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Short Communication

A Colorimetric Enzyme-Linked On-bead Assay for Identification of Synthetic Substrates of Protein Tyrosine Kinases

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Abstract: Protein tyrosine kinases play key roles in the progression of numerous human diseases including several types of cancers. We report here a simple colorimetric assay for tyrosine kinase activity employing synthetic peptide substrates prepared on Tentagel synthesis beads. Phosphorylation of compounds on beads was detected with an antiphosphotyrosine antibody complexed with a secondary antibody–alkaline phosphatase conjugate. This assay may prove useful for the identification and characterization of synthetic substrates of this important class of enzymes.

Keywords: protein tyrosine kinase; solid-phase; antiphosphotyrosine; ELISA

INTRODUCTION

Protein tyrosine kinases (PTKs) catalyse the transfer of the gamma phosphoryl group of adenosine triphosphate (ATP) to tyrosine residues of substrate proteins. These enzymes play critical roles in the regulation of cellular growth, differentiation and division.[1] Mutations that aberrantly activate PTKs are correlated with the emergence and progression of numerous diseases that include cancer, diabetes, atherosclerosis and psoriasis.[2] As a consequence, compounds that inhibit PTK activity or influence PTK-regulated signalling pathways are currently under intense investigation as potential drug candidates.[3–10]

We are investigating natural and non-natural substrates of Abl tyrosine kinases, which are oncogenic enzymes involved in the proliferation of murine and human leukaemias.[11–13] We report here a novel enzyme-linked solid-phase 'on-bead' assay that enables the visual detection of synthetic Abl substrates prepared on PEG-polystyrene (Tentagel) synthesis resin. Other previously reported on-bead assays for detection of tyrosine kinase activity have typically radiolabelled beads with [γ-^{32}P] ATP followed by exposure of beads to film for analysis.[14–19] More recently, detection of phosphate through molecular recognition by a specific dye was described.[20]

Abbreviations: Ac_2O, acetic anhydride; ATP, adenosine triphosphate; BSA, bovine serum albumin; ddw, distilled deionized water; DIEA, diisopropylethylamine; DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MeOH, methanol; PBS, phosphate buffered saline; PEG, polyethylene glycol; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

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We report that v-Abl kinase phosphorylates tyrosine-containing peptides linked to Tentagel synthesis beads. This phosphorylation can be readily detected by addition of an antiphosphotyrosine antibody combined with an alkaline-phosphatase (AP)-conjugated secondary antibody. This secondary antibody stains beads bearing phosphotyrosine (pTyr) upon addition of a colorimetric AP substrate. Related AP-conjugated protein reagents have been employed to detect non-covalent interactions for on-bead screening of combinatorial libraries.[21–23] Additionally, similar strategies have been employed for the detection of PTK activity in a microtitre-based format.[24–27] This approach enables the simple and rapid visual detection of kinase substrates, while obviating the reagent and disposal costs associated with radioactive materials.

MATERIALS AND METHODS

Reagents and solvents were obtained from Aldrich, Alfa Aesar or VWR. Fmoc-protected amino acids and Rink amide resin (75–150 mm, 0.7 mmol/g) were purchased from Novabiochem. Amino Tentagel resin (80–100 mm, 0.3 mmol/g) was obtained from Advanced Chemtech. Abl tyrosine kinase was purchased from New England Biolabs. BM Purple AP substrate was from Boehringer Mannheim GmbH. Monoclonal mouse anti-phosphotyrosine IgG and AP-goat anti-mouse IgG conjugate were obtained from Zymed Laboratories Inc. Mass spectra were obtained on a Mariner mass spectrometer (Perceptive Biosystems, Framingham, MA) with electrospray ionization (positive ion) at a spray tip potential of +3500 V. Samples were introduced by loop injection at 0.1 ml/min. Peaks are reported as m/z. HPLC analysis employed a Zorbax StableBond C18 column on a Hewlett Packard HP1100 instrument (1 ml/min flow rate using a gradient from 9:1:0.001 ddw/CH_3CN/TFA to 0.9:9.1:0.001 ddw/CH_3CN/TFA over 40 min). Micrographs were captured through Zeiss CP-Achromat (10X or 20X) objectives by a Zeiss Axiocam digital camera interfaced to a Zeiss Axiovert S100TV microscope. Images were processed with Adobe Photoshop 5.0.

Peptide Synthesis

Peptides were prepared with an Advanced Chemtech FBS-357 automated batch-mode synthesizer. Peptide synthesis employed standard N_α-Fmoc methodology with *t*-butyl ester and *t*-butyl ether side-chain protection. Coupling and Fmoc deprotection steps were monitored with the ninhydrin test for free amines. Fmoc carbamates on Rink amide resin (NovaBioChem, 50 mg, 35 μmol) were deprotected by addition of 30% piperidine in DMF (2 × 1 ml for 5 min followed by 1 ml for 20 min). N_α-Fmoc-protected amino acids (AA) (100 mmol) were coupled by sequential addition to the resin of the following reagents in DMF: AA + HOBt (200 μl, 0.5 M of each component) followed by PyBOP (200 μl, 0.5 M), and DIEA (400 μl, 0.5 M). The resin was subsequently shaken at room temperature for 30 min. Capping employed the stepwise addition of 30% Ac_2O in DMF (0.5 ml), then DIEA (0.5 ml, 0.5 M in DMF) for 10 min. Side chains of Tentagel-linked peptides **2** and **4** were removed by treatment with TFA/H_2O (95 : 5, 1 ml) for 3 h, followed by washing with TFA (1 ml), DMF (4 × 1 ml), CH_2Cl_2 (4 × 1 ml), MeOH (4 × 1 ml) and H_2O (4 × 1 ml). Cleavage of peptides **1** and **3** from the resin with TFA/H_2O (95 : 5) for 3 h was followed by analysis by HPLC and ESI-MS mass spectrometry.

Reagents and Conditions for the On-bead Assay

Tentagel beads **2** and **4** (1 mg) were treated with a solution of Abl tyrosine kinase in buffer (1 μl Abl enzyme was added to buffer comprising 18.5 μl ddw, 2.5 μl Abl reaction buffer (New England Biolabs), 2.5 μl 10 × BSA (New England Biolabs) and 0.5 μl 100 mM ATP (New England Biolabs)) for 24 h at 30 °C. The beads were then heated to 90 °C for 10 min to denature the enzyme. The beads were washed (3 × 100 μl for 5 min) with PBS-Tween (150 mM NaCl, 10 mM NaH_2PO_4, 0.1% Tween 20) followed by washing (3 × 100 μl) with PBS (150 mM NaCl, 10 mM NaH_2PO_4). The beads were then dispersed in blocking buffer (100 μl, 1% dry non-fat milk in PBS) and incubated for 0.5 h at 37 °C. Mouse anti-phosphotyrosine IgG (1 mg/ml, 2 μl) was added to the suspension, which was held at room temperature for 1 h. Beads were washed with PBS-Tween (3 × 100 μl for 5 min) followed by washing with PBS (3 × 100 μl). Blocking buffer (100 μl) was added and the suspension warmed to 37 °C for 0.5 h. AP-goat anti-mouse IgG conjugate (diluted from 1 mg/ml to 0.5 mg/ml by addition of glycerol, 4 μl) was added and the suspension held at room temperature for 1 h. Alternatively, a mixture of mouse anti-phosphotyrosine IgG (1 mg/ml, 2 μl) and AP-goat anti-mouse IgG conjugate (diluted from 1 mg/ml to

0.5 mg/ml by addition of glycerol, 4 µl) was added and the suspension held at room temperature for 1 h. Beads were washed with PBS-Tween (3 × 100 µl for 5 min) followed by washing with PBS (3 × 100 µl). BM Purple AP substrate (used undiluted from the manufacturer, 100 µl) was added to the bead suspension. After 30 min (Figure 4A, B) or 1 h (Figure 4C–F), the substrate was removed by washing the beads with ddw (3 × 100 µl for 5 min).

RESULTS AND DISCUSSION

Synthetic peptides **2** and **4** were synthesized on amino Tentagel resin for the on-bead assay (Figure 1). Analogous soluble peptides **1** and **3** were prepared for analysis using Rink amide resin. Peptide synthesis employed N_α-Fmoc methodology,[28] commercially available Fmoc-protected amino acids and PyBOP as the coupling reagent.[29] The coupling and Fmoc deprotection steps were monitored by detection of any residual free amines with ninhydrin.[30] Synthesis of soluble peptides **1** and **3** on Rink amide resin enabled side chain cleavage and concomitant release from the resin upon treatment with TFA. This permitted analysis of the corresponding soluble products by HPLC and mass spectrometry (Figure 2), which confirmed the identity of the expected products and indicated overall yields of >70%. Peptides **2** and **4** were synthesized in parallel and were retained on the resin after side-chain deprotection with TFA. Peptide **5** corresponds to the product derived from treatment of **2** with v-Abl kinase and ATP. These peptides were designed based on the optimal Abl peptide substrate EAIYAAPFA (E: glutamic acid, I: isoleucine, Y: tyrosine, A: alanine, P: proline, F: phenylalanine), which was previously identified from a combinatorial peptide library.[31] The phenylalanine analogue **4** served as a negative control.

Phosphorylation of peptides on beads and detection of phosphotyrosine products was accomplished as shown in Figure 3. Briefly, beads were treated with Abl, heated to 90 °C to denature the enzyme, washed to remove soluble protein, and subsequently treated with the mouse anti-phosphotyrosine antibody. Exposure to goat anti-mouse AP conjugated secondary antibody typically constituted a separate step, although the antibodies could be pre-mixed

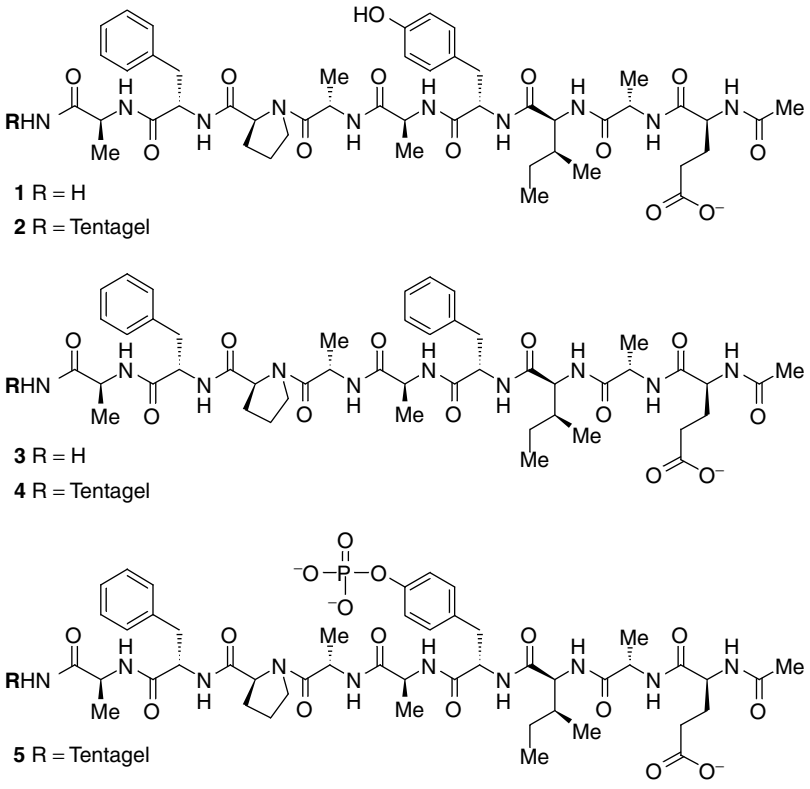

Figure 1 Structures of soluble and bead-linked peptides.

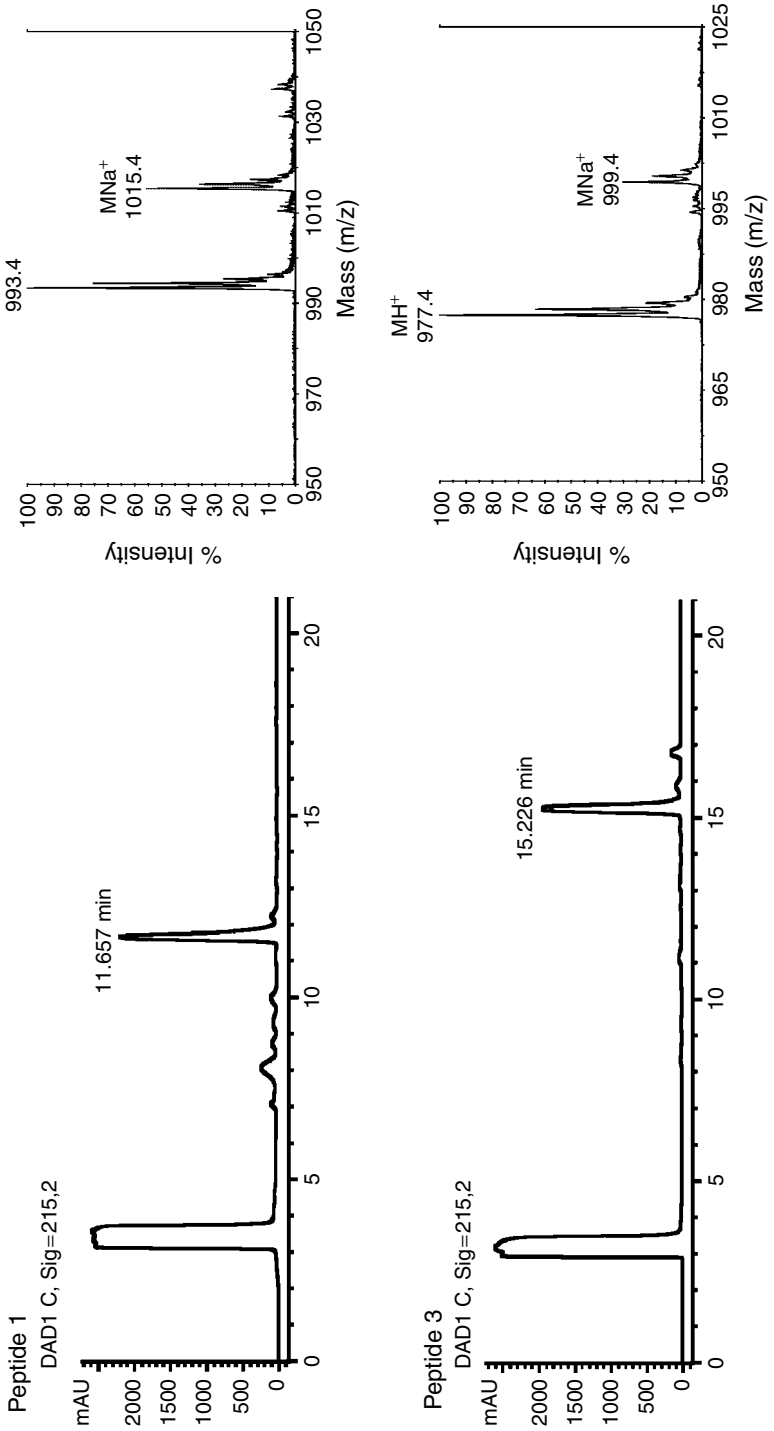

Figure 2 HPLC and mass spectral analysis of soluble peptides **1** and **3**.

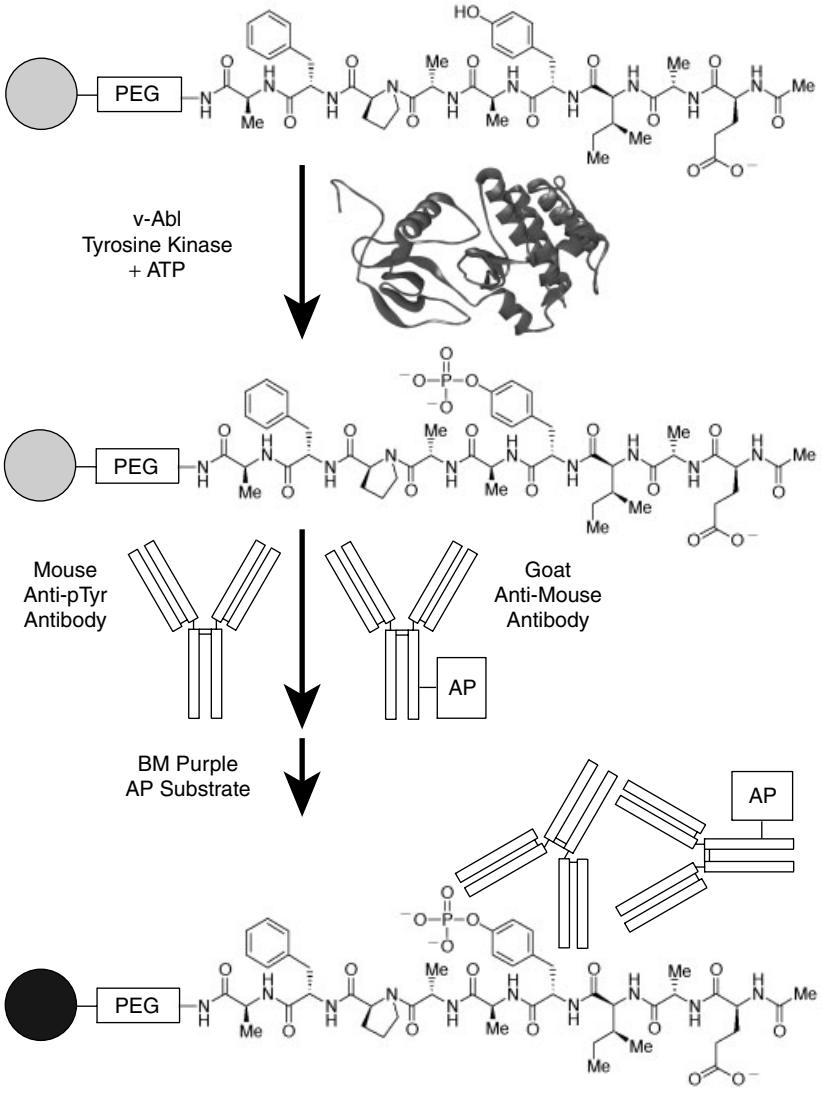

Figure 3 Schematic depiction of on-bead phosphorylation and detection of phosphotyrosine.

to yield similar results. Beads were then treated with BM Purple AP substrate, a proprietary dye related functionally to 5-bromo-4-chloro-3-indolyl phosphate,[32] which unmasks an insoluble blue dye upon phosphate hydrolysis by alkaline phosphatase. Representative micrographs of bead staining are shown in Figure 4. These results demonstrated that beads bearing the phosphotyrosine-containing peptide (**5**) can be readily distinguished from those bearing the phenylalanine control (**4**) or the tyrosine-containing precursor peptide (**2**). Analysis of phosphorylation of a ca. 1 : 1 mixture of **2** and **4** confirmed that tyrosine-containing beads can be manually isolated by pipette from beads bearing the phenylalanine analogue indicating that this method could be useful for the identification of PTK substrates from combinatorial libraries.

CONCLUSIONS

The simplicity of the assay described herein should facilitate elucidation of the specificities of uncharacterized protein tyrosine kinases. The development of improved methods for characterizing the activity and specificity of tyrosine kinases will enhance our understanding of the mechanisms of biological signal transduction involved in the progression of numerous diseases.

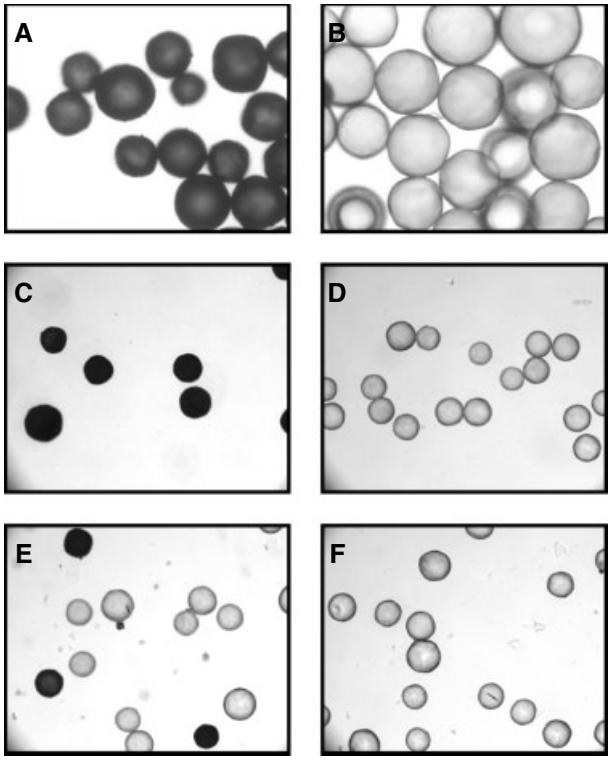

Figure 4 Micrographs of chemically modified synthesis beads treated with detection reagents. (A) Treatment of **2** with v-Abl/ATP followed by addition of mouse anti-pTyr antibody, goat anti-mouse-alkaline phosphatase antibody and BM Purple substrate (20X objective). (B) Treatment of phenylalanine control **4** with the conditions described for panel A (20X objective). (C) Beads shown in panel A imaged with a 10X objective. (D) Beads shown in panel B imaged with a 10X objective. (E) Treatment of a ca. 1 : 1 mixture of **2** and **4** with the conditions described for panel A (10X objective). (F) Treatment of **2** with ATP, mouse anti-pTyr antibody goat anti-mouse-alkaline phosphatase antibody and BM Purple substrate alone (10X objective).

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