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Probing the Soybean Bowman–Birk Inhibitor Using Recombinant Antibody Fragments

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Supporting Information

ABSTRACT: The nutritional and health benefits of soy protein have been extensively studied over recent decades. The Bowman–Birk inhibitor (BBI), derived from soybeans, is a double-headed inhibitor of chymotrypsin and trypsin with anticarcinogenic and anti-inflammatory properties, which have been demonstrated in vitro and in vivo. However, the lack of analytical and purification methodologies complicates its potential for further functional and clinical investigations. This paper reports the construction of anti-BBI antibody fragments based on the principle of protein design. Recombinant antibody (scFv and diabody) molecules targeting soybean BBI were produced and characterized in vitro ($K_D \sim 1.10^{-9}$ M), and the antibody-binding site (epitope) was identified as part of the trypsin-specific reactive loop. Finally, an extremely fast purification strategy for BBI from soybean extracts, based on superparamagnetic particles coated with antibody fragments, was developed. To the best of the authors' knowledge, this is the first report on the design and characterization of recombinant anti-BBI antibodies and their potential application in soybean processing.

KEYWORDS: *soybean proteins, Bowman–Birk inhibitor, antibody engineering, superparamagnetic particles, purification*

■ INTRODUCTION

Soybeans constitute all three macronutrients, proteins, carbohydrates, and fat, and are widely used in human foods in a variety of forms including infant formulas, soy flour, soy fibers, soy sauce, and tofu. Fat-free (defatted) soybean meal is a primary, low-cost source of protein for animal diets.[1−3] The quality of soy protein is equal to that of milk and meat proteins, as evaluated under guidelines adopted for assessing the quality of different proteins. Protease inhibitors are responsible for ~6% of the total protein content of soybeans. The Bowman–Birk inhibitor (BBI), initially identified in the 1940s[4,5] and later purified by Birk,[6] is the major protease inhibitor present in soybeans and almost all monocotyledonous and dicotyledonous seeds. This inhibitor contributes to the nutritional quality of soybeans due to its high cystine content. Soybean BBI is a small, water-soluble protein containing 71 amino acids, with a molecular size in the range of 8 kDa.[6−8] The BBI protein is characterized by two separate protease reactive sites. Each domain comprises a β-hairpin (antiparallel β-sheet and a *cis*-proline-containing type VIb reverse turn) with a short segment making a third strand of antiparallel β-sheet. The N-terminal subdomain (loop 1, residues Lys_{16}–Pro_{20}) neutralizes trypsin activity, and the C-terminal subdomain (loop 2, residues Leu_{43}–Ala_{47}) is involved in the binding to chymotrypsin. BBI is an extremely robust protein with seven disulfide bridges,[7−9] making the molecule very stable within the pH range encountered in most foods. Additionally, BBI can withstand boiling water temperature for 10 min and is resistant to low pH and proteolytic enzymes of the gastrointestinal tract. The potential therapeutic value of BBI has been explored over the past 25 years. Animal studies have shown that BBI is able to prevent the development of malignancies in different animal tumor model systems.[10] More recently, a soybean preparation enriched in BBI (named BBI concentrate or BBIC) has received drug status by the U.S. FDA and has been evaluated in human phase IIa clinical trials.[2] BBIC has shown a very favorable safety profile in clinical trials in patients with benign prostatic hyperplasia or precancerous conditions, such as oral leukoplakia and ulcerative colitis. No major clinical evidence of toxicity has been observed to date.[11] Furthermore, BBI has recently been proposed as an oral delivery treatment for multiple sclerosis,[12] and its biotechnological potential as a molecular building block is still being explored.[13,14]

Polyclonal, anti-BBI antibodies have been developed, but their apparent affinity against the BBI was too low; therefore, the resulting immunoassays were not sensitive enough to detect BBI in complex samples such as processed foods.[15,16] More recently, a small number of monoclonal antibodies, specific for the native BBI structure, have been used to analyze soy products.[17−19] Of particular interest is the mouse hybridoma cell 238, which secretes a specific anti-BBI (mAb) $IgG_1\kappa$. It has a subnanomolar affinity for soybean BBI and does not interact with BBI isolated from chickpea and lima.[16,19,20] The antibody–antigen interaction has been well characterized, using enzyme-linked immunosorbent assays (ELISA), allowing nanomolar concentration of active BBI in soybean cultivars to be successfully detected.[21] Furthermore, it is the only anti-BBI IgG available commercially for the specific detection of BBI in soy products. Its paratope targets the tryptic loop (conformational epitope) of BBI. More interestingly, its reactivity with other soybean-derived protease inhibitors (i.e., Kunitz) is very

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low[22] and nonexistent with BBI derived from other sources. Therefore, mAb 238 seems to be the ideal scaffold on which to develop antibody-based affinity purification methodology.

Recombinant molecular binders based on antibody structure have major advantages when compared to conventional polyclonal or monoclonal antibodies derived from hybridoma technology. They are genetically obtained without animal facilities, either by phage display, a highly efficient in vitro selection strategy,[23−25] or by transformation of an existing hybridoma cell line. Full-length antibodies have been reduced in size, dissected into minimal binding fragments (i.e., scFv), and rebuilt into multivalent molecules (i.e., diabody, triabody, ...), providing a significant increase in their functional affinity or avidity for their target antigen.[26] Antibody fragments have been genetically or chemically fused to a wide range of molecules, including radioisotopes for in vivo molecular imaging, enzymes for pro-drug therapy and targeted cell killing, and colorimetric or fluorescent tracers for one-step biodetection. Recombinant antibodies can exhibit subnanomolar affinity and high specificity for their target as they usually retain the binding behavior (affinity, specificity) of the parental IgG from which they are derived.[27,28] In addition, their structural and functional properties can be further improved by genetic engineering and antibody evolution procedures. The cost of manufacturing recombinant antibodies is decreasing extremely quickly, and their purification is often performed in a one-step procedure using grafted tags. Finally, the use of recombinant antibodies as affinity ligands is well accepted by the scientific community.[29]

Interestingly, no major advances in biomaterial science and molecular engineering have been proposed for the isolation of BBI during food processing until now. Therefore, the purification of BBI is still based on extensive and time-consuming analytical methods, initially developed by Birk[6] for structural and functional studies of isolated BBI. Minor modifications have been proposed,[30] but the BBI isolation process remains challenging.[3,31]

In the present study, we have cloned VH and VL domains from the hybridoma cell mAb 238 and assembled them together as single-chain Fv (scFv) and diabody (scDb) molecules. Recombinant antibody fragments were bacterially expressed in *Escherichia coli* cells and purified at a level of ∼500 μg L^{-1} of culture. Binding to soybean BBI was demonstrated by immunoassays, and binding kinetics were analyzed by surface plasmon resonance. The recombinant antibody fragments, derived from mouse IgG238, exhibit a high affinity (K_D ∼ 1.1 nM) for the purified BBI. Epitope−paratope interaction has been analyzed in silico and confirmed experimentally. To further explore their potential, the recombinant molecules were covalently grafted onto the surface of superparamagnetic particles for affinity-based separation from soy protein extracts. The protein eluted from the particles was identified as BBI and is fully functional, as determined by standard enzymatic assays, specific for BBI serine protease activity. This is the first experimental report for evidence of affinity-based purification of BBI from soybean extracts, using recombinant, tunable antibody molecules. Guided by the principles of antibody engineering, the further evolution of the antibody paratope (i.e., affinity, modulation of binding specificity, ...) and optimization of surface chemistries should improve the functional behaviors of the generated biomaterial.

■ MATERIALS AND METHODS

Materials. Hybridoma cell line 238 (ATCC HB 9657) has been derived from the fusion of the myeloma cell line P3-X64-Ag8.653 with splenocytes from mice inoculated with native Bowman−Birk inhibitor.[16,20,21] The 238 cell secretes a monoclonal IgG$_1$,κ directed against the tryptic loop of the soybean BBI. Soy (*Glycine max*) protein extracts and purified soybean BBI have been provided by Solae (Arhus, Denmark). Specifically, the soy whey comes from a waste stream during the isolate soy protein (ISP) process. The whey forms the liquid supernatant after the majority of proteins are precipitated (by lowering the pH to the isoelectric region) after extraction has been carried out (nitrogen extraction process using 0.03 M calcium chloride as the extracting agent). Ni-NTA agarose was from Qiagen (West Sussex, U.K.). All other reagents were purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland) of molecular biology research grade. All experiments were performed at least in duplicate.

Design and Construction of Recombinant Anti-BBI Antibody Fragments. mRNA was isolated from freshly subcloned hybridoma mAb238. Complementary DNAs (cDNA) encoding antibody variable domains were cloned and sequenced (GenScript, Piscataway, NJ, USA), as previously reported.[27] Mouse scFv and diabody genes were optimized for prokaryotic expression and created by de novo gene synthesis (Entelechon Gmb, Regensburg, Germany). For the scFv construction, the VH domain was fused to the VL domain by the (GGGGS)$_3$ linker. For the diabody design, the linker was reduced to GGGS. DNA sequences were optimized for *E. coli* expression. All constructions were cloned into pHEN1[23] between *Nco*I and *Not*I in frame with the *pel*B leader and downstream, a sequence encoding the c-myc and (His)$_6$ tags. The final constructed vectors (named pJMscFv238 and pJMscDb238) were verified by sequencing and cloned into the *E. coli* strain HB2151 (K12, *ara*, Δ(*lac-pro*), *thi*/F′ proA$^+$B$^+$, lac^{Iq} lacZΔM15) for recombinant antibody expression in bacteria. For mammalian cell expression, the recombinant plasmid encoding scfv238 was transiently transfected into 100 mL of suspension HEK293 cell cultures, and HiTrap IMAC captured the target protein from the cell supernatant. DNA manipulations were performed according to previously described techniques.[32]

Production and Purification of Antibody Fragments. For expression of the recombinant antibody fragments, bacteria harboring the desired plasmids were grown in 500 mL of 2xYT medium (Fisher, Dublin, Ireland) containing ampicillin (100 μg/mL) and 1% glucose at 37 °C under rotative agitation (180 rpm), until $A_{600\ nm}$ reached 0.6. The protein expression was then induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the medium, and incubation was continued for 16 h at 30 °C under agitation (180 rpm). Soluble antibody fragments were isolated from culture supernatants. The tagged antibodies were purified using a Ni-NTA agarose microcolumn, as previously described.[25] After extensive washing with PBS, the bound proteins were eluted with 0.1 M glycine−HCl (pH 2.2) in 0.5 mL fractions and immediately neutralized with 5 μL of Tris on ice. Fractions with $A_{280\ nm}$ > 0.2 were pooled and dialyzed against PBS overnight at 4 °C. The purity was evaluated by SDS-PAGE on a 15% gel followed by Coomassie brilliant blue staining and immunoblotting using antitag antibody.[32]

Immunoassays and SPR. To evaluate the binding activity of the rAb to BBI, 96-well plates (Fisher) were coated with 10 μg/mL BBI or BSA (negative control) (100 μL per well) overnight at 4 °C. Nonspecific binding sites were saturated with 3% nonfat dry milk for 90 min. The plates were then incubated with increasing concentrations of purified antibody preparations (100 μL) for 120 min. The plates were subsequently incubated with the anti-c-myc flag IgG (9E10) conjugated to HRP (100 μL, 1/500 in PBS) (Abcam, Cambridge, U.K.). Finally, the substrate solution (1 step-TMB) (100 μL) was added to each well for 5 min. The plates were monitored in a microplate reader (BioTek reader, Winooski, VT, USA) at OD_{650}, and the reaction was stopped by the addition of 2 M H_2SO_4, before another reading at OD_{450}. All incubations were carried out at room temperature. Five washes with PBS−Tween (PBST−BSA 1%) were performed between each of the intermediate steps. In competitive

binding assays, scFv and diabody 238 were mixed with increasing concentrations of BBI (0−100 μg/mL) before being added to BBI-coated microtitration wells. Surface-bound antibody was detected as described above.

The BIAcore T100 instrument and all of the reagents for analysis were obtained from GE Healthcare Ltd. Soluble purified BBI was immobilized (approximatively 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. Affinity-purified antibody fragments 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 μL min^{-1} at 25 °C. Glycine−HCl (10 mM, pH 2.0) was injected for 30 s at 20 μL min^{-1} to regenerate the sensor chip between successive samples. Kinetic constants (k_{on}, k_{off}) were deduced from the analysis of association and dissociation rates at four different antibody fragment concentrations, ranging from 5 to 40 μg.mL. The dissociation constant K_D was calculated from $K_D = k_{off}/k_{on}$. Sensorgrams were analyzed using the "biaevaluation" software. Guidelines for accurate kinetic analysis were employed, and data were double-referenced as described in ref 49. All experiments were carried out in duplicate at the UCD/NIBRT Biacore facilities (Dublin, Ireland).

Superparamagnetic Particle (SMP) Synthesis and Functionalization. SMPs were synthesized according to an emulsion solvent evaporation procedure developed in our laboratory[33] (also, Muzard, Platt, and Lee, to be published elsewhere). The surface of the iron oxide particle was functionalized with antibody molecules by EDC/NHS amine coupling chemistry. 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 μm with a CV of 20%. Antibody presence was qualitatively verified using a modified protocol described for immunoassays. Purified BBI was fluorescently labeled with Cy5 according to the manufacturer's instructions (GE Healthcare Ltd., Buckinghamshire, U.K.). Functionalized superparamagnetic particles (1×10^8/mL) were incubated with Cy5−BBI at room temperature for 2 h. After three washings in PBST, particles were analyzed on a FACS calibur flow cytometer (BD Biosciences, Oxford, U.K.) using dual color lasers (525 and 660−675 nm).

Immunomagnetic Isolation of BBI. Soy crude samples (∼100 mL) were incubated for 16 h at 20 °C with 500 μL of magnetic particles (10 mg/mL) coated with purified antibody fragments. After extensive washing with PBST until $A_{280\ nm}$ reached 0.001, bound proteins were eluted in 100 μL fractions of pH 2.2 glycine (100 mM) buffer and submitted to SDS-PAGE analysis and enzymatic assays. Identification of the silver-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.[34] The resulting tryptic peptides were desalted and concentrated using ZipTips (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. Peptides were resuspended in 12 μL of 0.1% formic acid. Each sample was loaded onto a Biobasic C18 Picofrit column (100 mm length, 75 mm i.d.) 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^{-1} on an Dionex Ultimate 3000 chromatography system incorporating an autosampler. 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 a potential of 1800 V applied to the frit. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300−2000 Da) was performed using the Orbitrap, followed by MS/MS analysis of the five most intense ions using the ion trap. PEAKS Studio version 5.2 (Bioinformatic Solutions, Waterloo, ON, Canada) was used for simultaneous matching to the NCBI nonredundant database, taxonomy *G. max* (soybean) and de novo sequencing of good-quality spectra. Trypsin was chosen as enzyme specificity, 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 and fragment tolerance at 0.5 Da.

Enzymatic Assays. Inhibition of the trypsin activity was measured at 25 °C in 0.4 M Tris-C1 and 0.01 mM CaCl, pH 8.1, and with 1 mM (*p*-tolylsulfony1)-L-argininme ethyl ester as substrate.[35] Inhibition of the chymotrypsin activity was determined at 25 °C in 0.04 M Tris-C1 and 0.05 M CaCl, pH 7.8, with 0.5 mM benzoyl-L-tyrosine ethyl ester as substrate.[35] For inhibition assays, recombinant antibody molecules were preincubated with BBI for 7−10 min at 37 °C. Readings were recorded every 20 s at 253 nm for the trypsin assays and at 256 nm for the chymotrypsin assays, in a UV−vis NIR spectrophotometer (Varian Cary model 6000i, Agilent Technologies, Palo Alto, CA, USA).

Computational Analysis and Bioinformatics. A three-dimensional structural model of 238 Fv domains was built using the Web Antibody Modeling facility (http://antibody.bath.ac.uk) with an updated version of the algorithm first implemented in AbM (Oxford Molecular, Oxford, U.K.).[36] Framework regions were modeled using highly homologous antibody templates. Complementary-determining regions (CDRs) from the KAPPA chain and hypervariable loops H1 and H2 from the heavy chain were built from homologous loops of the same canonical class. The CDR H3 loop was constructed using the CONGEN conformational search procedure and, finally, the whole model was energy minimized.[37] The rigid-body docking PatchDock server[38] was used for epitope−paratope modeling. A model of the Fv238 and the 3D structure of the BBI (Protein Data Bank entry 1BBI) [39] were docked together with the "antibody−antigen interaction" input parameter with a 4.0 Å cutoff. Observations and picture renderings were made using PyMol.[40]

■ RESULTS

The goals of this study were to (1) generate high-affinity ligands targeting BBI and (2) explore the potential of the designed probes covalently linked to SMPs for isolation and purification of BBI from crude soy extracts. We started from the mRNA of the anti-BBI cell line secreting the monoclonal mAb 238 previously characterized.[16,20,22] Then, monovalent (single-chain Fv, scFv) and multivalent (single-chain diabody, scDb) anti-BBI recombinant antibody fragments were developed using the principles of genetic engineering and protein design. Figure 1a contains the general strategy we used to clone and obtain each antibody construct as single recombinant proteins. The agarose gel analysis of PCR products shows a single band at ∼400 bp for both VH and VL domains as cloned separately (Figure 1b). The cDNAs encoding the VH and VL domains were sequenced and submitted to codon optimization for expression in *E. coli*. Anti-BBI recombinant antibodies were designed in the VH-linker-VL configuration, either as monovalent scFv (designated scFv238), in which the VH domain is fused to the VL domain through the flexible linker of 15 residues $(Gly_4Ser)_3$, or as a diabody (i.e., bivalent structures made of two subunits, designated diabody238), in which the two variable domains are linked together via a linker with a size limited to five residues (Gly_4Ser).[28] To avoid the risk of introducing mutations associated with the conventional PCR by overlap extension strategy for cloning antibody fragments, we chose de novo total gene synthesis for each construction. Gene synthesis offers a very fast, mutation-free process, without the need for intermediate time-consuming cloning steps. Full-length (∼800 bp) scFv and diabody238 genes, fully optimized for expression in bacteria, were finally cloned separately into the phagemid vector pHEN01[23] to give pJM-scFv238 and pJM-sdAb238 vectors, respectively (Figure 1). The integrity of the final constructions was verified by DNA sequencing. The deduced amino acid sequences of the VH and VL domains are reported in Figure 2a with the framework regions (FRs) and

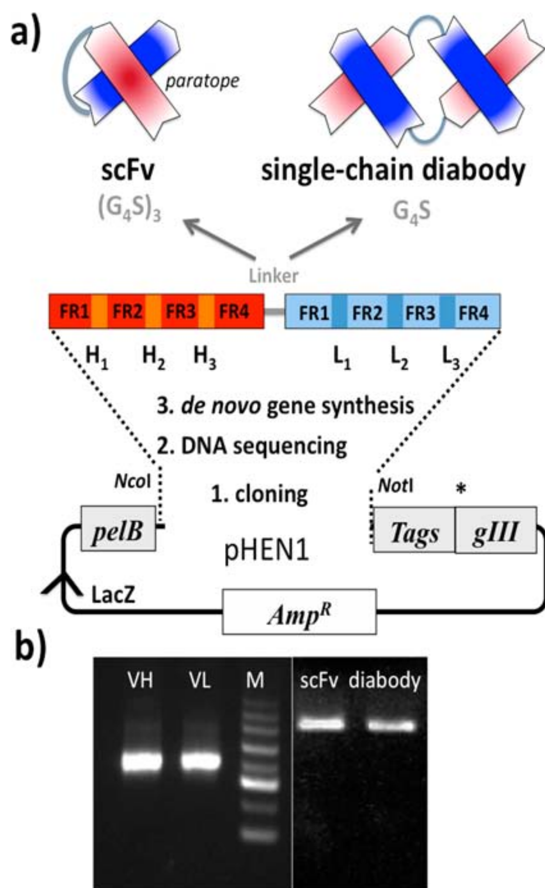

Figure 1. Design and cloning of recombinant anti-BBI antibody fragments. (a) After identification of cDNA encoding VH and VL domains and sequence optimization, gene synthesis was performed to assemble antibody domains and final constructs were cloned into the phagemid vector pHEN1 for bacterial expression. (b) Agarose gels of separately cloned antibody variable domains.

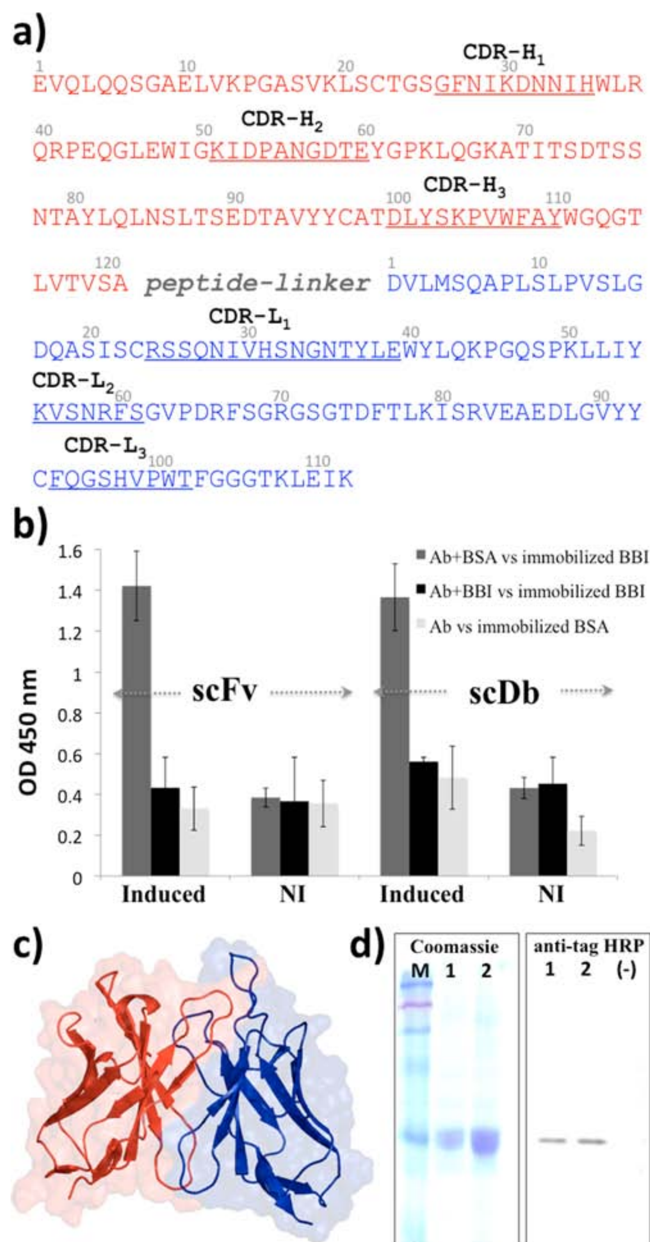

Figure 2. Engineering of recombinant anti-BBI monovalent and multimeric antibody fragments: (a) amino acid sequence of VH and VL domains (underlined are CDRs (L_1, L_2, L_3, H_1, H_2, and H_3)); (b) binding analysis of recombinant molecules to immobilized purified BBI in the presence/absence of BBI in solution; (c) structural model of Fv 238 (paratope); (d) SDS-PAGE and immunoblot analysis of purified recombinant antibody fragments from bacterial cultures (lanes: M, molecular weight marker; 1, scFv238; 2, diabody238; (−), negative control).

complementary determining regions (CDRs) (Kabat numbering[41]) being highlighted, as well as Collier de Perles representation, as described in the Supporting Information. Figure 2b provides the binding profiles by immunoassays from cells secreting antibody fragments (supernatants, before and after bacterial induction) against immobilized purified BBI in presence/absence of BBI in solution. A strong and specific binding is observed from supernatants derived from recombinant cells (harboring pJM scFv238 and pJM-sdAb238). Neither the cytoplasm nor periplasmic fractions contain activity (data not shown). A fully computed molecular homology model of Fv238 has been constructed according to the procedure used in our laboratory.[27,36,37] The resulting model of the paratope is provided in Figure 2c. Figure 2d corresponds to the electrophoresis and immunoblot of affinity-purified molecules. A single, homogeneous band at the expected size (~28 kDa) was observed for the affinity-eluted recombinant antibody 238 with no visible degradation products. We estimated the yield of purification to be ~500 μg of recombinant antibody proteins from a single liter of bacterial culture. This is in the typical range of other (purification yield) reports obtained with bacteria harboring the pHEN backbone vector.[25−27]

Figure 3a demonstrates the ability of the purified antibody fragments to specifically bind to immobilized BBI in a solid phase assay in which binding detection is performed using antitag antibodies. Significant binding to soy crude samples was also observed, and nonspecific binding interaction (irrelevant antibody) was insignificant (10%). Furthermore, antibody fragments binding to immobilized BBI were reduced by ~90% in a competition test in which antibodies were preincubated with free BBI (data not shown). Molecular binding interactions were analyzed in real time by surface plasmon resonance using Biacore technology. Kinetic analysis revealed a dose-dependent response binding of purified scFv238 and diabody238 to the BBI activated surface, and a

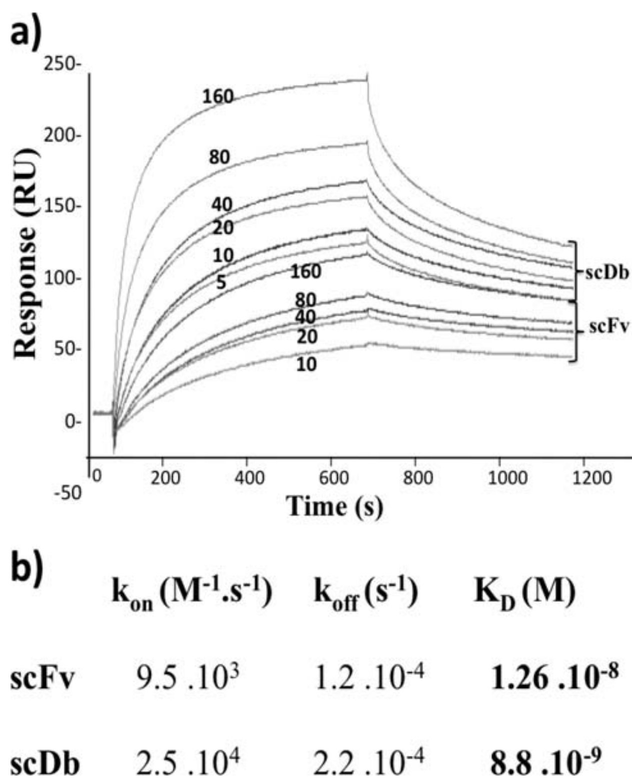

Figure 3. Antibody fragments−BBI binding characterization: (a) real-time (k_{on}, k_{off}) binding interactions by surface plasmon resonance spectroscopy for scFv and scDb (from 10 to 160 μg mL^{-1}); (b) binding kinetics determination.

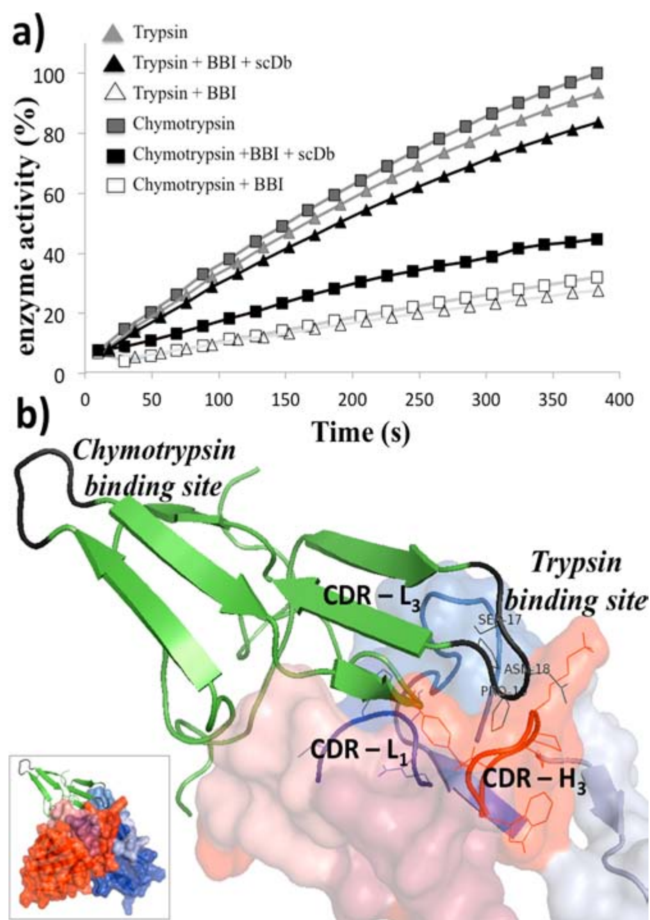

Figure 4. Determination of BBI protease activities and structural bioinformatics analysis: (a) effect of scDb238 on chymotrypsin and trypsin BBI activities; (b) cartoon rendering of the in silico high-resolution epitope−paratope modeling with emphasis on the binding contact interface. Side chains involved in contact with the trypsin reactive loop are indicated. A full-size image of the side view of Fv VH (red) and VL (blue) in complex with BBI is also shown.

dissociation constant (K_D) of 10 nM (k_{off} = 1.1 × 10^{-1} s^{-1}; k_{on} = 1.1 × 10^{6} M^{-1} s^{-1}) (Figure 3b), with a slight shift for scDb binding as expected (Table 1). As antibody fragments usually

Table 1. Kinetics for BBI−Antibody Fragments Binding Molecular Interactions As Determined by Surface Plasmon Resonance

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
scFv238	9.5 × 10^{3}	1.2 × 10^{-4}	1.26 × 10^{-8}
diabody238	2.5 × 10^{4}	2.2 × 10^{-4}	8.8 × 10^{-9}

retain their affinity from their parental IgG, this value seems to be in the range of the one determined previously for the mAb 238 by the radioimmunoassay technique.[20,42]

The protease-inhibitory activity of BBI was determined after preincubation of the soy protein with our purified recombinant antibodies. Consistent with Figure 4a, binding of scFv238 or diabody238 had no major effect on the activity of chymotrypsin, but more importantly, trypsin inhibition could be fully blocked by the recombinant antibody molecules (Figure 4a) as seen with the native IgG.[16,20,22] We concluded that (1) expression as antibody fragments had no effect on the binding to the native BBI structure and (2) the trypsin loop corresponds, at least partially, to the epitope recognized by the antibody fragments. On the basis of the experimental evidence that the trypsin-reactive site corresponds to the epitope of the mAb238, we studied next the epitope−paratope interaction in silico. Fv238 was submitted to a rigid-body docking procedure with the 3D structural details of BBI (1BBI.pdb) as obtained by NMR.[39] A total of six loops, usually supported by FRs, was specifically involved in the antibody−antigen contact. In our antibody−antigen structural model, CDRs formed a single cleft with CDR-H$_1$, -H$_3$, and -L$_3$, which dominated ligand interactions. Residues Lys^{16}−Ser^{17}−Asn^{18}−Pro^{19}−Pro^{20} corresponded to the trypsin active site on the BBI structure, and residues Leu^{43}−Ser^{44}−Tyr^{45}−Pro^{46}−Ala^{47} were involved in the binding to chymotrypsin (pdb entry 1D6R[43]). As suggested by the computed in silico epitope−paratope characterization, Tyr^{102}, Ser ^{103}, and Lys^{104} from the CDR-H$_3$, Asn^{33}, and Asn^{35} in the CDR-L$_1$ of the Fv238 were in close proximity with residues Asn^{18}−Pro^{19}−Pro^{20}−Gln^{21} from the BBI and could easily form bonding interactions. CDR-L$_1$ and -L$_3$ mediated the recognition of the segment Asp^{53}−Glu^{60} (Figure 4b). Thus, we conclude that the epitope recognized by 238 was conformationally distributed through the segments Asn^{18}−Gln^{21} and Asp^{53}−Glu^{60}.

Finally, we designed a pilot immunopurification of BBI from soy crude extracts using magnetic particles functionalized with antibody fragments. Carboxyl-coated SMPs were synthesized using emulsion directed iron oxide nanoparticle assembly technology, resulting in particles with an average size of 1 μm and CV of 20% (Figure 5a). The primary advantage of this synthetic strategy lies in the magnetic properties of the resulting microparticles, which typically possess a very high saturation

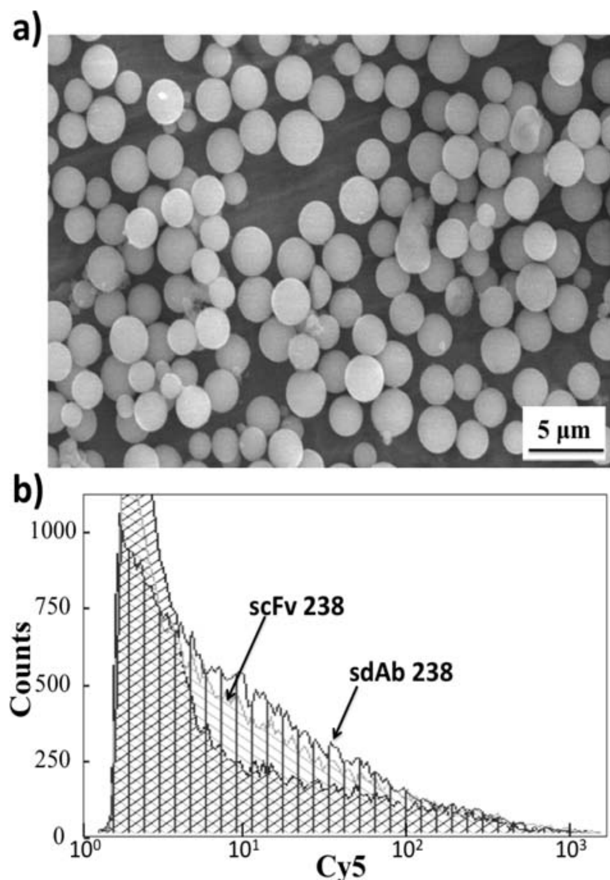

Figure 5. Synthesis of SMPs and functionalization with recombinant antibody fragments: (a) SEM image of SMPs synthesized in-house; (b) flow cytometry analysis of BBI binding to magnetic particles (~10^8 mL^{-1}). Fluorescently labeled cy5-BBI (50 $\mu g\ mL^{-1}$) was incubated with magnetic beads (uncoated, scFv coated, and diabody coated) for 60 min. After washing in PBST, particles were analyzed for fluorescence.

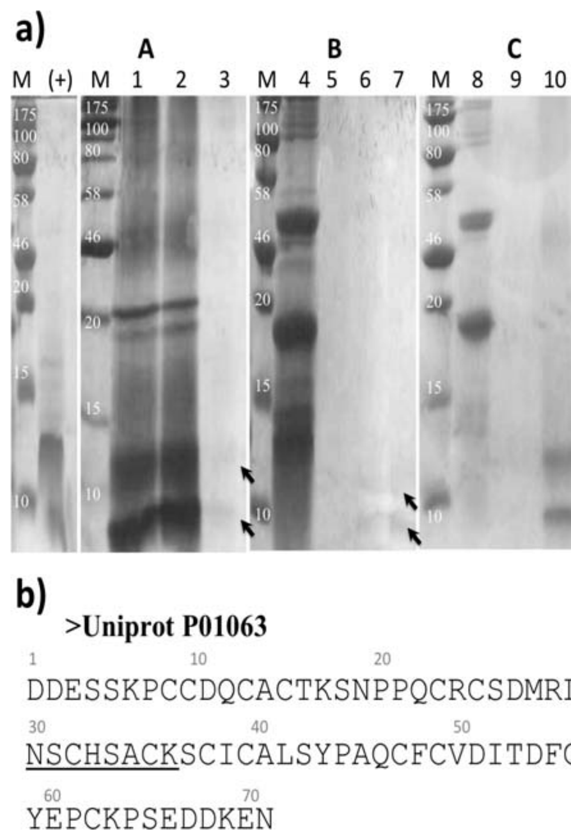

Figure 6. Magnetic-based immunopurification of BBI from soybean crude protein extracts: (a) SDS-PAGE analysis of three independent experiments (A, B, and C) (lanes: M, molecular weight marker; (+), migration control (purified BBI); 1, 4, and 8, crude soybean total protein extract; 2, unbound fraction (A); 5 and 8, final washes before elution (B and C); 3, 6/7, and 10, affinity-purified fractions from matrix); (b) confirmation of BBI presence in the eluted fraction[10] by tandem mass spectrometry (the amino acid sequence of BBI (isoform E) is shown in Fasta format with the identified peptide underlined).

magnetization (of approximately 55–60 emu/g). Consequently, these particles can be rapidly separated from viscous solution with a simple permanent magnet. Those SMPs were functionalized with anti-BBI recombinant antibody fragments, and Figure 5b demonstrates the ability of coated magnetic particles to bind the fluorescently labeled BBI in flow conditions. Figure 6a shows an SDS-PAGE analysis of the soybean protein extract before purifications as well as of the eluted fraction after acidification of the diabody238-coated particles. We notice a batch-to-batch variation of the BBI presence and protein profiling for the soy extracts, due to the mode of preparation and factors (temperature, ...) affecting the protein composition (lanes 1, 4, and 8). Whereas the magnetic-based capture of the BBI process was not fully optimized (i.e., low level of bound proteins on particles, samples A, B, and C), in the eluted fractions (lanes 3, 6/7, and 10), the gel shows one component at the expected size (~8 kDa), alongside a band at the approximate molecular size for a BBI dimer (at around 14 kDa). The propensity for BBI to self-associate has previously been observed by us and others, with or without SDS, and in the presence of β-mercaptoethanol and urea.[3,9,31] After separation on the SDS-PAGE gel, proteins immunoprecipitated by magnetic particles were then analyzed by direct tandem mass spectrometry after in-gel digestion with trypsin and reverse-phase separation of the tryptic peptides. The prominent band was identified as the soybean trypsin inhibitor (Figure 6b). Chymotrypsin and trypsin enzyme activity assays were finally performed with the purified fraction and confirmed the presence of the serine protease inhibitor in the affinity-purified fraction (data not shown). Thus, we conclude that our affinity-based magnetic purification fully preserves the functional activity of the BBI. Further experiments will focus on cross-reactivity studies against different BBI variants and sources. This is the first report of (1) novel anti-BBI recombinant antibodies and (2) a rapid (~60 min time) potent affinity purification strategy based on molecular probes grafted onto SMPs.

■ DISCUSSION

Over the past 20 years, recombinant antibodies have found success in a wide range of applications from biomedical research to molecular diagnostics and therapeutics. An entire spectrum of antibody fragments has been identified through protein-engineering studies, many showing remarkable potential.[47] Smaller recombinant antibody fragments, the monovalent Fab and scFv, and engineered variants (diabodies, triabodies, minibodies, and single-domain antibodies) are emerging as novel forms of diagnostics and therapeutics that are less costly to manufacture while still retaining the targeting specificity of full-length IgGs.[48] They possess unique and superior properties

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for a range of diagnostic and therapeutic applications. As compared with full IgG proteins, genetic engineering also provides the advantage to modulate the characteristics (affinity, specificity) of recombinant molecules, allowing optimization of paratope−antigen interactions. Surprisingly, the advancement of recombinant antibody technology is under-represented in food research and industry, an area where there is a need for improved analytical and purification methodologies.[22,31]

Just as soy consumption patterns vary greatly across the world, so too do cancer incidences. An inverse relationship between soybean intake and cancer incidence has been reported, with Asian countries where soy forms an important part of the diet, such as Japan, displaying the lowest incidences of cancer. The anticancer effects of soy have been attributed to the BBI, a seemingly valuable protein with therapeutic implications in multiple health defects. To date, the widely used purification method for BBI consists of ammonium sulfate fractionation, acid precipitation, and classical gel filtration chromatography.[5] More recently, hydrophobic chromatography methods have been proposed for BBI isolation from soybean total proteins.[30] In general, such approaches are time-consuming and costly and require the use of robust analytical equipment such as chromatographs. This study introduces a novel concept in the isolation of BBI from soybean protein extracts. Our strategy explores the use of recombinant antibodies derived from an IgG-secreting hybridoma and the benefits of paramagnetic particle technology. The effective isolation and purification of proteins from complex mixtures is the most crucial step for successful protein purification when only minute amounts are available. Whereas conventional purification methods such as dialysis, ultrafiltration, or protein precipitation often lead to a marked loss of protein, manipulation of magnetic particles is a powerful alternative. Magnetic particles have been increasingly used in a variety of applications such as single-molecule research, biosensing, nucleic acids purification, and biomaterial scaffolds for in vivo magnetic resonance imaging.[43−46] Magnetic handling is extremely fast and efficient for a wide range of target molecules. All steps take place in a single tube, without the need for centrifugation or the use of chromatography equipment. Due to the magnetic core, the sample preparation can also be automated using robot systems, resulting in minimized handling errors during the protein purification process as compared with other purification procedures, such as column chromatography. With the increasing implementation of magnetic particles with novel surface textures/chemistries and enhanced protein-binding capacities, the investigation of magnetic-based purification methods can also be extended to large-scale downstream processing.[46]

We describe here our recent efforts for bacterial synthesis of soluble antibody fragments using the phagemid vector pHEN01. We report the design and recombinant production of monomeric and multimeric single-chain antibody fragments as dictated by the principles of molecular antibody design. Binding data suggest that the multimeric diabody construct has a higher affinity for the BBI over the monomeric scFv fragment. The multivalent diabody construct may present itself as the favorite candidate due to the increase in functional affinity, or avidity, observed upon the generation of multivalent molecules. Optimization efforts are still required for maximum coverage of antibody fragments on particles that will increase yields. Of particular interest, will be the production of the described antibodies starting from other expression systems. The developed BBI immunopurification protocol is fast, which minimizes protein degradation, is capable of isolating BBI from prefiltered crude whey samples early in the processing stages, and is based on inexpensive reagents for cost-effective BBI product isolation. Finally, the technology proposed in this paper is fully compatible with robotic and high-throughput technology, ensuring fast and maximum reliability. Recombinant antibody molecules derived from mAb 238 can be useful tools for the soy food processing community.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Designed the study: G.U.L. and J.M. Performed the experiments and analyzed the data: J.M., C.F., G.U.L., and J.J.O'M. Contributed materials/reagents: J.M. and C.F. Wrote the paper: J.M. and G.U.L.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, immunoglobulin G; kDa, kilodalton; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PBS-T, PBS Tween 20; scFv, single-chain variable fragment; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMPs, superparamagnetic particles.

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