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**Isolation of Bowman-Birk-Inhibitor from soybean extracts using novel peptide probes  
and high gradient magnetic separation**

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## **Abstract**

Soybean proteins offer exceptional promise in the area of cancer prevention and treatment. Specifically, soybean Bowman-Birk inhibitor (BBI) has the ability to suppress carcinogenesis *in vivo*, which has been attributed to BBI's serine protease activity, made possible by the presence of two distinct binding sites for trypsin and chymotrypsin. The lack of molecular tools for the isolation and identification of this protein has made it difficult to work with, limiting progress as a significant candidate in the treatment of cancer. This study has successfully identified a set of novel synthetic peptides targeting the Bowman-Birk inhibitor, using a phage display screening approach, and has demonstrated the ability to isolate BBI from crude mixtures. These peptide probes have been covalently immobilized on superparamagnetic (SPM) microbeads to allow for high gradient magnetic purification from crude soy whey mixtures in a single step. Our ultimate goal is for the described probe to be utilised to facilitate the isolation of this therapeutically relevant protein for low cost, scalable analysis and production of BBI.

## **Keywords**

Soybean proteins; Bowman-Birk Inhibitor; phage display; superparamagnetic particles; purification; soluble peptide binders.

## 1. Introduction

The soybean derived Bowman-Birk Inhibitor (BBI) has long been recognised as an anti-cancer agent with abundant epidemiological evidence pointing at diets rich in soybean-derived products resulting in lower incidences of cancer (Fournier, 1998). Areas in Asia, especially Japan, have particularly low cancer mortality rates, a phenomenon that has been attributed to the large-scale consumption of soybean products in this region. The suppressive effects of BBI on the carcinogenic process were initially described in 1983 (Yavelow, Finlay, Kennedy, & Troll, 1983) and have since demonstrated similar effects across different species and multiple organ systems. BBI has also been implicated in the treatment of multiple sclerosis, where daily oral administration of a concentrated form of BBI, BBIC, has consistently suppressed multiple sclerosis in animal models (Gran et al., 2006). Unsurprisingly, in 1992, BBI gained Investigational New Drug status from the Food and Drug Administration (FDA) (Kennedy, 1998) and later entered human trial stages, producing promising results and low toxicity.

Soybean proteases account for 6% of the total protein content of soybeans (Rackis, Wolf & Baker, 1986) attributed largely, to the two main inhibitors; BBI and Kunitz trypsin inhibitor (KTI). Multiple BBI isoforms have been described in soybean cultivars, each differing in molecular weight and the extent of interaction with their substrates (Tan-Wilson, Chen, Duggan, Chapman, Obach & Wilson, 1987). BBI is a serine protease inhibitor with a molecular weight of 7.8 kDa. Its 71 amino acids are cross linked by seven, conserved disulfide-bridges, making it a robust protein (Wu & Sessa, 1994), allowing it to withstand digestive system acidity and remain stable at 100°C for up to 10 min (Losso, 2008). The double-headed protein contains distinct, kinetically independent antitryptic and antichymotryptic domains with which BBI can form a 1:1 complex or a ternary complex with both substrates (Werner & Wemmer, 1991). Each domain comprises a conserved motif of three  $\beta$ -strands and evidence suggests that divorcing these domains by scission yields two

enzymatically active components. Specifically, BBI binds to trypsin and/or chymotrypsin *via* a conserved, exposed protease binding loop observed in all BBI variants. The surface-exposed loop, constrained by the presence of cysteine residues, is complimentary to the protease active site and resides to inhibit protease activity (Bode & Huber, 1992). This inhibition is believed to account for the anti-cancer properties of the BBI. Despite knowledge of its substrates, the exact mechanism by which BBI elicits its anti-cancer effects remains a mystery.

Mouse monoclonal antibodies against BBI have been raised laboriously using the hybridoma approach, allowing quantitative determination of BBI in soy protein mixtures (Brandon, Bates et al. 1989; Seerama & Gowda, 1997; Mao et al., 2005) but the lack of probes and purification methodologies has impeded progress. To date, tedious and time consuming, multi-step chromatographic procedures have been the convention for BBI purification. In addition, no affinity-based peptide ligands for BBI have been identified, making the isolation and consequently the study of this protein difficult.

Here, by designing appropriate synthetic peptides and manipulation of molecules using SPM microbeads, we detail a procedure to purify BBI from crude soy whey extracts that would not be possible with classical chromatography, given the tendency of the particulate matter to block the column. A set of novel BBI peptide binders has been identified by phage display, a highly efficient affinity-selection technology. Subsequent assays have confirmed the capacity of isolated peptides to selectively bind to the BBI, thereby acting as specific molecular probes. We detail our efforts on the isolation of BBI from complex soy protein extracts based on magnetic particles functionalized with these synthetic peptide scaffolds. This approach circumvents both the time and multi-step issues inherent previously in the chromatographic purification of BBI. Bioseparation using magnetic particles coupled to affinity ligands offers a rapid, efficient and scalable alternative to column chromatography. We believe the described probe will contribute to the evaluation of BBI and the efforts to fortify food with its

chemopreventive properties, leading ultimately to the development of functional and medical foods.

## **2. Materials and methods**

### *2.1 Materials*

Purified BBI and soy extract samples were provided by the Solae Company (Aarhus, Denmark). The Ph.D.-12™ phage display library ( $\sim 1.2 \cdot 10^9$  independent clones) was purchased from New England Biolabs (Hitchin, Hertfordshire, UK). *Escherichia coli* ER2738 (F<sup>+</sup> strain) was used for M13 phage propagation and was cultured at 37 °C on Luria–Bertani agar or broth, supplemented with tetracycline. Mouse monoclonal HRP labelled anti-M13 antibody was purchased from GE Healthcare (Buckinghamshire, UK). Ultrasensitive streptavidin coupled to peroxidase was from Sigma Aldrich (Dublin, Ireland). Bovine serum albumin and 1-Step ultra TMB (3,3',5,5'-tetramethylbenzidine) were from Fisher Scientific (Dublin, Ireland). DNA extraction was performed using a QIAprep spin M13 kit from Qiagen (West Sussex, UK) and sequencing was performed using the 96 gIII primers (5' d(CCCTCATAGTTAGCGTAACG)3') from New England Biolabs (Hertfordshire, UK). Protein standards were procured from Alpha-technologies (Wicklow, Ireland). All other reagents were purchased from Sigma-Aldrich Ireland Ltd (Dublin, Ireland) of molecular biology research grade. DNA manipulations were carried out using conventional molecular biology procedures (Sambrook, 1989). All binding and purification experiments were performed at least in duplicate. DNA translations were performed using the online translation tool (ExPASy) and sequences aligned using the Clustal program (EBI).

### *2.2 Affinity selection of BBI binding peptides using phage display*

The detailed biopanning procedures are available at the New England Biolabs website (<http://www.neb.com>). Briefly, 130 µL of purified BBI (Solae Inc, St. Louis, MO) was

immobilized in microtiter wells at a concentration of 50 µg/mL in NaHCO<sub>3</sub> buffer (0.1M, pH 8.6) overnight at 4 °C. After removal of the coating solution, microtiter wells were filled with blocking buffer, i.e., 2.5 % v/m BSA, for 90 min at 4 °C. The blocking solution was discarded and wells were washed rapidly six times with TBST (TBS + 0.1% Tween-20) before adding 130 µL of approximately 2x10<sup>9</sup> phage particles in TBST buffer to each well for 60 min at room temperature with gentle agitation. Non-binding phage were discarded. Bound phage were subsequently eluted in 100 µL of a low-pH glycine buffer (200 mM, pH 2.2) for 15 min at room temperature. Eluates were immediately neutralized by the addition of 15 µL of Tris buffer (1 M, pH 9.1) and amplified by infection of an early-logarithmic phase culture of *E. coli* (ER2738) in Luria-Bertani medium supplemented with tetracycline (LB-Tetracycline) for 4.5 hours at 37°C. Recombinant phage were purified by double polyethylene glycol (PEG)-NaCl (20% w/v PEG, 2.5 M NaCl) precipitation. In subsequent rounds of panning, the concentration of immobilised BBI was reduced to 20 µg/mL in the second round and 10 µg/mL in the final two rounds while increasing the number of washing steps.

### 2.3 Immunoscreening

After 4 rounds of selection, 32 isolated individual phage clones were selected for purification and submitted to binding experiments by direct solid-phase immuno-assay (ELISA) with all four amplified eluates (phage pool) from the previous rounds of selection and wild type M13 virus acting as a control. Briefly, 100 µL of purified BBI (100 µg/mL in 100 mM NaHCO<sub>3</sub>) was immobilized onto a 96-well microtiter plate and incubated overnight at 4°C. Excess target solution was poured out and each well was filled with 2% BSA in PBS for 90 min at room temperature to block free binding sites. Each well was washed 5 times using PBST (0.1%) and approximately 10<sup>10</sup> phage particles in PBST were added to each well. 100 µL of HRP-conjugated anti-M13 pVIII monoclonal antibody (1:2500) was incubated in each well for 60 min. The wells were washed 5 times as before, followed by the addition of 100 µL of TMB substrate to each well. The reaction was terminated by the addition of 30 µL of 1 M sulphuric

acid. Absorbance was measured at 450 nm on a microtiter plate reader (Biotek EL808, Winooski, USA).

#### *2.4 Peptide design, synthesis and characterization*

Peptide selection for synthesis (Entelechon, Germany) was based on the strong, reproducible and specific reaction of phage clones against BBI in the phage ELISA assays (described previously). The 12-mer linear peptides (clone 1, 2, 3 and negative control: VAMVLPGVMGTL) were synthesised at >90 % purity. The C-terminus was elongated with a GGGS-CONH<sub>2</sub> tail to mimic the GGGS-peptide spacer between the random peptide sequence and the phage protein pIII and to block the negative charge of the carboxyl terminus. The biotinylated peptides and multimeric structures (“*Dendrimer*”) ((GAMHLPWHMGTL)<sub>4</sub>K<sub>2</sub>KGSGCG) were synthesised at >95 % purity, with an additional cysteine-biotin (underlined) added to the C-terminus. All synthetic peptides were reconstituted in sterile deionised H<sub>2</sub>O to a concentration of 2 mg.mL. The ability of biotinylated synthetic peptides at a concentration from 0 to 200 µg.mL to bind to the immobilized purified BBI, soy pulp, soy whey extracts and BSA (as a negative control) was analyzed in solid phase assays. In this case, the biotinylated synthetic peptides were incubated on BBI coated wells (100 µg.mL) and detected using a streptavidin-HRP conjugate (1:500). Binding and absorbance readings were finally carried out using the TMB substrate as described before.

#### *2.5 Surface Plasmon Resonance*

The BIAcore T100 instrument and all the reagents for analysis were obtained from GE Healthcare Ltd. (Buckinghamshire, UK). Soluble purified BBI was immobilized (approximately 500 RU) on a carboxymethyldextran CM5 sensor chip activated with a 1:1 mix of N-hydroxysuccinimide (50 mM) and N-ethyl-N-(dimethylaminopropyl)- carbodiimide (200 mM) by a 7 min pulse. BBI specific peptides were then passed over the BBI surface in



HBS-EP buffer [0.01 mM Hepes (pH 7.4), 0.15 mM NaCl, 0.005% polysorbate 20 (v/v)] at a flow rate of 20  $\mu\text{L}\cdot\text{min}^{-1}$  at 25 °C. Glycine-HCl (10 mM, pH 2.0) was injected for 30 sec at 20  $\mu\text{L}\cdot\text{min}^{-1}$  to regenerate the sensor chip between successive samples.. The dissociation constant  $K_D$  was calculated from  $K_D = k_{\text{off}} / k_{\text{on}}$ . All experiments were carried out at the UCD/NIBRT Biacore facilities (Dublin, Ireland).

## *2.6 Magnetic particle synthesis, functionalisation and characterisation*

Superparamagnetic particles were synthesized according to an emulsion solvent evaporation procedure developed in our lab (Shang, 2006; Muzard, Platt and Lee, to be published elsewhere). The magnetic properties of the particles were determined using a Quantum Design Superconducting Quantum Interference Device (SQUID). The diameter of the particles was determined (using a Hitachi S-4200 SEM) to be 1  $\mu\text{m}$  with a CV of 20 %. The surface of the iron oxide particle was functionalized with multimeric peptide 1 using sulfo-SMCC coupling chemistry as follows: 1 mL of 12 mg/mL carboxyl coated particles was washed three times with MES buffer pH 6.0 and 6 mg of both EDC and Sulfo-NHS were added before the sample was sonicated for 30 sec and incubated for 1 hour at room temperature. The sample was washed three times with carbonate buffer pH 8.2, before 5% PEI (Mw 1300) in carbonate buffer pH 8.0 was added and the solution was incubated for 2 hours. To couple the PEG to the PEI, the sample was washed with carbonate buffer pH 8.2, 0.6 M  $\text{K}_2\text{SO}_4$  before 12 mg of Boc-PEG-NHS (Mw 3000) in 1 mL of the high salt carbonate buffer was added to the particles and the sample was then rotated at 50 °C for 12 hours. To couple the microparticles to the selected peptide the Boc groups were removed by incubating the sample in a 50 % v/v TFA solution for 5 min. The suspension was then washed three times with PBS pH 7.5 before a 4 mg/mL solution of Sulfo-SMCC in PBS was added to the particles for 40 minutes. The solution was then resuspended in 1 mL PBST pH 6.75 and immediately added to the multimeric peptide 1 (~1 mg) overnight. Finally, the multimeric peptide linked magnetic particles were washed five times with PBST and stored at 4 °C before

use. Peptide immobilization on particles was analysed using a MALDI TOF mass spectrometer (SAI, UK) operating at 20 kV in linear mode with mass spectrometry grade sinapinic acid as a matrix. All zeta potential measurements were performed using a Malvern Zetasizer Nano ZS, using the DTS-1060 cells. 20 mL of particles were placed into a 1.5 mL centrifuge tube and washed three times with 1 mL of the required buffer before being resuspended in the appropriate buffer and pH.

### *2.7 Magnetic based purification of BBI from crude soy samples*

Soy crude protein extracts (~100 mL) were incubated for 16 h at 20 °C with 1 mL functionalized magnetic particles (10 mg.mL). After extensive washing with PBST until  $A_{280}$  reached 0.001, bound proteins were eluted in fractions of 100  $\mu$ L of pH 2.2 (glycine, 100 mM) and fractions > 0.1 being submitted to a MALDI-TOF assay, SDS-PAGE analysis and enzymatic assays. Identification of the stained proteins was performed by mass spectrometry. Bands of interest were excised out of the SDS-PAGE gel and submitted to tryptic digestion, according to the protocol described by Shevchenko et al (1996). The resulting tryptic peptides were desalted and concentrated using ZipTips (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. Peptides were re-suspended in 12  $\mu$ l of 0.1% formic acid. Each sample was loaded onto a Biobasic C18 Picofrit TM column (100 mm length, 75  $\mu$ m ID) and was separated by a 72 min reverse phase increasing acetonitrile gradient (0-50 % acetonitrile for 50 min) at a flow rate of 30 nL. min<sup>-1</sup> on an Dionex Ultimate 3000 chromatography system incorporating an auto-sampler. Nano-ESI LC-MS/MS was carried out on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer. The instrument was operated in positive ion mode with a capillary temperature of 200°C, a capillary voltage of 9 V, a tube lens voltage of 100 V and with a potential of 1800 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap, followed by MS/MS analysis of the 5 most intense ions using

the ion trap. PEAKS Studio version 5.2 (Bioinformatic Solutions, Waterloo, ON Canada) was used for the simultaneous database matching to the National Center for Biotechnology Information (NCBI) non-redundant database, taxonomy *Glycine Max* (soybean) and *de novo* sequencing of good-quality spectra. Trypsin was chosen as enzyme, up to three missed cleavages were allowed, and cysteine carbamidomethylation and methionine oxidation were set as variable modifications. Precursor tolerance was set at 10 ppm, fragment tolerance at 0.5 Da.

### *2.8 Enzymatic assays*

Inhibition of trypsin activity was measured at 25°C in 0.4 M Tris-Cl, 0.01 mM CaCl, pH 8.1, and with 1 mM (p-tolylsulfonyl)-L-arginine ethyl ester as substrate (Hummel, 1959). Inhibition of the chymotrypsin activity was determined at 25°C in 0.04 M Tris-Cl, 0.05 M CaCl, pH 7.8, with 0.5 mM benzoyl-L-tyrosine ethyl ester as substrate (Hummel, 1959). Readings were recorded every 20 sec at 256 nm in a UV/VIS NIR spectrophotometer (Varian Cary model 6000i, Agilent Technologies, Palo Alto, USA).

## **3. Results**

### *3.1 Identification of novel affinity ligands by phage display*

Novel, peptide-based binding reagents against purified soybean BBI were identified by screening a bacteriophage library displaying dodecapeptides (12-mer) containing  $\sim 10^9$  independent clones. Figure 1a presents the endpoint binding signal, as determined by direct immuno-assay of the amplified eluted phage pool obtained after each round of selection. The steady increase in the percentage of phage recognising the BBI as each round of selection proceeds is reflective of the enrichment of the whole population in favour of displayed peptides specific for the BBI.

From the enriched day 4 population, individual clones were isolated, amplified and screened for their ability to bind BBI, with each clone displaying a single peptide sequence on its surface which can be deduced by DNA sequencing. Figure 1b confirms the capacity of three isolated clones, from the day 4 population, to recognise and bind to the BBI. As expected, negative control (wild-type M13) binding was  $\sim 6\%$  of total detected binding and was not considered significant.

In total, 32 individual clones were examined for BBI binding. DNA sequencing of the 32 clones revealed 23 of the 32 individual clones to be of a single type, i.e., clone 1 (Figure 2). All the other peptides identified, i.e. sequences 2-9 in Figure 2, were only found once. The appearance of conserved amino acid motifs in high frequencies is commonly observed in phage display experiments. This is considered to be an important indication of the success of the selection procedure, together with the progressive enrichment in phage titers as increasing rounds of biopanning are performed. Analysis of the displayed amino acid sequences suggested an emergence of hydrophobic residues and a complete lack of cysteine residues.

### *3.2 Synthetic peptide structures and binding*

Based on the amino acid alignment and binding results from the individual clones, 3 peptide constructs were designed for synthesis with a C-terminal biotin moiety acting as a reporter for detection in binding experiments. During panning, the C-terminus of the displayed peptide was fused to the phage and did not possess a negatively charged carboxylate. As a result, care was taken to block the negative charge of the C-terminal carboxylate of the synthetic peptide by amidation to minimise any impact on binding. Additionally, a peptide spacer (Gly-Gly-Gly-Ser) sequence was added to the C terminus of the peptide to mimic the scenario when the peptide is fused to the phage. For further chemical conjugation of the peptide to a solid support, a C-terminal single cysteine residue was introduced, which added a unique functional group as there are no other cysteines present in the identified sequence.

Figure 3a corresponds to the solid phase binding assays using each of the individually synthesized biotinylated peptides incubated alongside immobilized BBI and soy whey extracts and followed by extensive washing and detection by streptavidin-HRP. The peptide 1 structure (GAMHLPWHMGTL) displayed the strongest intensity for the BBI. The two other synthetic peptide modules, i.e., peptides 2 (KFLGPLNHRDRH) and 3 (ANYFLPPVLSSS), also displayed intensity notably higher than the control peptide. However, given the greater binding profile, synthetic peptide 1 was chosen as the best candidate for subsequent purification of BBI from crude soy whey mixtures.

Figure 3b shows a dose-dependent binding of the selected soluble peptide 1 to the purified BBI. A lower interaction with BBI in the soy whey and soy pulp extracts was observed with 70-80% of total binding detected. Non-specific interaction with immobilized BSA was ~10% of total detected binding, which is very low considering the complexity of the sample matrix.

Real-time binding interaction analysis by surface plasmon resonance (BIAcore) was used to evaluate the affinity of the soluble peptides. The affinity of peptide 1 (GAMHLPWHMGTL) was found to be  $K_D \sim 2.5 \cdot 10^{-6}$  M (*Materials and methods 2.5*).

Incubation of the anti-BBI peptide alongside chymotrypsin and trypsin confirmed that no inhibitory effect on the active enzymatic sites was observed, thereby eliminating these regions as the sites recognised by the peptide structure (supplementary, Fig1a & b).

### *3.3 Isolation of BBI from crude soy protein extracts using magnetic particles*

Figure 4a presents a scanning electron micrograph of the 1  $\mu$ m superparamagnetic particles. The surface of the particles was monitored at each stage of the chemical modification using zeta potential analysis, which demonstrated the successful covalent immobilization of the multimeric soluble peptides to the magnetic particles (Figure 4b). The PEI polymer was chosen because it is very effective at generating a high density of amines on the particle surface, as reflected by the dramatic shift from negative to positive zeta potential (Figure 4c). The PEG layer permitted a clean separation and acted to minimise fouling. This is reflected in the high purity of the first eluted fraction analysed by SDS-PAGE (Figure 5b).

Branched, multimeric peptides often offer enhanced avidity towards their target antigen. As a result, a multimeric form of synthetic peptide 1 was designed for use in purification experiments with the general formula: (GAMHLPWHMGTL)<sub>4</sub>K<sub>2</sub>KGSGCG. The presence of multimeric peptide on the magnetic particle surface was confirmed using direct MALDI-TOF (Figure 5a) and approximately 80% binding efficiency was achieved when 10—20 times peptide excess was used. The first peak at  $\sim 6101$  m/z corresponds to the molecular size of the synthesized multimeric soluble peptide immobilized on the magnetic particles using SMCC chemistry (Figure 4B). Following incubation with soy crude protein extracts, a second peak was detected at  $\sim 7866$  m/z on magnetic particles and corresponds to the theoretical molecular

size of the BBI computed from the amino acid sequence of BBI (BBI-E isoform, 7872.8 Da, multimeric peptide, 6094.3 Da).

This confirms the successful capture of the BBI with our synthetic peptide-magnetic particle construct. In the SDS gel in Figure 5b two bands are clearly evident in the first eluted fraction, i.e, 8 and 13 kDa in lanes 2 and 3, respectively, that coincided with the expected migration profile for the BBI. The double band is a common feature when running BBI on polyacrylamide gels (Bawadi, Antunes, Shih, Losso, 2004; Birk, 1985). The ability of BBI to self-associate has previously been observed, even in reducing conditions (Sessa & Wolf, 2001). The ability of the eluted protein to inhibit the activity of chymotrypsin and trypsin was confirmed using the standard spectrophotometric assays in order to observe whether the protein had retained its post-purification biological activity, and consequently, to confirm the nature of the eluted protein as the BBI (data not shown).

We have demonstrated a recovery of ~150µg of pure BBI starting from 10mg of functionalised 1 µm sized magnetic particles. After separation on the SDS-PAGE gel, proteins immunoprecipitated by magnetic particles were then analysed by direct tandem mass spectrometry. The prominent band at ~ 8500 Da apparent molecular weight was identified as the Bowman-Birk protease inhibitor by two different peptides. The MS/MS spectrum of one of these peptides is shown in Figure 5c.

#### 4. Discussion

Naturally-occurring BBI has emerged as a therapeutically significant candidate in the treatment of multiple health defects. Perhaps its greatest potential lies in its ability to suppress carcinogenesis. With the capability of irreversibly suppressing the carcinogenic process, eliminating the need for it to be continuously present, and a favourable safety profile, it is a high potential therapeutic in the field of cancer prevention. Encouragingly, the amount of BBI reaching internal organs after oral administration has been shown to be within the range capable of preventing malignant transformation *in vitro*. (Yavelow, Collins, Birk, Troll & Kennedy, 1985). BBI is an ideal protein to work with, in terms of handling, on account of its resistance to temperature extremes and acidification. However, its extraction and purification from total soybean proteins is not straightforward. To date, the widely used purification method for BBI consists of ammonium sulfate fractionation, acid precipitation, and classical gel filtration chromatography (Bowman, 1946). More recently, hydrophobic chromatography methods have been proposed for BBI isolation from soybean total proteins (Yeboah et al, 1996). In general, such approaches are time- consuming, costly, and require the use of robust analytical equipment such as chromatographs.

New separation and isolation strategies are required to accelerate the processing of soybeans and extract the valuable target molecules amidst vast amounts of accompanying material. Conventional column and expanded bed chromatography are let down by slow adsorption and separation of the target molecules in the stationary phase. Variations in column length and sample volume mean adsorption and separation can take anywhere from hours to a day, or even more. By coupling BBI affinity ligands to magnetic supports as described here, these processes are achieved within minutes. Furthermore, chromatographic column systems will not tolerate particulate material and are therefore not suitable for isolation in the early processing stages where minute suspended solids, as is the case in crude soy whey samples, may be present and block the column.



This study introduces a novel concept in the isolation of BBI from soybean protein extracts which overcomes many of the undesirable steps present previously. To highlight this, the described magnetic separation assay for BBI takes place in a single tube in just minutes, yielding a highly pure product. Our strategy explores the use of novel peptide structures acting as affinity ligands grafted onto magnetic particles, allowing rapid purification of the BBI.

We report the identification and characterisation of the first dodecapeptide probe targeting BBI, achieved by phage display technology. Peptide-based structures serve as ideal probes, being simple to synthesise and generally small in size, allowing easy target accessibility. After four rounds of affinity selection of a sufficiently diverse phage library, the selection process appeared to select phage displaying a significant number of hydrophobic residues tethered to the pIII coat protein. Interestingly, BBI exhibits some structural peculiarities, namely exposed hydrophobic patches on the surface and the presence of charged amino acids and water molecules on the interior (Voss et al., 1996). This is in contrast to the majority of proteins where a hydrophobic core and exposed polar amino acids are typically observed. It is possible that the described peptide is binding to the BBI via a hydrophobic interaction between its side chains and the exposed hydrophobic patches on the BBI surface. As the peptide has no effect on the enzyme active sites, we can eliminate these regions as the site of contact.

With the U.S. alone producing over  $9.1 \times 10^8$  kg of soybean seed coats annually (Sessa & Wolf, 2001), sourcing BBI is not a problem. We face a dilemma in that probes for this chemopreventive agent are limited, making it a difficult protein to work with. If the effective, anti-cancer component of the soy can be rapidly sequestered by high-affinity magnetic-based peptide probes, this will facilitate the study and ultimately, the treatment and/or prevention of cancer and tumour growth.

The need for additional BBI probes is always on the increase and it is our belief that the described BBI probe will be a useful research tool in the processing of soybeans in the food industry. Our magnetic approach to BBI purification using immobilised affinity peptide ligands provides a new, faster alternative to the purification of this relevant protein and will contribute, we hope, to the speed of evaluation of BBI as a functional food source. The final product is very pure and the gentle nature of the magnetic separation process has allowed the magnetic particle-peptide construct to remain effective for multiple separation cycles, demonstrating its potential for reusability. It is possible also that the described probe can be used by investigators analytically to quantitatively determine the presence of BBI in food products claiming to possess this anti-cancer agent. Further efforts will be necessary such as increasing the binding properties and functionalities of the magnetic particles (higher affinities leading to increased surface coverage) to scale up and optimise the purification of BBI from soy protein extracts. In this manner, researchers and consumers alike can benefit from functional foods enriched with BBI at a cost-effective price.

### **Author contributions**

Designed the study: GL, JM. Performed the experiments and analyzed the data: CF, JM. Contributed materials/reagents: JM, CF, JM. Wrote the paper: CF, JM and GL.

### **Competing interests**

The author(s) declare that they have no competing interests.

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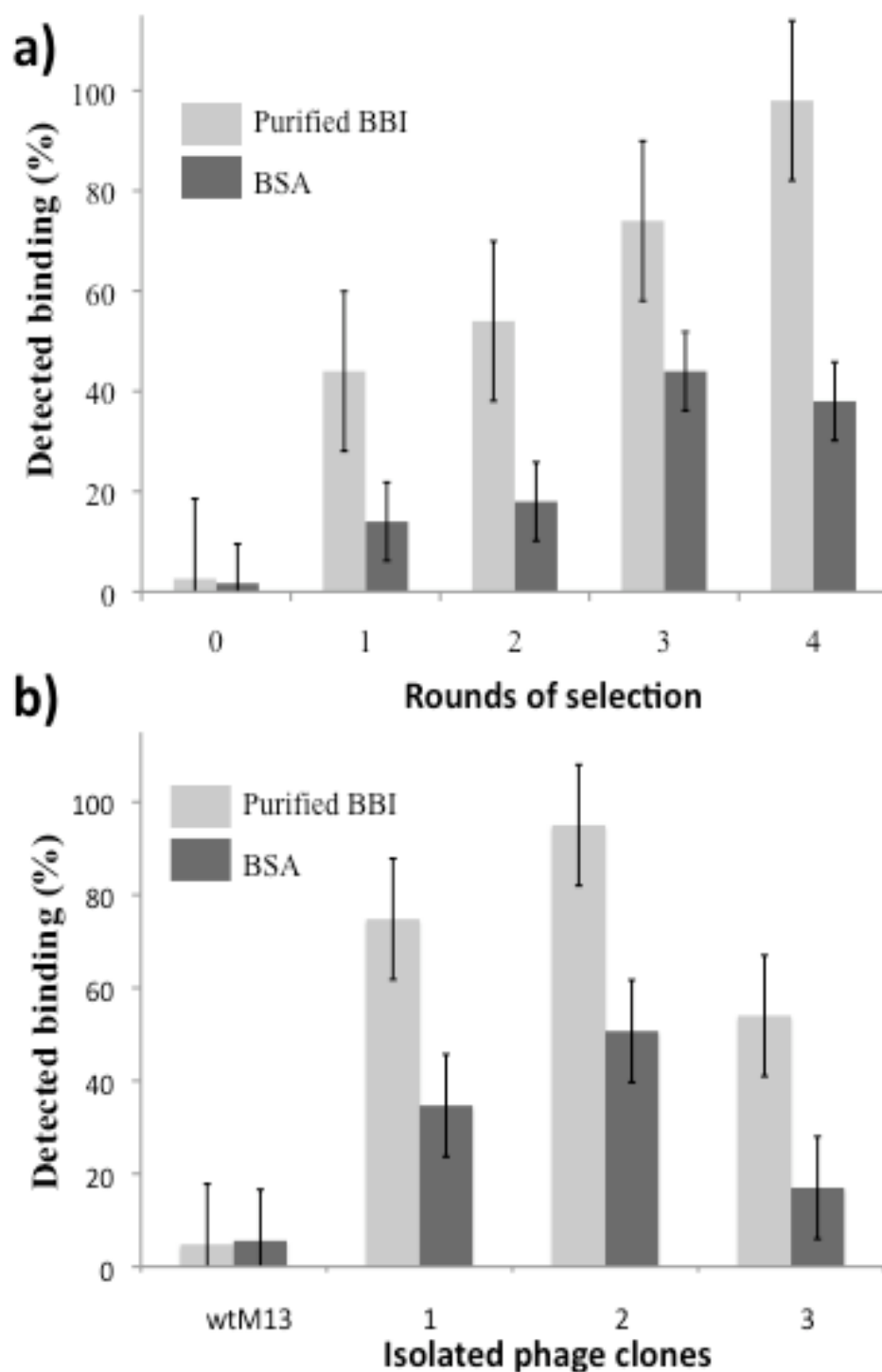
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## Figures



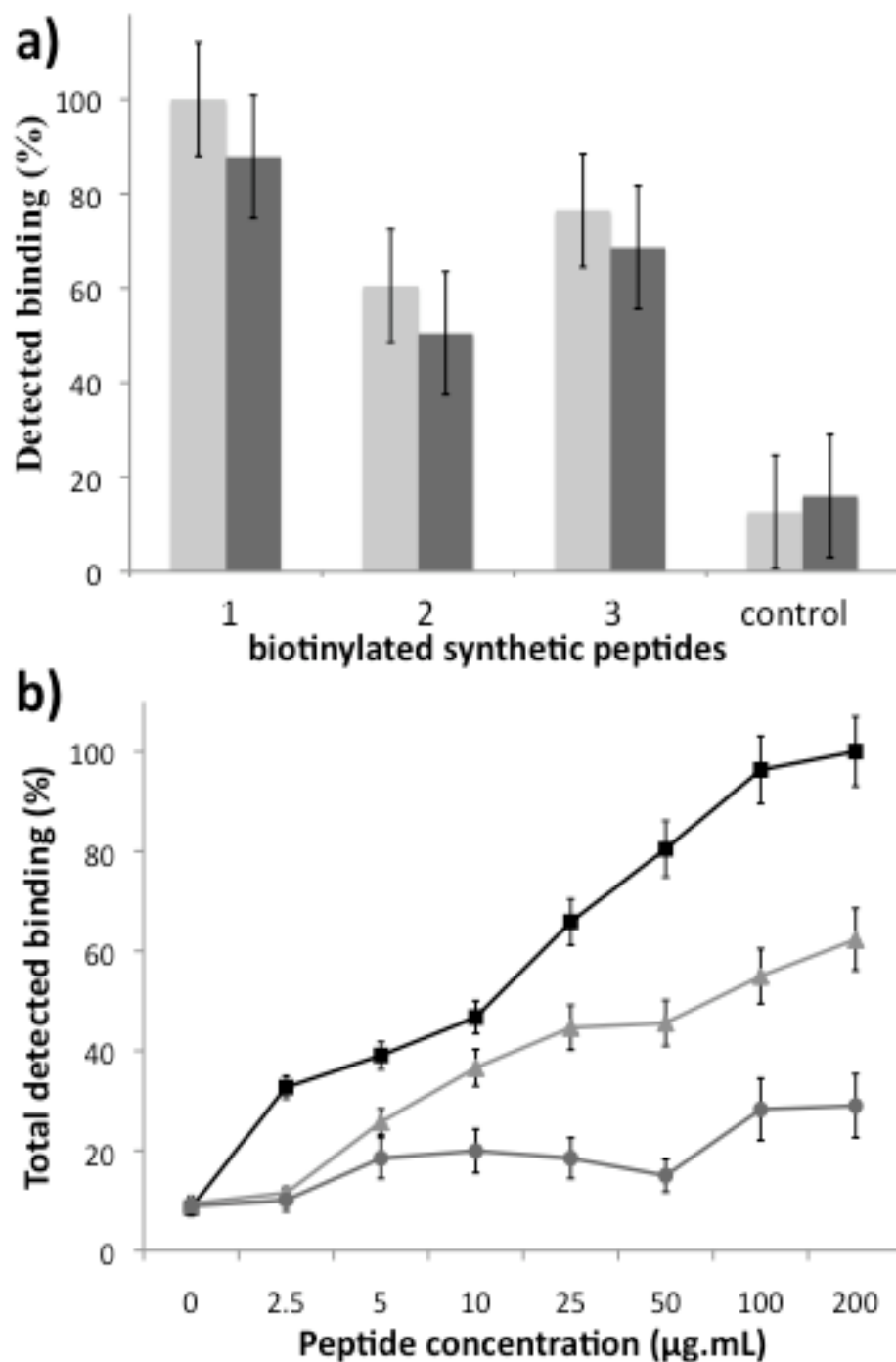
**Figure 1. Selection of recombinant phage specific for BBI.** a) Increased phage pool recognition of BBI after four successive rounds of biopanning where 100% binding was assigned to the highest colorimetric intensity b) Binding of three selected, isolated clones from the fourth round of biopanning displaying peptides specific for BBI. Clone 1 had the

sequence GAMHLPWHMGTL, clone 2 was KFLGPLNHRDRH, and clone 3 was ANYFLPPVLSSS.

**Figure 2. Amino acid sequences of isolated, individual recombinant phage clones. a)**

Amino acid sequences of the displayed peptide insert from 32 isolated phage clones. Sequence 1 which corresponds to the same phage clone was observed in 23 of the 32 clones. Selected peptides for total synthesis are indicated by the \* symbol. Amino acid residues are coloured according to the Clustal colour scheme, based on sequence conservation and similarity.

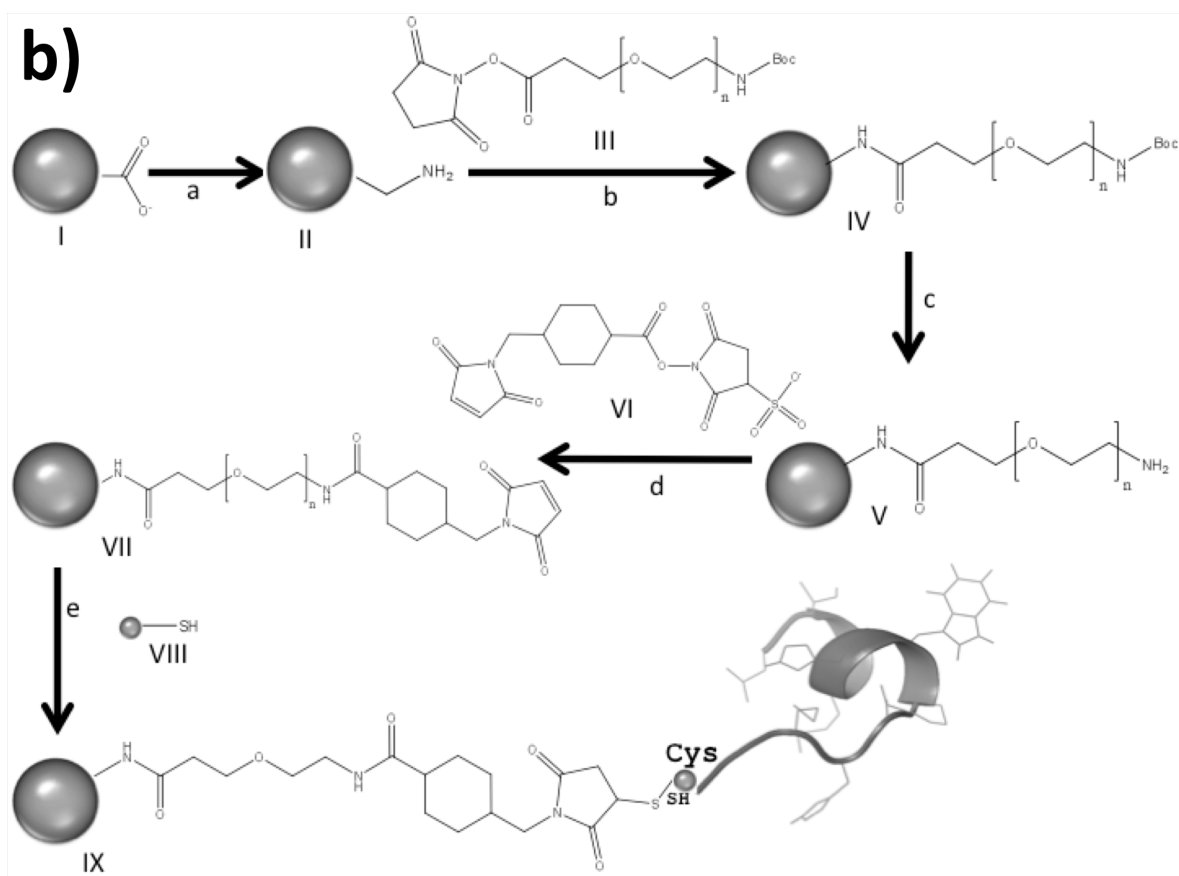
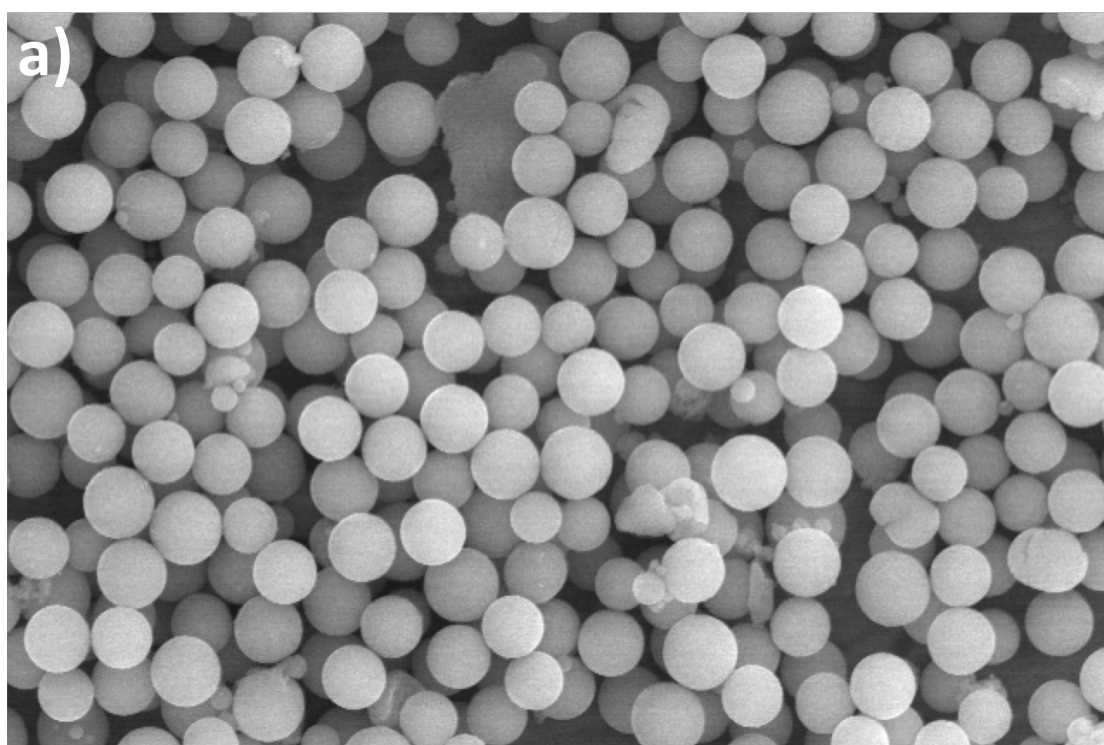




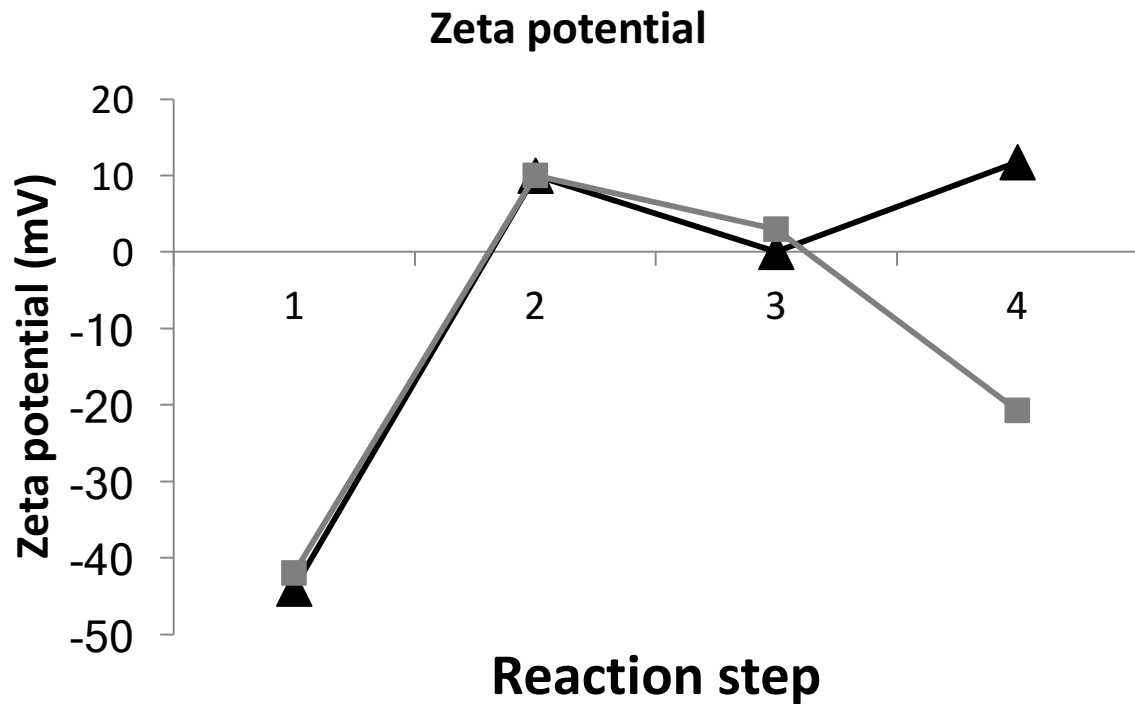
**Figure 3. Synthetic peptide screening.** a) BBI binding of each of the three designed synthetic peptide constructs (1: GAMHLPWHMGTL, 2: KFLGPLNHRDRH, 3: ANYFLPPVLSSS) and control peptides were tested by ELISA procedures using immobilized BBI (black, 100 µg/mL) or crude soy whey extract (grey) b) Dose-dependent binding of the best candidate, synthetic biotinylated peptide 1 (GAMHLPWHMGTL), to purified BBI

(black squares, 100  $\mu\text{g/mL}$ ), crude soy whey (light grey triangles) and soy pulp (grey circles).

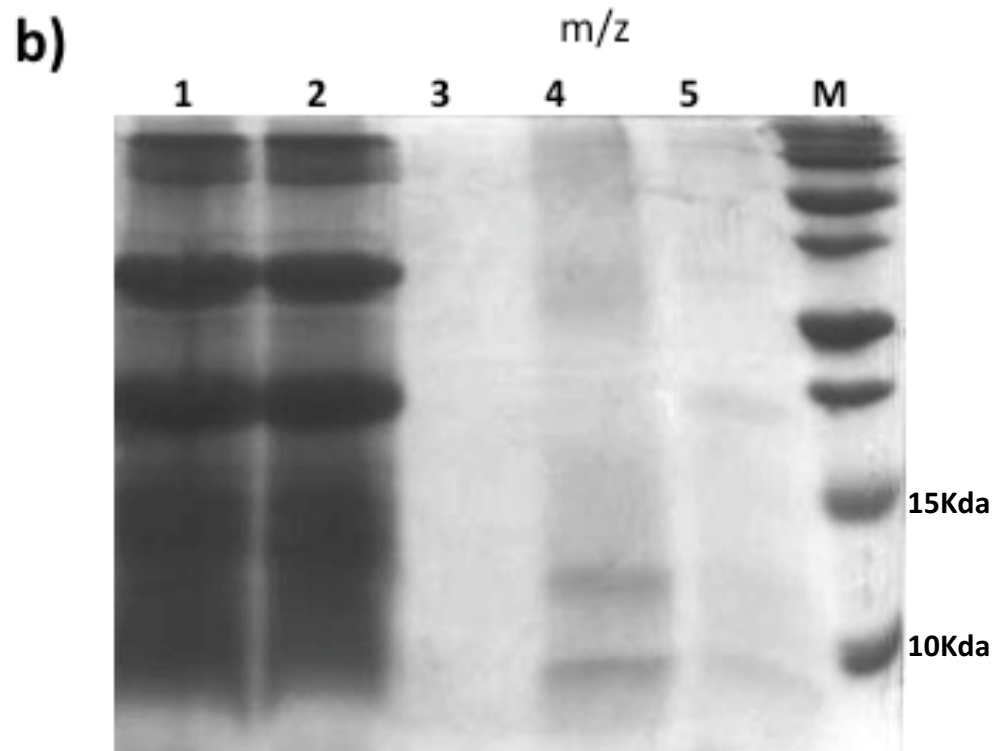
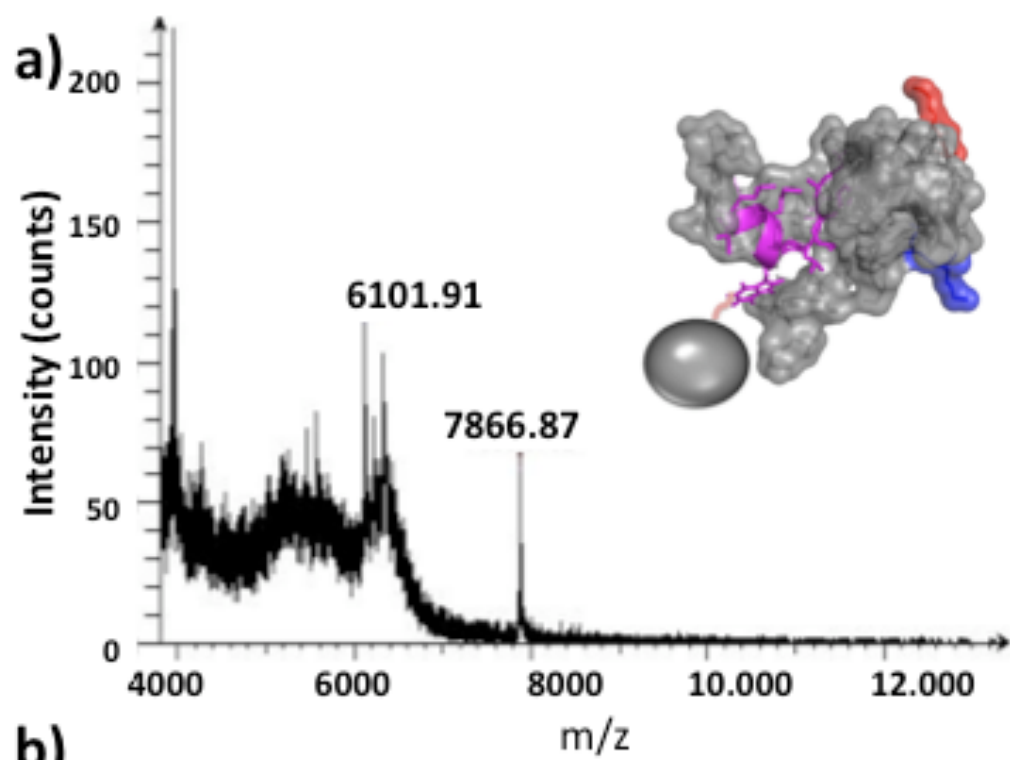
In each case, maximum percentage binding was assigned to the highest colorimetric reading.

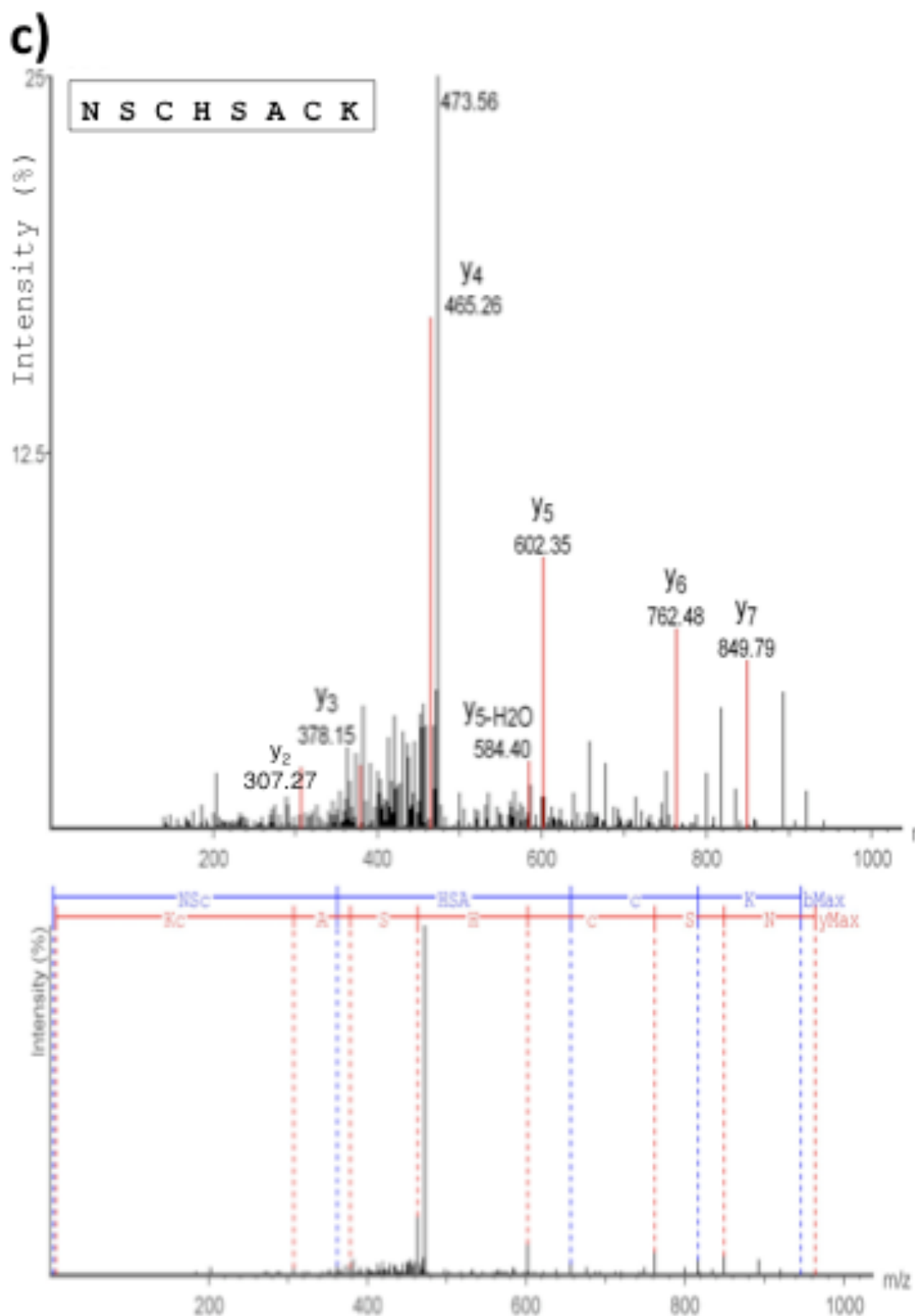


C)



**Figure 4. Particles characterization.** a) SEM image of 1  $\mu\text{m}$  superparamagnetic particles that have a CV of 20%. b) Strategy for functionalizing magnetic particles with synthetic peptides. The synthesis starts with the addition of EDC and Sulfo-NHS followed by PEI to PAAMA-coated particles (I) to generate PEI-coated particles (II). Addition of Boc-PEG (3000)-NHS (III) results in the formation of Boc-PEG coated particles (IV) before removal of the Boc groups by the addition of TFA to generate amine PEG coated particles (V). Sulfo-SMCC (VI) is then added and SMCC conjugated particles are formed (VII). Finally, the cys-labelled peptides (VIII) are added, resulting in the formation of peptide-conjugated particles. c) Zeta potential monitoring of particles surface at each stage of the functionalization (pH 6.0: black; pH 8.0 grey).



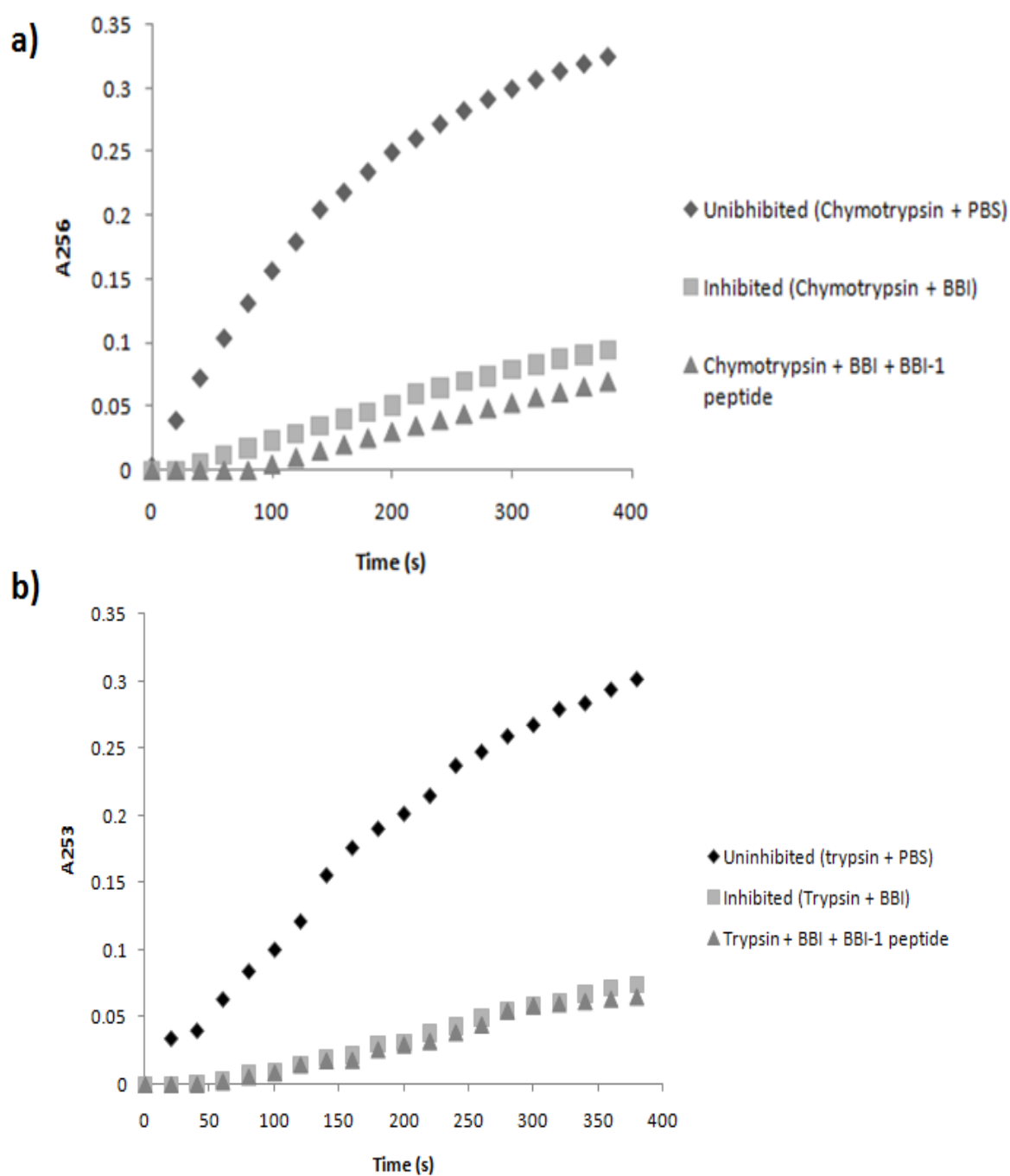


**Figure 5. Magnetic capture and purification of soybean derived BBI.** a) direct MALDI-TOF spectrum onto magnetic particles functionalized with the synthetic multimeric peptide. b) SDS-PAGE analysis of eluted fractions. Lane 1: Crude soy whey sample, Lane 2: Non-adsorbed fraction, Lane 3: Final wash, Lane 4: First elution using multimeric BBI-1 peptide-



covered particles, Lane 5: First elution using a construct not described here, Lane 6 Molecular weight standards. c) Tandem MS spectrum of the peptide NSCHSACK from the BBI (Uniprot P01063). Upper panel, matched y-ions are marked in red and annotated. Intensity of spectral lines is 4x magnified respect to base peak intensity. Lower panel, *de novo* sequencing of the same MS/MS spectrum.

Figure 1



**Figure 1. Effect of anti-BBI peptide on BBI enzymatic activity.** a) Pre-incubation of peptide with BBI does not affect the ability of BBI to inhibit chymotrypsin activity. b) Pre-incubation of the peptide with BBI does not affect BBI trypsin inhibitory activity.