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EVALUATION OF RESINS FOR ON-BEAD SCREENING: A STUDY OF PAPAIN AND CHYMOTRYPSIN SPECIFICITY USING PEGA-BOUND COMBINATORIAL PEPTIDE LIBRARIES

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Abstract: TentaGel, ArgoGel and PEGA resins were evaluated for on-bead biological screening, using a fluorescently-labelled peptide attached to each and assayed for papain activity. Peptide attached to PEGA was cleaved in near quantitative yield at the expected sites, whilst an identical sequence on TentaGel and ArgoGel beads was hydrolysed in very low yields and nonspecifically on ArgoGel. The compatibility of PEGA with enzymes was further demonstrated by the determination of subsite specificities of papain and chymotrypsin using PEGA-bound peptide libraries, which proved to be similar to those observed in free solution.

Introduction

The technique of solid phase synthesis using resin beads offers many advantages over solution phase methods and has thus become the preferred method of preparing peptides and an increasing number of other organic compounds. Whilst combinatorial libraries are generally synthesised on solid supports, biological screening is most commonly carried out in solution. For soluble biological targets, however, screening of the library while still attached to the support offers many advantages over assay in solution. In solution, a mixture of a relatively small number of compounds is assayed, which generally involves a process of deconvolution to identify the active components. Moreover, the possibility of cooperative effects between the different compounds in a solution phase mixture is difficult to avoid.

The validity of on-bead screening has however remained a subject of debate. Concerns over the permeability of resins to biomolecules and the possibility that their specificity may be perturbed, have led many researchers to favour screening in solution. We have evaluated the compatibility of three resins: TentaGel (TG), ArgoGel (AG) and PEGA,[1] in the screening of proteinase activity towards peptide substrates. AG and TG resins consist of a polystyrene backbone, 1-2% cross linked with divinyl benzene and grafted with polyethylene glycol (PEG). The extensive use of TG in solid-phase synthesis can be attributed to the mechanical stability of the beads along with their swelling properties in organic and aqueous media.[2] ArgoGel displays similar characteristics to TG but swells more extensively than TG in aqueous environments, owing to a higher PEG content.[3] It was therefore expected that AG might offer greater accessibility to biomolecules. PEGA resin is based on a more hydrophilic, polyacrylamide backbone copolymerised with a PEG spacer which imparts a more open structure.[4]

Evaluation of resins for on-bead screening

Papain (M_r 23 kDa) is a well-characterised member of the cysteine proteinase family and has a preference for substrates carrying a phenylalanine residue at the P_2 position.[5,6] The behaviour of the enzyme towards the resin-bound peptide sequence GGFGLGGG has been investigated; the Phe residue was expected to direct enzymatic

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cleavage to the Gly-Leu bond, whilst Leu was incorporated at the S_1' site as it has been reported to bind well at this position.[7] The sequence was synthesised on each of the three resins *via* a hydroxymethylbenzoic acid (HMBA) linker. The peptide was dansylated at the N-terminus (Fig. 1). Fully dansylated resin beads appeared very bright when examined under a fluorescence microscope and could be distinguished readily from beads which were unlabelled or only partially labelled.[8] Visualisation of the beads by this method therefore allowed a rapid, qualitative estimation of the extent of enzymatic cleavage of the resin-bound peptide. The use of 2-[4-(dimethylamino)phenylazo]benzoic acid (methyl red) as an alternative (visible) N-terminal label also proved effective in monitoring cleavage, though the fluorescent dansyl label was favoured as it was found to offer a clearer distinction between labelled and unlabelled beads.

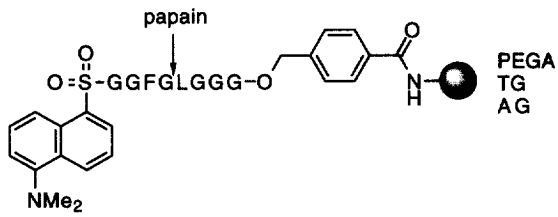

Fig. 1: Resin-bound peptide substrates labelled with dansyl fluorophore, showing the expected site of cleavage by papain.

The three sets of beads were each treated with enzyme solution. The rinsed beads were then re-examined by fluorescence microscopy to assess the change in brightness. Treatment of the dansyl-peptide-PEGA beads with 5.5 μM pH 6.6 buffered papain for 1 h resulted in a significant reduction in the fluorescence of the beads, suggesting that enzymatic cleavage had occurred. Cleavage was confirmed by N-terminal Edman sequencing. The sequencing yields indicated that partial cleavage had occurred at two distinct sites, *i.e.* at the FG-L and the LG-G bonds.[9] The major site of cleavage (FG-L) was consistent with the well-documented specificity of papain for Phe at P_2 in solution.[6] The secondary site of cleavage resulted from the occupation of the S_2 pocket by the hydrophobic Leu side chain. This is also consistent with literature observations, which showed that the S_2 pocket was able to accommodate Leu, albeit less effectively than Phe.[10]

In contrast, identical substrate sequences attached to TentaGel and ArgoGel beads appeared unaffected even after prolonged treatment with papain, the beads remaining unaltered when visualised by fluorescence microscopy. Despite this lack of discernible change, partial enzymatic cleavage was indicated by the release of dansyl fluorophore, which was detected in solution by fluorimetry. This low level of hydrolysis is consistent with the "shaving" experiments of Lebl and co-workers, which suggested that only peptide at the surface sites of TentaGel beads are cleaved by proteinases.[11-13] N-terminal Edman sequencing of the treated ArgoGel beads revealed a complex mixture of peptide fragments.[14] In further contrast to the results obtained using PEGA, enzymatic cleavage appeared to occur nonspecifically, suggesting that the ArgoGel resin had affected the specificity of papain.

These results suggest a marked difference in enzymatic activity towards substrates bound to TG and AG resins compared with those on PEGA. Quantitative cleavage of the PEGA-bound substrate indicated that the interior of the beads is freely accessible to macromolecules such as papain, as reported previously by Meldal *et al.*[15-17] Moreover, the sites of cleavage correlated with those hydrolysed in solution, indicating that the resin did

not interfere with the specificity of the enzyme. A disadvantage of PEGA, however, is that its 'open' structure imparts fragility to the beads, which must be stored and handled whilst swollen in methanol or water. A second drawback is that the beads are only commercially available in wide size distributions, *i.e.* diameters of 150-300 μm and 300-600 μm. Despite these drawbacks, we have investigated the subsite specificities of both papain and the serine proteinase chymotrypsin using 'one-bead, one-peptide' libraries of PEGA-bound substrates.

Subsite specificity of papain

Very few investigations have been made into the S' subsite specificities of proteinases, with most of the published studies being limited to comparisons between a small number of different amino acids at the P_1' and P_2' positions. We synthesised the library of peptides (**1**), in which X represents one of the 20 natural amino acids. The peptides were assembled using standard Fmoc methodology, and the sequences verified by Edman sequencing prior to dansylation, indicating a level of functionalisation of 60-180 pmol/bead. Since the specificity of papain is governed primarily by the binding of Phe at S_2, cleavage was expected to occur at G-X, with any specificity for particular X residues being reflected by differences in cleavage rates.

Dansyl-GGFGXGG–PEGA
(1)

Dansyl-GGGF$X_A X_B$GGGG-HMBA–PEGA
(2)

The 20 peptide sequences were assayed separately with papain using the wells of a "MultiScreen" microplate,[18] containing a 5 μm filter at the base of each well. Approximately a dozen beads of each sequence were treated with 22 μM papain for 25 min. The wells were then drained and the beads rinsed thoroughly using a vacuum manifold unit. Examination of the beads under a fluorescence microscope revealed a range of bead brightnesses, with the results suggesting the highest degree of cleavage for X = Ser, Leu and Ala, whilst X = Met and Pro remained bright. The observed S_1' preference for the hydrophobic side chains of Leu and Ala is consistent with literature studies[7, 19, 20] whilst a specificity for the more polar Ser residue is also supported by published results.[20, 21]

The approach was extended to the study of a larger library to explore the S_1 and S_1' subsites of papain simultaneously. The library of peptides (**2**), in which X_A and X_B represent one of the 20 natural amino acids, was synthesised as an array of 400 individual sequences in MultiScreen microplate wells, using standard Fmoc-amino acid couplings. Each successive coupling was verified using Kaiser test reagents[22] or a trinitrobenzene sulphonic acid test.[23] The synthesis was confirmed by Edman sequencing prior to dansylation at the N-terminus.

Samples of each peptide-bead[24] were incubated separately with 11 μM papain for 2 h, then rinsed and examined for dansyl fluorescence. This circumvented the subsequent need for sequence analysis, since the identity of each sequence could be deduced by its position in the array. As with the previous assay, beads of the same sequence showed a consistent level of brightness, whilst a range of fluorescence intensities was observed across the assay. Assessment of the extent of cleavage using a standard fluorescence microscope does not allow a quantitative comparison between the different sequences. The variation in brightness for the different sequences nevertheless enabled a clear distinction to be made, and beads were classed into one of five categories according to brightness. Owing to the subjective nature of this method of analysis, the beads were then re-examined to obtain a second set of results, which proved identical to the first.

Consistent with the known, broad specificity of papain, a large number of the sequences were shown to have been cleaved. Table 1, in which the amino acids are arranged roughly in order of polarity and size, indicates some degree of preference for polar amino acids or those with small, hydrophobic side chains in both the P_1 and P_1' sites. Peptides containing cysteine, which is rarely a specific residue for proteinases, consistently underwent very little hydrolysis. These general observations support the literature results,[19, 21] although the lack of a clear trend in favour of any particular amino acid serves to confirm the relatively indiscriminate nature of the S_1 and S_1' subsites of papain.

Subsite specificity of chymotrypsin

Similar assays using libraries (1) and (2) were performed with chymotrypsin. The phenylalanine residue, present in all the sequences, was expected to bind in the S_1 pocket of chymotrypsin, directing cleavage to the F-G and F-X_A bonds in the two libraries respectively.

Table 1: Classification of fluorescence of beads from peptide library (2) after assay with 11 μM papain for 2 h.

X_A \ X_B	F	L	M	I	H	P	A	G	C	T	S	N	E	D	K	R
F						●				●		●		●		
Y			×	×	●			●		●			●	●	●	
W			×	×							○	●	●	○	○	
L					●	●	○	●	○	●		●	●	●		
M					●	●	○	●	○	●	●	●	●	●		
I					○	●				●			●	●		○
V										●		●	●	●	●	●
H		●	×	×	●	●	●			●	○	●	●	●	●	
P	●	●	●	●	●					●	○	●		●	●	
A		●			●					●	●	●		●	●	●
G	●	●	●	●	●	●	●			●	●	●	●	●	●	○
C			×	×							○		○			
T	●	●	×	×	●	●	●			●	●		●	●		●
S			×	×	●		●	●	●	●	●	●	●		●	●
Q	●	●	●	●	●		●		●	●	●		●	●	●	●
N	●	●	●	●	●	●	●		●	●	●	●	●	●	●	○
E	●	●	×	×	●	●	●	●				●	○		●	
D	●	○	×	×	●	●	●	●					○	●		
K	●	●	×	×	●		●	●	●	●	○	●	●	○	●	
R	○		×	×			●		○	●	○	●	●	○		

Table 2: Classification of fluorescence of beads from peptide library (2) after assay with 8 μM chymotrypsin for 23 h.

X_A \ X_B	F	L	M	I	H	P	A	G	C	T	S	N	E	D	K	R
F								○				○		○	●	
Y		●	×	×			●	●		●	●		●	●		
W			×	×								○				
L														●	○	
M					○					○				●	○	
I					○			○						●		
V										○					●	
H			×	×	○		○			○		○	○		○	
P		○						○								
A										○				●	●	
G		●	●				○							●	○	
C			×	×										○		
T		●	×	×	●		○			●				●	●	
S	●	●	×	×	●		●	○		●		●	○	●	●	
Q								○	○	○	○					
N	●													●		
E	●		×	×				○		○		○			○	
D			×	×		○		○				○		○		
K	●	●	×	×		○	●	●			●	●		●	●	
R	○		×	×												

key: ● dim beads ● moderately dim beads medium beads
○ moderately bright *(blank box)* bright beads × not determined

Library (1) was assayed using a mixture containing 5 beads of each sequence. Treatment of the beads with 8 μM chymotrypsin for 5 h resulted in a range of bead fluorescence intensities. In contrast to the papain experiment, however, a much smaller proportion of the beads were affected by the enzyme. Of the 82 beads recovered intact, the majority exhibited a fluorescence level comparable to that of the untreated beads. A significant reduction in brightness was observed for 14 beads, of which 9 appeared very dim. This indicated that chymotrypsin imposes a specificity for a limited range of amino acids at the S_2' site. Edman sequencing of four

of the dimmest beads confirmed cleavage to have occurred exclusively at the F-G bond, and identified X as Thr in two cases and Glu and Leu in the other samples.[25]

As with papain, library (2) was assayed against chymotrypsin as an array of individual sequences. Fewer sequences were cleaved than in the papain assay, again indicating the more specific nature of this enzyme (Table 2). The peptides most extensively cleaved by chymotrypsin were those containing Ser or Lys at X_A, and corresponded to the residues most favourably accommodated in the S_1' subsite. These observations are consistent with the results from the acyl transfer experiments in solution of Schellenberger *et al.*, in which Ser was the most specific amino acid at P_1',[26] but the experiments of Schellenberger *et al.* demonstrated a significant interdependence of the specificity of the S' subsites.

Some cleavage was observed with several other residues at P_1', though this appeared to be influenced by the adjacent P_2' amino acid. Data for peptides in which X_A = Tyr and X_A = Phe were discounted from consideration of S_1' and S_2' specificity owing to our earlier studies which showed preferential cleavage at the Y-X_B bond.[27]

Variation of X_B (*i.e.* P_2') appeared also to affect susceptibility to cleavage, with the dimmest beads most frequently containing Leu, Asp, Ala and Lys at this position. The trend of S_2' specificities was however less distinct than that for the S_1' subsite.

Summary

The results from proteinase specificity assays demonstrate the suitability of PEGA resin for on-bead bioassays and are consistent with literature results based on solution studies, indicating that the resin did not interfere with the specificity of the enzyme. By contrast TentaGel and ArgoGel resins were shown to restrict access and alter the specificity of papain towards bound substrates. These results emphasize the importance of the choice of resin for on-bead biological screening.

Acknowledgement

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9. Edman sequencing results: Cycle 1: L (44), G (32); Cycle 2: G (44); Cycle 3: G (25). Figures in brackets denote yields in pmol/bead, and are average values calculated from duplicate analyses of single bead samples. PEGA beads of a diameter range 150-300 μm were used, with a peptide loading of 60-180 pmol/bead as determined by Edman sequencing. Prolonged treatment of the beads with papain showed quantitative levels of cleavage.
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14. Edman sequencing results: Cycle 1: G (4.2), L (1.7), F (1.6); Cycle 2: G (4.3), F (2.4); Cycle 3: G (3.5), F (1.5); Cycle 4: G (2.7), L (1.7); Cycle 5: G (2.8), L (1.3); Cycle 6 (2.6). Figures in brackets are average yields in pmol/bead, calculated from the duplicate analyses of samples comprising 13 beads. Beads were of mean diameter 176 μm, with an original peptide loading of approx. 140 pmol/bead determined by Edman sequencing.
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24. The assays with library (**2**) were limited to 300 sequences due to a logistical problem during synthesis.
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Sample 1: Cycle 1: G (20); Cycle 2: T (4); Cycle 3: G (18); Cycle 4: G (16).
Sample 2: Cycle 1: G (20); Cycle 2: T (4); Cycle 3: G (16); Cycle 4: G (15).
Sample 3: Cycle 1: G (84); Cycle 2: L (77); Cycle 3: G (69); Cycle 4: G (59).
Sample 4: Cycle 1: G (13); Cycle 2: E (8); Cycle 3: G (9); Cycle 4: G (10).
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