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SERRS-Based Enzymatic Probes for the Detection of Protease Activity

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Surface enhanced resonance Raman scattering (SERRS) has proven to be a powerful analytical tool for the detection of enzymatic activity. Recent cases have included phosphatase, peroxidase,[1−3] lipase,[4] and now for the first time determination of protease activity, as reported herein.

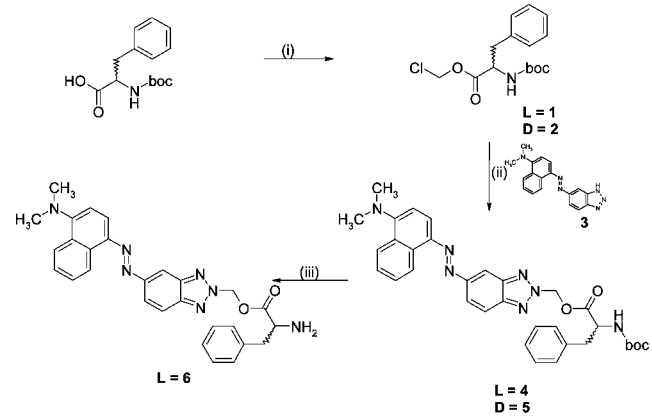
From the human genome data, it is now known that more than 500 genes encode proteases.[5] Proteases mediate nonspecific protein hydrolysis, but also have a vital role in post-translational modifications of proteins, performing selective and efficient cleavage of specific substrates.[6] Synthetic probes for the detection of protease activity are therefore essential, and most probes use fluorescence detection. Fluorescence can provide very sensitive monitoring, but the technique can suffer from background fluorescence when analyzing cellular extracts.

A detection technology whereby rapid, simultaneous detection of multiple enzyme activities could be measured at concentrations found in cellular environments would be of great interest. Surface-enhanced resonance Raman spectroscopy (SERRS) has the potential to meet these criteria. SERRS has been used extensively for DNA detection,[7−12] but there have been few reported cases of its use as a detection method for enzymatic activity. Phenylenediamine can be transformed into a SERRS-active compound by reaction with peroxidase and has been used to detect peroxidase-conjugated antibody for use in immunoassays.[1−3] Ruan et al. report that 5-bromo-4-chloro-3-indolyl phosphate (BCIP) forms a SERRS active species upon reaction with alkaline phosphatase.[13] BCIP is traditionally used for enzyme detection by colorimtery, and it is likely it is the indigo dye product that is SERRS active.

Previously we reported a SERRS-based technique for lipase detection, where the surface seeking moiety of a dye, in this case benzotriazole, was covalently alkylated, thus binding to the metal surface was prevented and surface enhancement of the Raman scattering was limited.[4,14] By careful design of the covalent mask, it can be recognized and selectively cleaved by specific enzymes, thus regenerating a surface seeking species and accordingly an increase in SERRS response clearly detectable.

This study shows how the technique has been adapted to the synthesis of masked protease substrates. An amino acid ester, in this case phenylalanine, was covalently attached to a SERRS active species via chloromethyl ester **1** and **2** (scheme 1). The chloromethyl ester was prepared by reaction of chloromethyl chlorosulfate (CMCS) with Boc-protected phenylalanine under phase-transfer conditions.[15] Both D and L isomers of phenylalanine were attached by this method. Furthermore a deprotection protocol for the removal of Boc from **4**, to afford amino analogue **6**, was developed. It was found that Boc deprotection would proceed smoothly in neat TFA after 15 min treatment. The compound is stable if the amine is quaternized as the TFA salt, but the free amine species is unstable if stored for longer than 24 h. Compatibility with a Boc deprotection strategy demonstrates the potential to incorporate masked amino acids such as **4** into a longer synthetic peptide sequence, which

Scheme 1[a]



[a] Conditions: (i) CMCS, $NaHCO_3$, Bu_4NHSO_4, DCM/H_2O, 99%; (ii) (a) NaOH, H_2O; (b) acetone, 13%; (iii) TFA, 0 °C, 99%.

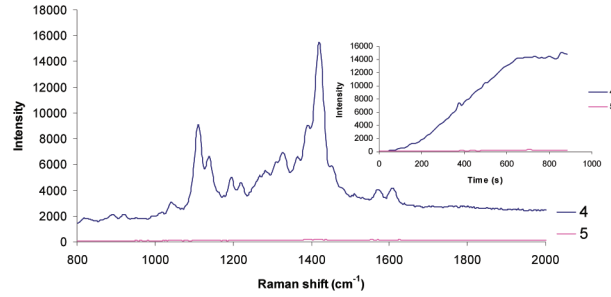


Figure 1. SERRS obtained from **4** and **5** upon treatment with Subtilisin carlsberg for 30 min. (Inset) Monitoring emergence of peak at 1419 cm^{-1} over time for **4** and **5** upon exposure to Subtilisin carlsberg 0.1 mg ml^{-1}.

may be necessary if the protease in question requires a specific multiple amino acid sequence. A colloidal silver suspension containing either masked enantiomer **4** or **5**, at a concentration of 10^{-7} M gave no SERRS spectra under the experimental conditions employed. Upon treatment with Subtilisin carlsberg the SERRS response of **4** was significantly increased (Figure 1). This was attributed to the generation of the SERRS active compound **3** by enzymatic cleavage, the spectra being identical to that obtained in previous enzymatic studies where the same masked SERRS substrate had been used,[4] and also identical to SERRS spectra recorded of **3** alone. When the experiment was repeated with D-Boc-phenylalanine enantiomer **5**, no detectable increase in SERRS was observed, suggesting that no enzymatic hydrolysis of **5** had taken place. This is not unexpected as the enzyme should have enantiospecificity for the natural substrate and means that the D isomer can be used as a negative control in future experiments.

When tested against a selection of proteases, a broad range of SERRS responses was observed after 30 min incubation (Fig-

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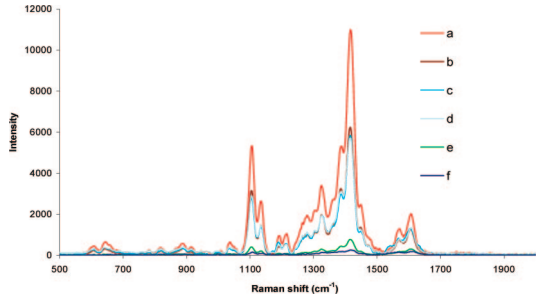

Figure 2. SERRS obtained from **4** upon 30 min treatment with (a) α-Chymotrypsin, (b) Subtilisin carlsberg, (c) Trypsin, (d) Proteinase K, (e) Pepsin, (f) blank, in each case with the enzyme concentration at 0.01 mg ml^{-1}.

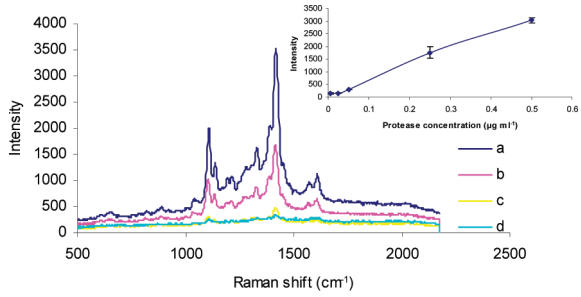

Figure 3. SERRS obtained from **4** upon exposure to Subtilisin carlsberg, at concentration of (a) 0.5, (b) 0.25, (c) 0.05, and (d) 0.005 $\mu g\ ml^{-1}$. (Inset) Enzyme concentration against SERRS response at 1419 cm^{-1}. Reaction time is 30 min.

ure 2). This is indicative of varying rates of hydrolysis by the respective enzymes and is an important result as it shows that substrate **4** can discriminate between protease classes. This discriminating ability, coupled with the demonstrated capability for in situ spectroscopic monitoring, could be potentially applied in a high-throughput screening format, where multiple protease variants could be rapidly analyzed for modulations in reactivity.

A determination of the lowest enzyme concentration that could be used and still observe a detectable response was made. The protease from Bacillus licheniformis, also known as Subtilisin carlsberg, was selected. When monitoring the increase in intensity at 1419 cm^{-1} (Figure 3), a clearly detectable response was observed at enzyme concentrations as low as 50 ng ml^{-1}.

The detection of protease activity using SERRS and modified substrates has been successfully achieved. The substrate was shown to be L selective, selective for proteases over lipases, and for subtilisin the sensitivity of the technique was at least 50 ng ml^{-1}. Incorporation of different amino acid masking groups will be straightforward, and any Boc-protected amino acid (with side-chain protection where appropriate) can be used, most of which are commercially available. This approach offers a highly sensitive screening method for activity of protease variants, for example generated through a directed evolution format. This preliminary study offers the prospect of multiplexed protease activity measurements by incorporation of varying amino acid sequences attached to SERRS dyes with different chromophores.

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Supporting Information Available: Full protocols for enzyme reactions, spectroscopic experiments, additional spectroscopic experiments using lipases and synthesis of masked substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

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