immobilized on carbon electrodes integrated into microtiter plates. Subsequent challenge of the immobilized oligopeptides with the catalytic domain of MMP3 revealed catalytic rate constants (K_{cat}/K_m) in agreement with published values. The use of the MMP3-specific inhibitor, NNGH, confirmed the specificity of cleavage. The assay is robust using as little as 1 picomole of substrate, minimizing the cost of substrate. Both the immobilized substrate and the quenched reaction are stable over many hours facilitating easy adaptation to automation. This novel platform will facilitate high-throughput discovery of protease-specific inhibitors.

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Multi-Array™ Technology

Unified technology platform with instruments, plates and reagents for drug discovery.

Combines the power of microarrays with the sensitivity of electrochemiluminescence.

96-, 384- and 1536 microplate formats.

Multi-Spot™ plates with high density arrays for multiplexing.

Sector HTS™ Instrument: High resolution imaging detection and robotic integration for HTS and large-scale proteomics.

Sector PR™ Instrument: Medium throughput benchtop reader for assay development, cellular and molecular biology, research in therapeutic areas, secondary screening, QC. Assays developed on Sector PR port to Sector HTS.

Sector HTS

Sector PR

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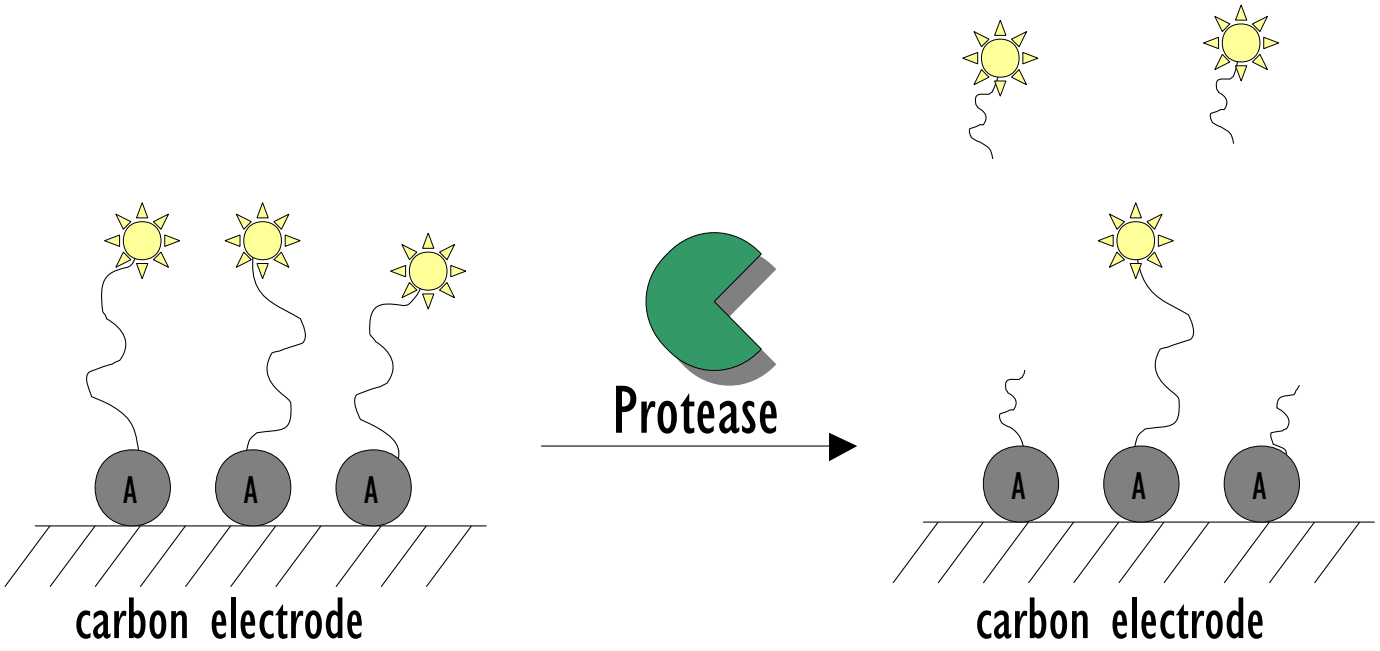
Schematic of the Protease Assay



An oligopeptide, modified with biotin at one end and an electrochemiluminescence label (a derivative of Ruthenium (II) tris-bipyridine, ☼) at the other, is immobilized to an avidin(A)-coated electrode. Cleavage of the peptide by a sequence specific endopeptidase results in a decrease in the amount of label proximal to the surface and thus a decrease in signal. The active, catalytic domain of MMP3 protease was purchased from CalBiochem.

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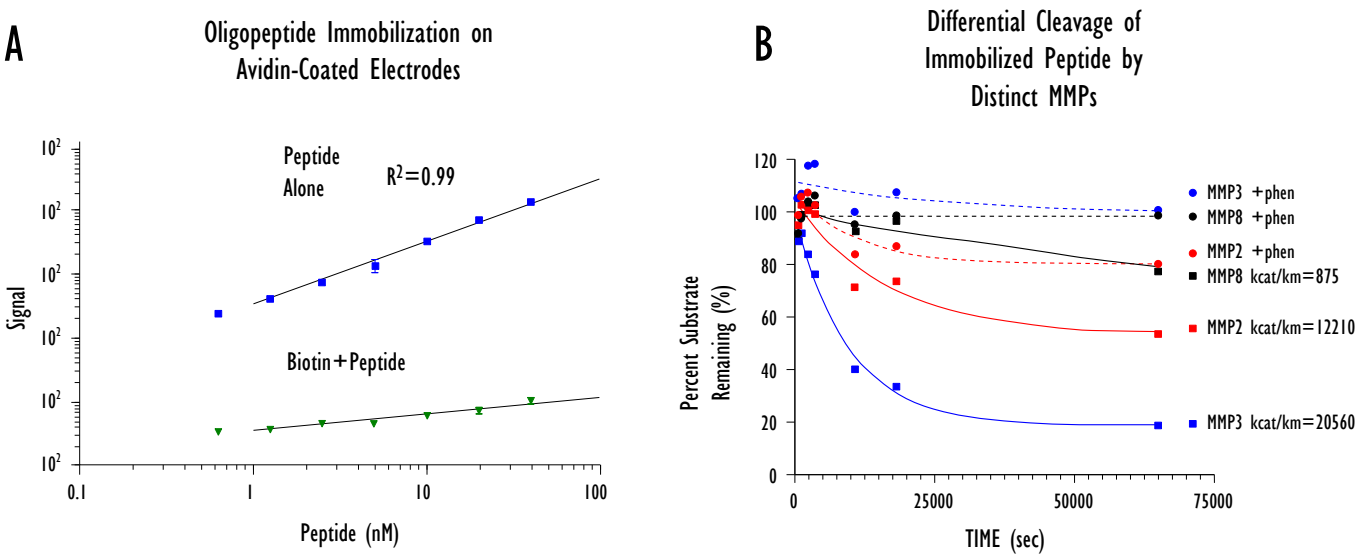
Biotin-Dependent Immobilization & Enzyme-Specific Cleavage of an Oligopeptide



A) A control experiment showed that immobilization of the peptide was biospecific (i.e. mediated by avidin/biotin interactions). Dual-labeled oligopeptide was incubated above avidin-coated carbon electrodes at varying concentrations in the absence (blue) or presence (green) of a vast molar excess of free biotin. Signal output increases linearly over the concentration range studied and correlates with quantitative immobilization of the substrate.

B) One picomole of dual-labeled oligopeptide was immobilized on avidin-coated carbon electrodes and challenged with 5nM of each enzyme in the presence or absence of 10nM 1,10-phenanthroline. The reactions were conducted in MES buffer (50mM MES pH6.0, 10mM $CaCl_2$, 0.05% Brij35,1uM $ZnSO_4$, 150mM NaCl).

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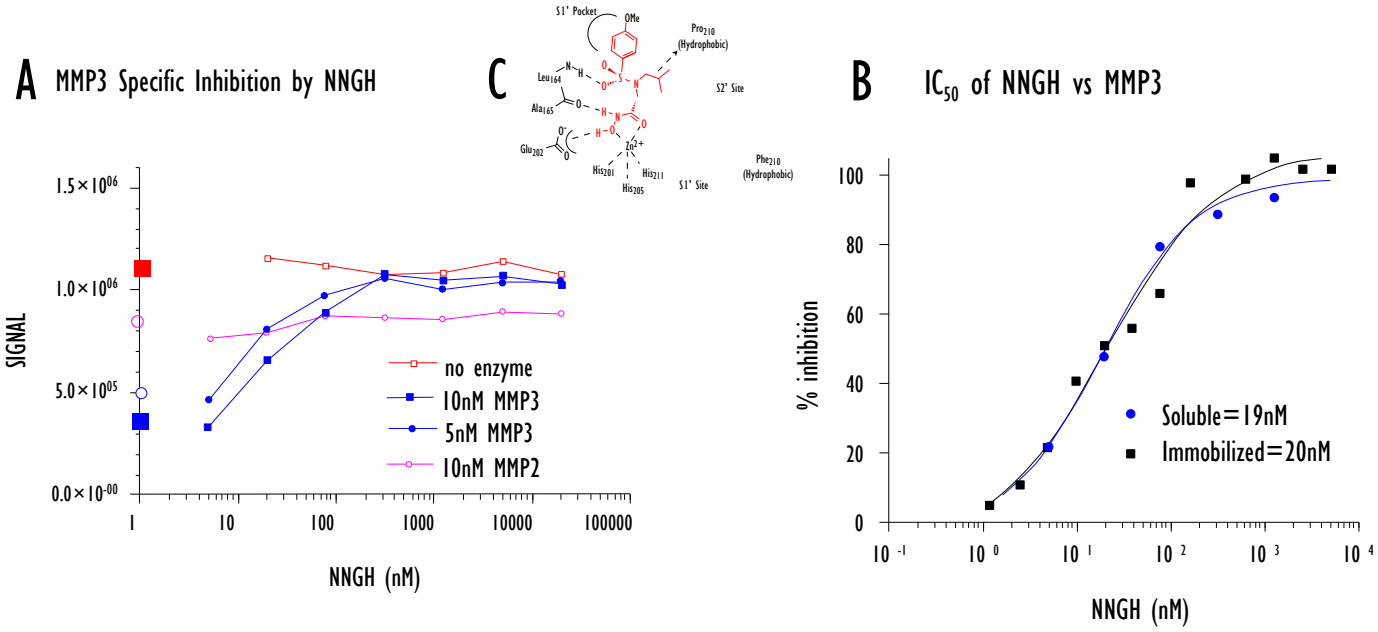
The MMP3 Specific Inhibitor NNGH Exhibits Characteristic Properties in the Assay



A) Immobilized, dual-labeled oligopeptide was challenged with MMP3 or MMP2 in the presence of varying concentrations of the known, MMP3 specific inhibitor (c) N Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH). The signals observed in the absence of inhibitor are plotted on the ordinate. Note that MMP3 is inhibited at higher concentrations, while MMP2 is not.

B) The percent inhibition observed over a range of NNGH concentrations was examined for MMP3 cleavage of the dual-labeled peptide either free in solution or immobilized to avidin-coated carbon electrodes. The IC_{50} values are in agreement.

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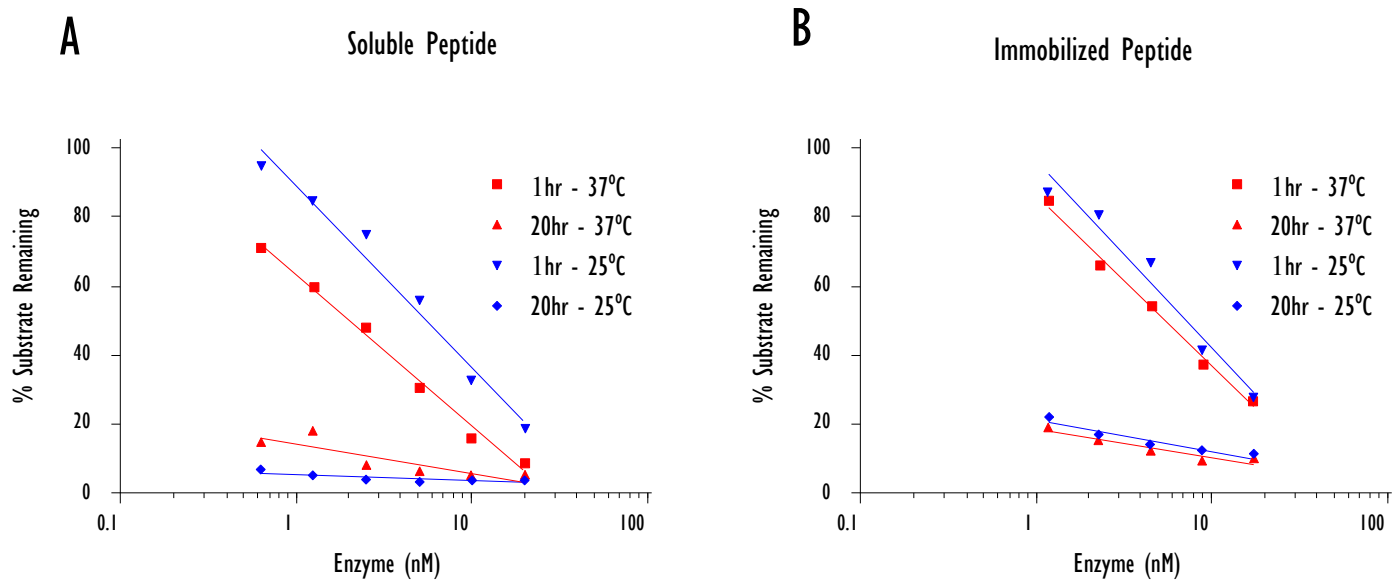
Enzyme Kinetics are Unaltered by Substrate Immobilization



The dual-labeled oligopeptide was incubated with active MMP3 enzyme either in solution (A) or after being immobilized through binding to an avidin-coated carbon electrode followed by incubation with the enzyme (B). In A, intact peptides and cleaved fragments were captured to the electrode after the cleavage reaction. The percentage of substrate remaining is plotted as a function of enzyme concentration at 25°C and 37°C after 1hr or 20hrs. For both the soluble and immobilized substrates, the rate of substrate consumption is linear over the range of enzyme concentrations studied.

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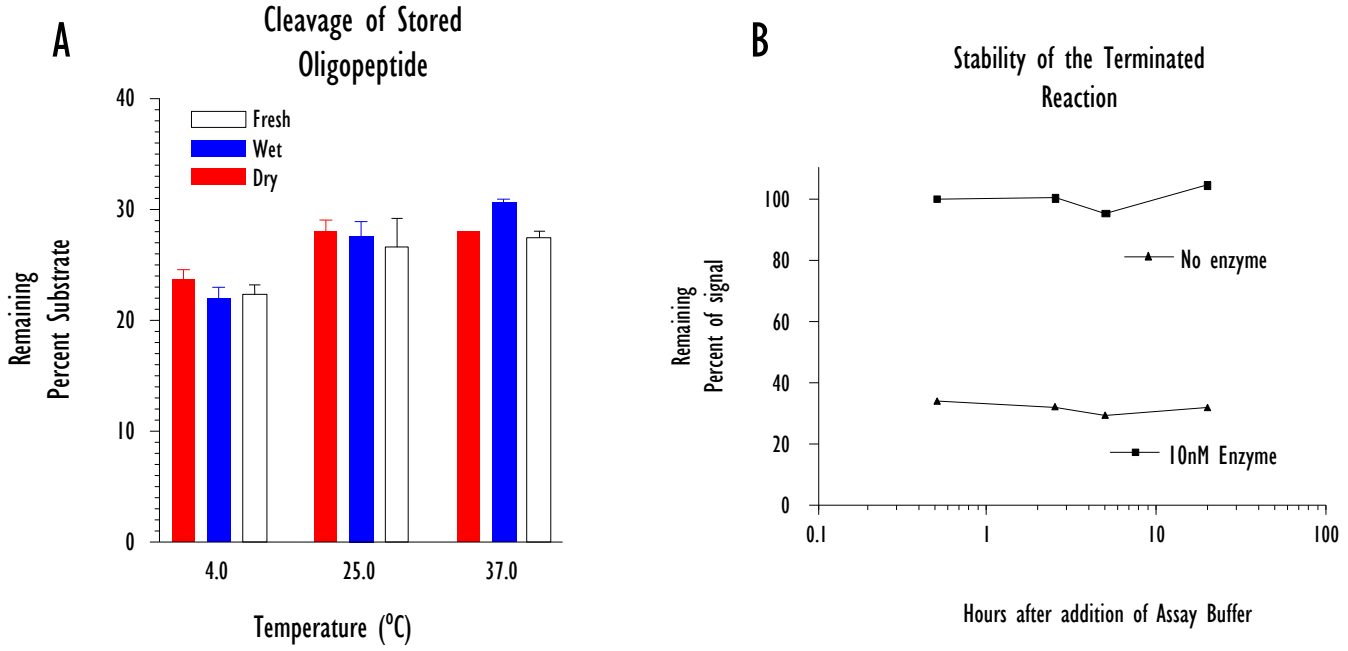
Short Term Stability of Immobilized Peptide and the Terminated Reaction



A) Dual-labeled oligopeptide was immobilized on avidin-coated carbon electrodes and stored 24 hrs., wet or dry, at various temperatures. Subsequently, the immobilized peptide on the stored and, for comparison, freshly prepared plates was challenged with 20nm MMP3 for 2 hrs. at 25°C.

B) The stability of the terminated reaction was determined through quantification of the signal remaining over a 24 hr period after the reaction was stopped.

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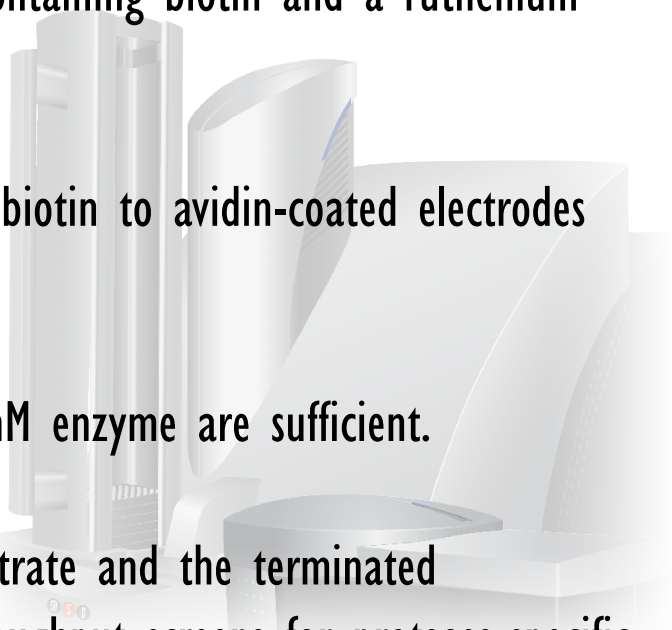
Conclusion

A protease assay has been developed on a novel platform that utilizes carbon electrodes arrayed in disposable microtiter plates.

Synthetic, dual-labeled oligopeptides, containing biotin and a ruthenium metal chelate serve as substrate.



Immobilization of the peptide via the biotin to avidin-coated electrodes does not alter enzyme kinetics.

As little as 1pMol of substrate and 5nM enzyme are sufficient.

The stabilities of the immobilized substrate and the terminated reaction are compatible with high throughput screens for protease-specific inhibitors.

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