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Real-Time Imaging of Protease Action on Substrates Covalently Immobilised to Polymer Supports

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Abstract: We report for the first time single bead spatially resolved activity measurements of solid-phase biocatalytic systems followed in real-time. Trypsin cleavage of Bz-Arg-OH and subtilisin cleavage of Z-Gly-Gly-Leu-OH each liberate a free amino group on aminocoumarin covalently immobilised to $PEGA_{1900}$ beads [a co-polymer of poly(ethylene glycol) with molecular mass of 1900 cross-linked with acrylamide]. This restores fluorescence which is imaged in optical sections by two-photon microscopy. For trypsin cleavage, fluorescence is restricted initially to surface regions, with more than 1 hour needed before reaction is fully underway in the bead centre, presumably reflecting slow enzyme diffusion. In contrast, for subtilisin cleavage fluorescence develops throughout the bead more quickly.

Keywords: $PEGA_{1900}$; protease cleavage; real-time imaging; solid-phase biocatalysis; spatial resolution; two-photon microscopy

Certain enzymes such as cellulases have uniquely evolved to be enzymatically active at solid surfaces.[1–3] However the use of other enzymes such as serine proteases which have not evolved to be active at solid surfaces but in solution, has recently gained interest.[4–11] The use of such enzymes in this non-conventional manner where the substrate is covalently attached to a solid support has many applications in compound library generation[7] and screening technologies.[12–13]

Solid-phase synthesis on porous polymer supports is a well established technique for peptides, oligonucleotides and is increasingly applied to other organic molecules. As water-compatible porous resins have become available,[14–15] enzyme catalysis on solid supported substrates has been used for solid phase synthesis.[4–6,16] and also for assaying enzyme activity.[17–21] A concern in this area has been the effect the interaction of the porous polymer with the enzyme has on catalytic rates, effects which are very poorly understood. Rates and yields of such enzyme-catalysed reactions are often quite different from the equivalent reaction in solution.[4] This is particularly true where access of the large enzyme molecules to the bead interior may be a limiting factor.[22] This limiting factor can also be used to spatially segregate sites within individual polymeric beads as shown by Vágner et al. in 1996.[23] Indeed in this study chymotrypsin action on amino-protected sites on a polyoxyethylene-polystyrene (tentagel) polymer was shown to only occur on the outside layers of the bead with cleavage yields of <0.5–$2.3\,\%$ quoted for different peptides.[23] Given the intrinsic heterogeneity of the reaction system, biocatalytic reaction on solid phase can only be fully characterised with spatially and temporally resolved analytical methods, ideally designed to act as a real-time measurement. There are reports of the use of IR, confocal Raman and fluorescence spectroscopy but currently there is a lack of on-line measurement techniques which would allow for spatially and temporally resolved activity measurements on a single bead.[9,23–27]

Fluorescence techniques have long been widely used in the biochemical and life sciences. One such technique which is widely used in the life sciences (i.e., tissue staining) is confocal two-photon microscopy (TPM) and allows for optical sectioning at different sample planes. TPM relies on a fluorophore being

excited by a laser which has approximately double the usual single-photon excitation wavelength. Excitation can only result from non-linear two-photon absorption events, which only have significant probability for the extremely high intensities at the focal point of the laser beam. Fluorescence will, thus, only originate from that part of the sample which interacts with the focal volume (i.e., the 3-dimensional region that defines the focal point). Away from the focus, the light passes through the sample essentially unaffected (in the absence of another chromophore able to absorb at the long wavelength). The focal point is scanned through the sample, in three dimensions, and the fluorescence detected used to construct optical section images. This technique thus avoids the artefacts of absorption which have been described for confocal fluorescence microscopy.[24] Because there is no significant absorption away from the focal volume, light penetrates fully, deep into the sample. There is some loss of signal intensity with depth, probably due to loss of focus through aberration, but this can be corrected for with a simple linear function.[28]

PEGA is a polyethylene glycol co-polymer cross-linked with polyacrylamide and is widely used in solid phase synthesis[14–16] and combinatorial screening.[18–21] Previously we reported how two-photon microscopy can be used to monitor fluorophore concentrations at different positions within an uncut $PEGA_{1900}$ bead, with a linear relationship between concentration and fluorescence.[28] Hence we were able to make spatially resolved measurements of the extent of reaction.[22] However, the previous method required derivatisation of the beads after stopping the reaction, whereas real-time monitoring would be much more preferable. We now report initial data on spatially resolved real-time systems, based on enzyme-catalysed release of a peptide/amino acid from an aminocoumarin fluorophore (see Figure 1) covalently attached to $PEGA_{1900}$ beads.[7–8,29]

Fluorescent aminocoumarin derivatives are used extensively in biological assays for determining enzyme activity in solution. These substrates exploit the high shift of the emission spectrum of the free amine upon hydrolysis of the acyl derivative.[7,29] The systems reported here rely on the use of a heterobifunctional coumarin [7-aminocoumarin-4-acetic acid (ACA)]. The aminocoumarin-carboxylic acid is coupled to the $PEGA_{1900}$ amine support *via* a hexa-glycine linker,[30–31] which has been found optimal for enzyme action on $PEGA_{1900}$. A peptide/amino acid substrate is coupled to the aminocoumarin which leads to a quenching of the fluorescence on the bead. A protease is then used to cleave the amide bond between the amino acid/peptide substrate and the aminocoumarin which leads to an increase in fluorescence (see Figure 1). Blank experiments where the PEGA substrate was incubated in buffer containing

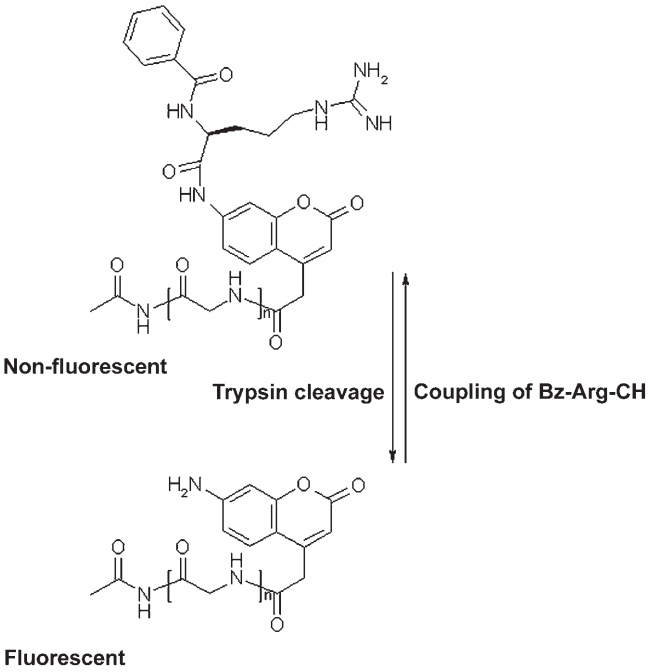

Figure 1. Schematic of coupling and enzymatic reaction processes on $PEGA_{1900}$ beads. Bz-Arg-OH is replaced with Z-Gly-Gly-Leu-OH for subtilisin reaction.

no enzyme showed no hydrolysis: no amino acid/peptide was released into solution (HPLC analysis) and there was no change in bead fluorescence (TPM).

In Figure 2, images from the equatorial cross-section of Bz-Arg-ACA-Gly_6-$PEGA_{1900}$ beads are presented. Image one is of an ACA-Gly_6-$PEGA_{1900}$ bead showing intense fluorescence which is homogenously spread throughout the bead (verified by averaging the pixel intensities of different annular portions of the image).

The second image is the Bz-Arg-OH capped aminocoumarin and the reduced fluorescence is clearly seen. On addition of acetylated trypsin, images at different time intervals over 150 min (for the same

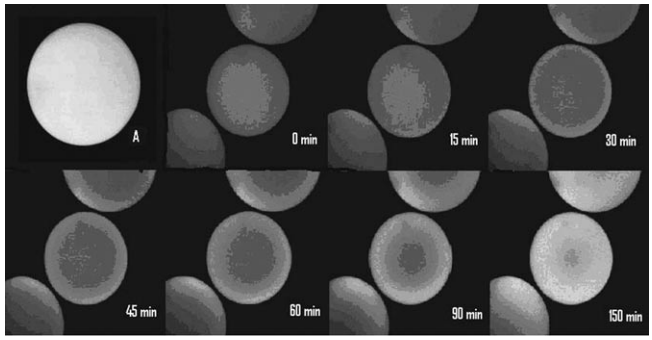

Figure 2. Two photon cross section images of Bz-Arg-ACA-Gly_6-$PEGA_{1900}$ beads, A is an uncapped amino coumarin bead. The other images are the same bead at different times of reaction with acetylated trypsin at 20 °C.

bead) show the biocatalytic reaction occurring on the support. As can be seen fluorescence appears in an annular ring around the outside of the bead, indicating that enzymatic reaction is confined to this region. Figure 3 shows the progress of the reaction in shells

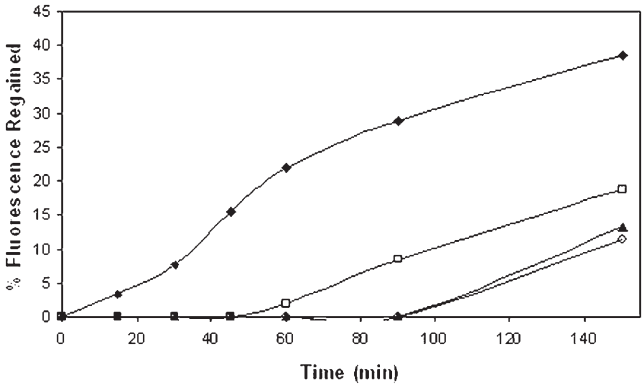

Figure 3. Percentage fluorescence intensity regained upon reaction of Bz-Arg-ACA-Gly_6-$PEGA_{1900}$ with acetylated trypsin versus time. Each plot represents different zones within a single bead where, ◆ is 0–53 μm from the bead edge to 53 μm inside the bead, □ is 53–105 μm, ▲ is 105–158 μm and ○ is 158 μm–centre of the bead. The bead diameter was 350 μm.

at different distances from the bead surface, monitored by averaging pixels in annular rings from images like those shown in Figure 2. As time progresses the reaction continues near the surface, but significant fluorescence begins to appear deeper into the bead. However, reaction near the bead centre is delayed by more than 90 min compared to that at the surface. This is slower than the relative progress observed by Bosma et al.[22] for the similarly sized enzyme thermolysin penetrating into beads of the same support. Most likely these observations reflect slow diffusion of the enzyme into the support beads. If the enzyme diffusion coefficient were close to that in dilute aqueous solution, it would take only about a minute to approach the final concentration at the centre of beads of this size. Hence, as Bosma et al.[22] noted, this implies an effective diffusion coefficient for the enzyme inside the beads more than an order of magnitude lower. After 150 min the extent of reaction at the surface is still double that at the core of the bead (Figure 3). Indeed it takes over 3 h before the fluorescence values of the inner core converge with those in the outer layers. This extremely slow enzyme diffusion may be explained by electrostatic repulsion of the positively charged trypsin (pI 8.69[32]) and the positively charged immobilised arginine substrate.

The reaction progress assessed by fluorescence can be compared with that monitored by HPLC analysis of the released Bz-Arg-OH. HPLC analysis necessarily gives the average reaction rate across the volume of a population of beads. For comparison the fluorescence intensity was averaged over the whole volume of 5 randomly selected beads. There was reasonably good agreement, but the extent of reaction assessed by HPLC was normally somewhat higher. For example, after 24 hour reaction, HPLC indicated 26% release of Bz-Arg-OH, while fluorescence regained was 18% of that from the uncapped bead. The inherent heterogeneity of the beads will affect this comparison. Initial studies of beads of different diameters have shown lower cleavage yields from larger beads. Another factor that might account for the discrepancy would be cleavage by the enzyme of the peptide linkage between the aminocoumarin and the glycine linker. However, the HPLC analysis showed no evidence of peaks from the aminocoumarin or of products other than Bz-Arg-OH. Furthermore, upon reaction of ACA-Gly_6-$PEGA_{1900}$ with acetylated trypsin there was no apparent decrease in fluorescence over 3 days of reaction.

The same approach can be used to monitor, subtilisin activity on Z-Gly-Gly-Leu-ACA-Gly_6-$PEGA_{1900}$ as the immobilised substrate. Figure 4 shows some

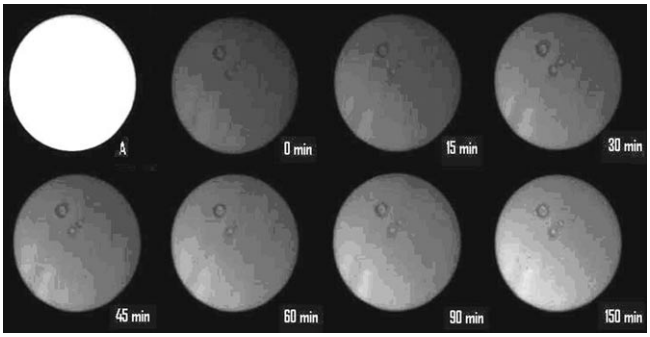

Figure 4. Two photon cross section images of Z-Gly-Gly-Leu-ACA-Gly_6-$PEGA_{1900}$ beads, A is an uncapped amino coumarin bead. The other images are the same bead at different times of reaction with Subtilisin Carlsberg at 20 °C.

sample images obtained for this system, again monitoring a single bead in real time. Figure 5 shows the progress of the subtilisin reaction in different shells of the bead. The images and graph show a very different profile for subtilisin within the polymer. The differences between the fluorescence values in the outer segment and the core of the bead are less obvious than with the trypsin system. This suggests that enzyme diffusion is fast and that it is not rate limiting. If the diffusion coefficient were close to the value for aqueous solution, enzyme molecules would be able to diffuse throughout a bead of 100 μm radius in about 1 min.[22] Therefore it is not inconceivable to have such fast enzyme penetration into the bead core, although it is unusual, even though the PEGA beads are composed

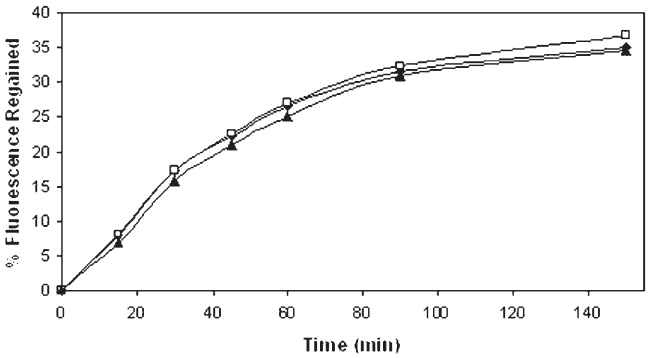

Figure 5. Percentage fluorescence intensity regained upon reaction of Z-Gly-Gly-Leu-ACA-Gly_6-$PEGA_{1900}$ with Subtilisin Carlsberg versus time. Where □ is 0–50 μm from the bead edge to 50 μm inside the bead, ▲ is 50–100 μm and ● is 100 μm–centre of the bead. The bead diameter is 300 μm.

of 90% water. After 16 h of reaction, HPLC indicated 50% release of Z-Gly-Gly-Leu-OH, while fluorescence regained was 39% of that from the uncapped bead. When subtilisin was reacted with ACA-Gly_6-$PEGA_{1900}$ a reduction of 9.2% in the fluorescence intensity was noted after 16 h of reaction. No free aminocoumarin was detected by HPLC analysis.

In summary, the method described here allows for the study of real-time spatially resolved kinetics of enzyme action on substrates which have been immobilised within a solid polymer bead support. It should be noted that this method allows the complete progress of the reaction to be monitored on a single bead, as in the example of Figure 2 and Figure 4. The TPM system used allows for images of a single section to be taken every 5 seconds if this was necessary to follow kinetics. This will allow for more extensive studies of such enzymatic systems and is suitable for studying heterogeneity within a population of beads, a topic that is currently under further study.

Experimental Section

Materials

N-Hydroxybenzotriazole (HOBt) and benzoyl-L-arginine-OH (Bz-Arg-OH) were from NovaBiochem. Fmoc-Gly-Gly-Gly-OH (Fmoc-Gly_3-OH), Z-Gly-Gly-Leu-OH were from Bachem. 1,3-diisopropylcarbodiimide (DIC) (99%), *N*,*N*-diisopropylethylamine (99%) (DIPEA), oxalyl chloride, picrylsulfonic acid, trifluoroacetic acid (TFA) (99% spectroscopic grade), acetone and methanol (both spectroscopic grade), acetic anhydride, ultrafree MC microcentrifuge filters, acetylated trypsin type V-S bovine pancreas, subtilisin Carlsberg type VIII *Bacillus lichenformis* were from Sigma. Piperidine and dimethylformamide (DMF), both peptide synthesis grade, were from Rathburn Chemicals Ltd, Walkerburn, Scotland, EH43 6AU. All other solvents were from Fischer. $PEGA_{1900}$ resin [a copolymer of poly(ethylene glycol) with molecular mass of 1900 and acrylamide with bead size ~300 μm] was supplied by Polymer Laboratories, (UK), wet in ~90% methanol, the free amine content was 200 μmol·g^{-1} dry resin.

Resin Preparation

$PEGA_{1900}$ beads (1 gram wet) were swollen and washed with 3×25 mL of each of the following in sequence: DCM, THF, DCM, MeOH/DMF (50:50 v:v) and dry DMF. PEGA was then equilibrated in dry DMF for 30 min.

Linker Chain Attachment

This used the standard coupling chemistry essentially as recommended in the catalogue from NovaBiochem. Fmoc-Gly_3-OH (5 equivs.) was coupled directly to the swollen/washed beads (1 g wet) with HOBt (6 equivs.) and DIC (4 equivs.). Two couplings for 3 h each at 30 °C in 4 mL dry DMF were performed. The resin was washed (3×25 mL) DMF and the excess NH_2 groups were acetylated with 100μL of acetic anhydride in 4 mL DMF for 30 min. The Fmoc group was cleaved in 4 mL 20% piperidine/DMF (v/v) for 30 min at 30 °C on a blood rotator. The resin was washed (3×25 mL) DMF and incubated for 30 min in DMF. The presence of free amino groups was confirmed using standard TNBS test (picrylsulfonic acid two drops, added to resin in 5% DIPEA/DMF). A second Fmoc-Gly_3-OH was then coupled using the same procedure, followed again by cleavage of the Fmoc. The Fmoc released was analysed by HPLC (C_{18} with a gradient from 10% ACN, 0.1% TFA in deionised H_2O to 90% ACN, 0.1% TFA over 30 min at 1 mL·min^{-1}, detection at 254 nm and 280 nm), showing a loading of 160 μmoles·g^{-1} dry resin of Gly_6-NH_2 (80% yield based on original amino group content of $PEGA_{1900}$).

Synthesis of ACA

The 7-aminocoumarin-4-acetic acid (ACA) was synthesised using the method of Maly et al.[33] *via* a von Pechmann reaction using 3-*N*-(carbethoxy)aminophenol and highly pure 1,3-acetonedicarboxylic acid in 70% H_2SO_4. The ACA product (1 g, 54% yield) was NMR pure and NMR data were similar to the literature values.[33–35] ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 3.72$ (s, 2H), 5.97 (s, 1), 6.12 (br s, 2), 6.42 (d, 1, $J = 0.7$ Hz), 6.54 (dd, 1, $J = 8.6$, 2.2 Hz), 7.32 (d, 1, $J = 8.6$ Hz), 12.60 (s, 1).

Coupling of ACA to Beads

The ACA was coupled to the NH_2-Gly_6-PEGA (1 g wet) using standard HOBt/DIC coupling chemistry as described above for the Fmoc-Gly_3-OH couplings The beads were washed thoroughly to remove excess aminocoumarin (1× 25 mL 3% TFA/DMF, 3×25 mL DMF, 1×25 mL DMF, 30 min incubation). Note that the amino group of the aminocoumarin does not need to be protected for coupling.[30] Loading was determined by reacting Fmoc-Cl (3 equivs., that is, 160 μmoles·g^{-1} dry resin×3) with 6 equivs. DIPEA in 4 mL DMF for 60 min at 30 °C. Cleavage of the Fmoc and subsequent HPLC detection yielded 145 μmoles ACA g^{-1} dry resin (suggesting a coupling yield of 90%).

Coupling of the Substrates

Both Bz-Arg-OH and Z-Gly-Gly-Leu-OH (145 μmol) were first converted to acid chlorides by reaction with oxalyl chloride (40 equivs.) in 10 mL DCM (with a drop of DMF) at 25 °C for 3 h. The solvent was removed by evaporation under vacuum, and the acid chlorides (10 equivs.) then coupled to the aminocoumarin resin (1 g wet) with DIPEA (20 equivs.) in 4 mL DMF. Due to the weak nucleophilicity of the amine on the coumarin, four couplings were required each for 4 h at 30 °C on a blood rotator. The beads were washed with DMF (3×25 mL), 1×25 mL DMF for 30 min. The yields (based on fluorescence data from the TPM) were 72 % for Bz-Arg-OH and 53 % for Z-Gly-Gly-Leu-OH.

Resin Preparation for Biocatalysis

All beads were washed (3×25 mL) with buffer prior to use on the TPM. For Bz-Arg-ACA-Gly_6-PEGA, 20 mM aqueous Tris-HCl pH 8.0/100 mM $CaCl_2$ was used (i.e., for trypsin reaction). For Z-Gly-Gly-Leu-ACA-Gly_6-PEGA, 20 mM potassium phosphate buffer pH 7.0 was used (i.e., for subtilisin reaction). Beads were incubated in buffer for 3 h with subsequent washing with buffer (3×25 mL) prior to experiments. For experiments beads were pipetted (100 μL PEGA suspension) onto coverslips and focussed on the inverted two photon microscope. Reaction was initiated by pipetting (100 μL, 1 mg enzyme mL^{-1} buffer). Care was taken not to disturb the focussed bead while adding the trypsin. Excitation was at $\lambda = 750$ nm and a Kalman averaging of 6 images were used to obtain single images. The gain was set so that pixel intensities fell within the range detectable throughout the enzymatic reactions, and was kept the same for all comparisons. Pixel intensities were downloaded to an Excel spreadsheet using the METAMORPH imaging analysis (Universal Imaging) software. Distances to a manually drawn bead surface, and average intensities for different shells, were calculated using specially written macros.

HPLC Analysis of Products Released in Enzyme Solutions

For each enzyme reaction 20 mg (wet) of PEGA substrate was added to 900 μL of buffer. This was vortexed for 10 seconds to disperse beads. Reaction was initiated by addition of 100 μL of enzyme solution (1 mg mL^{-1}) beads were vortexed again for 10 seconds. Blanks were also carried out with PEGA substrate [20 mg (wet)] and buffer (900 μL) containing no enzyme to investigate non-enzymatic hydrolysis of the amino acid/peptide from the immobilised aminocoumarin. Reaction was carried out for the desired time at 30 °C with stirring on a blood rotator. Samples were then centrifuged at 6,400 rpm for 2 min and the supernatant removed. Enzyme was then removed by passing the supernatant (400 μL) through an Ultrafree MC microcentrifuge filter with molecular weight cut-off of 5,000 Da. Samples were centrifuged for 30 min at 6,400 rpm. Supernatant was then analysed by HPLC (C_{18} with a gradient from 10 % ACN, 0.1 % TFA in deionised H_2O to 90 % ACN, over 30 min at 1 mL min^{-1}, detection at 254 nm and 280 nm). Standards (50–500 μM) of Bz-Arg-OH and Z-Gly-Gly-Leu-OH in ACN, 10 % and 90 % dH_2O were used to generate standard curves.

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