USE OF COPPER-LOADED HIS-TAG BEADS IN THE SCINTILLATION PROXIMITY ASSAY

Julie Evans, Mike Looker, Gerard Brophy, Maxine Beveridge and Albert Santos*

Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England (Telephone +44 1222 526417, Fax +44 1222 526474, e:mail Cardiff.SPA.Group@eu.amershambiosciences.com)

Introduction

Porath in 1975 introduced the term Immobilised Metal Affinity Chromatography (IMAC) for the separation of proteins[1], originally using iminodiacetic acid (IDA) and tris(carboxymethyl)ethylene diamine (TED) based polymers, which bind a range of first-row transition metals such as Ni^{2+} and Cu^{2+}. Further chelators based on ethylenediamine tetraacetic acid (EDTA) have also been decribed in the literature[2]. These metal chelates in turn bind histidine residues via the latters' ε- or δ-nitrogen groups. The property of these metal-chelate complexes for use in purification of histidine-tagged fusion proteins and oligopeptide hybrid fusion products is well documented. Using this concept, the his-tag beads developed by Amersham Biosciences are novel scintillation proximity assay (SPA) bead formulations containing scintillant, where the outer surface of the bead has been modified by a coating of a chemical chelate containing bound copper. These beads enable the trapping and quantification of histidine-tagged fusion proteins and their binding partners using SPA technology.

In a **direct assay format**, the SPA beads could be used to trap and quantify the binding of a radiolabelled histidine (his)-tagged fusion protein, peptide or oligopeptide (such as a kinase substrate, using [^{33}P]ATP as the donor molecule).

In an **indirect format**, the SPA beads could be used to trap and quantify the association of a radiolabelled binding partner to a histidine (his)-tagged fusion protein, peptide or oligopeptide.

Amersham Biosciences evaluation studies have been performed using the direct binding to the SPA bead of a model [^{3}H]tyrosine-(histidine)$_6$-alanine ([^{3}H]YHHHHHHA) peptide. In addition a casein kinase II enzyme assay with a peptide substrate (HHHHHHEEESEEE) and [^{33}P]ATP as the donor molecule was also performed.

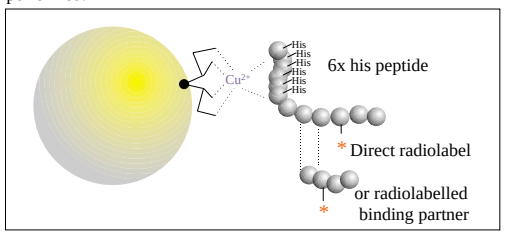

Figure 1. Copper-loaded his-tag SPA bead concept

Experimental and Results

Model Assay

The binding of a model peptide to both yttrium silicate (YSi) and polyvinyltoluene (PVT) copper-loaded his-tag beads was shown in this model assay. The assay was performed in a 96 well microplate using 0-22pmoles [^{3}H]YH$_6$A peptide and 10µg bead in a total volume of 110µl. The assay buffer for YSi beads was PBS, 0.2% (w/v) BSA and for polyvinyl toluene (PVT) beads, PBS, 0.5% (w/v) BSA. The assays were shaken (YSi for 1 hour and PVT for 2 hours). The YSi assay was counted after settling for 1 hour and the PVT assay was counted in suspension. The results, shown in Figure 2 indicate that between 5pmoles and 10pmoles [^{3}H]YH$_6$A peptide were required to saturate 10µg of both bead types.

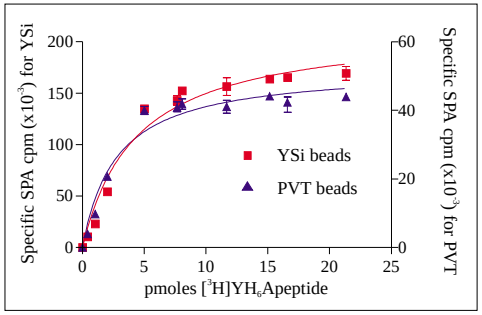

Figure 2. Saturation binding for YSi and PVT his-tag beads with [^{3}H]Y(H$_6$)A peptide using 10µg bead per well. All data points in this and subsequent figures are means of 3 replicates.

Competition binding between a di-iodo his peptide (di-iodo^{tyr})-YH$_6$A and the [^{3}H]YH$_6$A peptide to YSi and PVT his-tag beads is illustrated in Figure 3. EC_{50} values for YSi and PVT beads were 128.8nM and 150.5nM respectively.

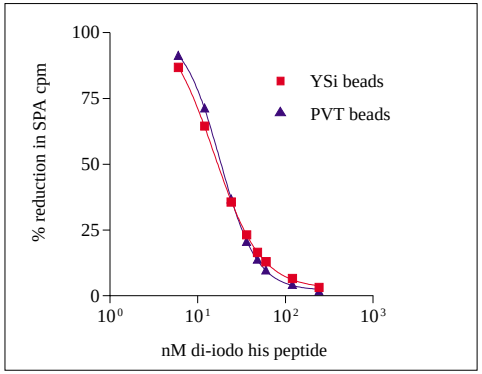

Figure 3. Competition binding curve for di-iodo his peptide against 4.2pmoles [^{3}H]YH$_6$A peptide using 10µg his-tag beads.

Casein kinase II Assay

Casein kinase II (CKII) is a protein serine/threonine kinase that is ubiquitously distributed in eukaryotes[3]. Evidence shows that CKII is an important component of signalling pathways that control growth and cell division. In particular, CKII is known to phosphorylate, and in some cases, regulate, the activity of a variety of regulatory nuclear proteins such as nuclear oncoproteins, transcription factors and enzymes involved in other aspects of DNA metabolism.

Due to the high energy of [^{33}P], the CKII assay requires bead settling or centrifugation[4]. This minimises non-specific proximity effects caused by unbound [^{33}P] in solution.

The SPA CKII kinase assay was performed in a 96 well microplate using CKII enzyme from rat liver. All reactions were carried out in 20µl volume at 37°C with 0.2µCi [^{33}P]ATP (Amersham Biosciences) and 2 µM peptide substrate (H EEESEEE) in 50mM MOPS, pH7.25, 10mM $MgCl_2$ and 150mM NaCl. Reactions were terminated by addition of 250µg YSi his-tag SPA beads in 200µl stop reagent (PBS, 0.2% (w/v) BSA, 50mM ATP, 1% (v/v) Triton™ X-100) or 500µg PVT his-tag SPA beads in 200µl stop reagent (PBS, 0.5% (w/v) BSA, 50mM ATP, 1% (v/v) Triton X-100). Assays were shaken, (YSi for 1 hour and PVT for 2 hours) followed by centrifugation for 5 mins at 1000rpm. The SPA signal was dependent on enzyme concentration as illustrated in Figure 4. Substrate depletion was observed beyond 5-10mU enzyme.

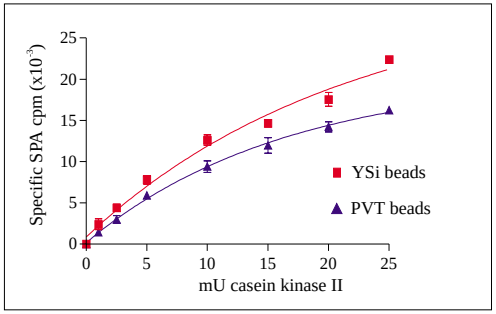

Figure 4. CKII enzyme concentration dependency using both YSi and PVT his-tag SPA beads and a range of 1mU to 25mU enzyme. Incubation was for 30 minutes at 37°C.

The SPA signal was found to be dependent on peptide concentration as illustrated in Figure 5. For both bead types, the graph is pseudo-linear up to 4µM peptide concentration and then starts to level off indicating enzyme depletion.

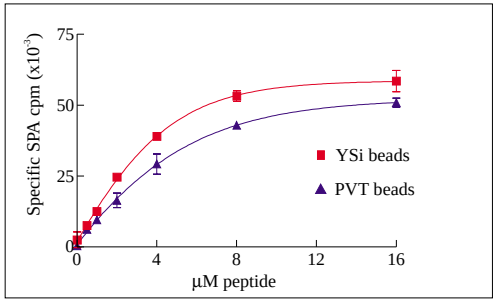

Figure 5. Specific SPA cpm as a function of peptide concentration using both YSi and PVT his-tag SPA beads. 0.5µM to 16µM peptide was incubated with 10mU CKII enzyme for 30 minutes at 37°C. 500µg YSi and 1mg PVT beads were used to capture the product.

CKII is inhibited by heparin as illustrated in Figure 6. The heparin used in this experiment was from porcine intestinal mucosa with an average molecular weight of approximately 6,000. The IC_{50} values calculated were 6.5nM for the YSi beads and 5.9nM for the PVT beads. This compares to an IC_{50} of 9.3nM in the literature[5] using heparin from bovine lung with an average molecular weight of 14,000.

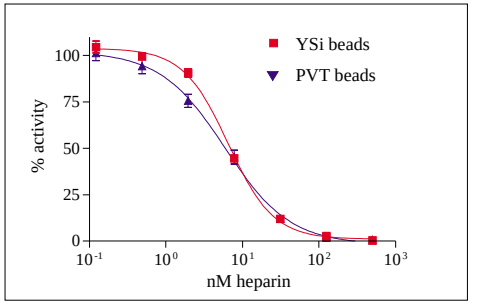

Figure 6. Inhibition of CKII by heparin using YSi and PVT his-tag beads. A range of heparin concentrations between 500nM and 0.5nM were added to the assay and incubated for 30 minutes at 37°C with 10mU of CKII enzyme.

Discussion

Amersham Biosciences has developed a copper-loaded his-tagged bead using metal chelate technology in conjunction with SPA. The beads have been shown to bind peptides in two model assays in the direct assay format.

The immobilisation of histidine-tagged fusion proteins onto SPA beads and the direct measurement of interaction with their binding partners has a number of potential advantages. The beads could be used to perform partial purification of his-tagged protein from crude preparations such as cell lysates. The number of manipulations in the assay is reduced because the link between the bead and the fusion protein is simplified. In addition, modification of the fusion protein itself is not required. The copper-loaded his-tag SPA beads are an important addition to the range of SPA bead types.

References

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