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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, Bowman-Birk Inhibitor (BBI) has the ability to suppress carcinogenesis *in vivo*, which has been attributed to BBI's inhibition of serine protease (trypsin and chymotrypsin) activity. The lack of molecular probes for the isolation of this protein has made it difficult to work with, limiting its progress as a significant candidate in the treatment of cancer. This study has successfully identified a set of novel synthetic peptides targeting the BBI, and has demonstrated the ability to bind BBI *in vitro*. One of those probes has been covalently immobilised on superparamagnetic microbeads to allow the isolation of BBI from soy whey mixtures in a single step. Our ultimate goal is the use of the described synthetic probe to facilitate the isolation of this potentially therapeutic protein for low cost, scalable analysis and production of BBI.

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, Erdman, & Gordon, 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) and later entered human trial stages, producing promising results and low toxicity (Kennedy, 1998).

Soybean protease inhibitors 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 et al., 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 disulphide-bridges, making it a robust protein (Wu & Sessa, 1994), and 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 β-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 there 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, & Friedman, 1989; 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 (Birk, 1961; Birk, 1985). In addition, no affinity-based peptide ligands for BBI have been

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identified, making the isolation and consequently the study of this protein difficult (Leite Nobrega de Moura et al., 2011).

Here, by designing appropriate synthetic peptides and manipulation of molecules using superparamagnetic microbeads (SPM) microbeads (Li, Mahmood, & Lee, 2011; Peter & Otto, 2010), 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 functionalised 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 (Lacroix, Ho, & Sun, 2010). 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$1.2.10^9 independent clones) was purchased from New England Biolabs (Hitchin, Hertfordshire, UK). *Escherichia coli* ER2738 (F^+ 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,′-tetramentylbenzidine) 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(CCCTCATAGTTAGCG TAACG)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, Fritsch, & Maniatis, 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 immobilised in microtiter wells at a concentration of 50 μg/ml in $NaHCO_3$ buffer (0.1 M, 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 2×10^9 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 neutralised 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 (Sidhu et al., 2000). Briefly, 100 μl of purified BBI (100 μg/m/ in 100 mM $NaHCO_3$) was immobilised onto a 96-well microtiter plate and incubated overnight at 4^0C. 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 five times using PBST (0.1%) and approximately 10^{10} 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 five 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 characterisation

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 (clones 1–3 and negative control: VAMVLPGVMGTL) were synthesised at >90% purity. The C-terminus was elongated with a GGGS-$CONH_2$ 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*”) ($(GAMHLPWHMGTL)_4K_2KGSGCG$) 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_2O 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 immobilised purified BBI, soy pulp, soy whey extracts and BSA (as a negative control) was analysed 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 immobilised (approximatively 500 RU) on a carboxymethyldextran CM5 sensor chip activated with a 1:1 mix of N-hydroxysuccinimide (50 mM) and N-ethyl-N-(dimethyl-aminopropyl)- 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