 DOI 10.1002/mnfr.200900122 *Mol. Nutr. Food Res.* 2010, *54*, 396–405

RESEARCH ARTICLE

The cytotoxic effect of Bowman–Birk isoinhibitors, IBB1 and IBBD2, from soybean (*Glycine max*) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases

Alfonso Clemente[1], *Francisco Javier Moreno*[2], *Maria del Carmen Marín-Manzano*[1], *Elisabeth Jiménez*[1] *and Claire Domoney*[3]

[1] Department of Physiology and Biochemistry of Nutrition, Estación Experimental del Zaidín (CSIC), Granada, Spain
[2] Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain
[3] Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich, UK

Received: March 19, 2009
Revised: April 23, 2009
Accepted: May 5, 2009

Bowman–Birk inhibitors (BBI) from soybean and related proteins are naturally occurring protease inhibitors with potential health-promoting properties within the gastrointestinal tract. In this work, we have investigated the effects of soybean BBI proteins on HT29 colon adenocarcinoma cells, compared with non-malignant colonic fibroblast CCD-18Co cells. Two major soybean isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their inhibitory domains, were purified in order to examine their functional properties, including their individual effects on the proliferation of HT29 colon cancer cells. IBB1 inhibited both trypsin and chymotrypsin whereas IBBD2 inhibited trypsin only. Despite showing significant differences in their enzyme inhibitory properties, the median inhibitory concentration values determined for IBB1 and IBBD2 on HT29 cell growth were not significantly different (39.9 ± 2.3 and 48.3 ± 3.5 μM, respectively). The cell cycle distribution pattern of HT29 colon cancer cells was affected by BBI treatment in a dose-dependent manner, with cells becoming blocked in the G0–G1 phase. Chemically inactive soybean BBI had a weak but non-significant effect on the proliferation of HT29 cells. The anti-proliferative properties of BBI isoinhibitors from soybean reveal that both trypsin- and chymotrypsin-like proteases involved in carcinogenesis should be considered as potential targets of BBI-like proteins.

Keywords:
Bowman–Birk inhibitors / Cell proliferation / HT29 cells / Protease inhibitory activity / Soybean

1 Introduction

Colorectal cancer (CRC) is a complex disease that reflects a combination of lifestyle factors and multi-step genetic alterations. It has become one of the major causes of morbidity and mortality in western countries; therefore, much attention has been focused on preventive strategies. One of the most effective means of preventing or reducing colon cancer risk is either directly or indirectly linked to

Correspondence: Dr. Alfonso Clemente, Department of Physiology and Biochemistry of Nutrition, Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, 18008 Granada, Spain
E-mail: alfonso.clemente@eez.csic.es
Fax: +34-958-57-27-53

Abbreviations: BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; **BBI,** Bowman–Birk inhibitors; **BBIC,** Bowman–Birk inhibitor concentrate; **BTEE,** *N*-benzoyl-L-tyrosine ethyl ester; **CIA,** chymotrypsin inhibitor activity; **CRC,** colorectal cancer; **GIT,** gastrointestinal tract; **IC_{50},** median inhibitory concentration; **IU,** inhibitor units; **K_i,** inhibition constant; **MT-SP1,** matriptase; **NR,** neutral red; **TIA,** trypsin inhibitor activity

appropriate diet and/or nutritional manipulation [1, 2]. The fact that certain dietary constituents can exert cancer chemopreventive properties has major public health implications and their widespread, long-term use should be promoted in populations at normal risk, based on understanding the scientific basis of their effects. Naturally occurring protease inhibitors of the Bowman–Birk family, a major protein inhibitor class in legumes such as soybean (*Glycine max*), pea (*Pisum sativum*) and chickpea (*Cicer arietinum*), have been linked to a possible protective effect against inflammation and cancer development within the gastrointestinal tract (GIT) [3–5]. Bowman–Birk inhibitors (BBI) have been shown to be structurally and functionally resistant to the challenges of the GIT *in vivo*. BBI from chickpea seeds can resist both acidic conditions and the action of proteolytic enzymes, and transit through the stomach and small intestine without major degradation, permitting significant amounts to reach the large intestine in active form [6]. Further studies have demonstrated that the protease inhibitory activities of soybean BBI are unaffected by the metabolic/proteolytic activities of faecal microbiota, thereby retaining activity potentially linked to CRC-preventive properties [7]. Such extraordinary stability seems to be linked to the presence of a highly conserved array of intra-chain disulphide bridges that stabilise a symmetrical structure of two tricyclic domains, each containing an independent serine protease binding site [8–10].

Several *in vitro* and *in vivo* studies have demonstrated that BBI proteins may exert a protective and/or suppressive effect in CRC development and associated inflammatory disorders. A soybean Bowman–Birk inhibitor concentrate (BBIC), an extract enriched in BBI, exerted a protective effect in dimethylhydrazine-treated animals, reducing the incidence and frequency of colon tumours in mice [11, 12] and rats [3]. In these studies, adverse side effects of BBIC were not documented for either animal growth or organ physiology. BBI-like proteins from field beans (*Dolichos lablab*) have been shown to be biologically active in suppressing benzopyrene-induced forestomach carcinogenesis in mice, following oral treatment [13]. The effectiveness of BBI in the reduction and/or suppression of inflammatory processes within the GIT has been reported also. Addition of BBIC to the diet of mice resulted in a suppression of inflammation in the dextran sulphate sodium model of ulcerative colitis [14] and such a beneficial effect could be related to the ability of BBI to inhibit serine proteases, such as leukocyte elastase, cathepsin G and mast cell chymase, released from inflammation-mediating cells.

In legume seeds, BBI are proteins with two inhibitory loops that can independently inhibit two enzyme molecules. These may be the same (trypsin-like) or different (trypsin- and chymotrypsin-like) enzymes [15, 16]; additionally, some BBI can inhibit leukocyte elastase [4]. Because of an apparent association of the chymotrypsin inhibitory binding site with anti-carcinogenic properties [17], it has been hypothesised that chymotrypsin-like proteases are likely to be involved in carcinogenesis [18]. Recently, we have demonstrated the effect of sequence variation within the chymotrypsin inhibitory domain of BBI from pea on their functional properties [19] as well as on their ability to inhibit the growth of human colorectal adenocarcinoma cells [20]. The relevance of the trypsin inhibitory domains of BBI on health benefits has not been examined specifically, and trypsin-like proteases involved in carcinogenesis should be investigated as potential targets of BBI-like proteins [4]. In this work, we demonstrate that soybean BBI, consisting of multiple isoinhibitors, inhibited the *in vitro* cell growth of HT29 colon adenocarcinoma cells as a consequence of their intrinsic ability to inhibit the proteolytic activities of serine proteases, where denatured BBI showed no such biological effect. We demonstrate that the cell cycle distribution pattern of HT29 colon cancer cells is affected by BBI treatment. In contrast, the growth of normal colonic fibroblast CCD-18Co control cells was unaffected by soybean BBI proteins. Two major soybean isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their inhibitory domains, were purified in order to evaluate their individual effects on the proliferation of HT29 colon cancer cells. Strikingly, the effective and positive contribution of the trypsin inhibitory domain to the anti-proliferative properties of BBI was revealed by evaluation of the double-headed trypsin inhibitor IBBD2. These data further advance our knowledge and understanding of the relevance of sequence variation within the inhibitory domains of BBI in relation to their colorectal anti-proliferative properties.

2 Materials and methods

2.1 Materials

BBI from soybean (T9777), trypsin (type III) and α-chymotrypsin (type VII) from bovine pancreas, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), DMEM, neutral red (NR) and additional cell culture-grade chemicals were obtained from Sigma (Alcobendas, Spain). The human colorectal adenocarcinoma HT29 and the normal colon fibroblastic CCD-18Co cell lines were supplied by the Cell Bank of the Scientific Instrumentation Centre at the University of Granada (CIC-UGR, Granada, Spain). Culture flasks and flat bottom 96-well microtitre plates were purchased from Corning Costar (Cambridge, MA, USA) and Nunc (Wiesbaden, Germany), respectively. All other chemicals were of analytical grade.

2.2 Measurement of protease inhibitory activities

BBI from soybean and their major constituent isoinhibitors, IBB1 and IBBD2 (see Section 2.4), were assessed for trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity

(CIA). TIA was measured using a modified small-scale quantitative assay, with BAPNA as specific substrate, and using 50 mM Tris, pH 7.5, as enzyme assay buffer. One trypsin inhibitor unit was defined as that which gives a reduction in absorbance at 410 nm of 0.01, relative to trypsin control reactions, in a defined assay volume of 10 mL [21]. CIA was measured using BTEE as specific substrate. One chymotrypsin inhibitor unit was defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to chymotrypsin control reactions, in a defined assay volume of 10 mL [19]. Specific TIA and CIA of major soybean BBI isoinhibitors, expressed as inhibitor units *per* milligram of protein, were calculated. The inhibition constants (K_i) of purified isoinhibitors for trypsin (at pH 7.5) and chymotrypsin (at pH 7.8), were determined from dose–response curves by competitive assays, using the chromogenic substrates BAPNA and BTEE, respectively [19]. The reactions were initiated by adding trypsin (108 nM) or chymotrypsin (28 nM) with the respective substrate concentrations determined by K_m measurements. The concentration of inhibitor required to achieve a half-maximal degree of inhibition (IC_{50}, median inhibitory concentration) was determined for each protease, using the GraFit software (GraFit Version 5, Erithacus Software, Horley, UK). K_i were calculated from IC_{50} values using the tight-binding equations for competitive inhibitors as previously described by Copeland *et al.* [22]. The trypsin and chymotrypsin inhibitory properties of soybean BBI were analysed furthermore on 4–16% zymogram blue casein gels (Invitrogen, Barcelona, Spain). Unfractionated BBI or individual isoinhibitors (16 or 32 μg, respectively) were loaded on zymogram gels for the detection of trypsin or chymotrypsin inhibitory activity. Following electrophoresis, and according to the manufacturer's instructions, gels were treated with zymogram renaturating buffer (Invitrogen) for 30 min at room temperature, equilibrated with zymogram developing buffer (Invitrogen), incubated with 10 mL of trypsin or chymotrypsin solution (0.2 mg/mL of zymogram developing buffer) at 37°C for 1.5 h, and washed with distilled water before the addition of acetic acid to stop the enzymatic reaction. Areas of the gels that remained blue indicated where trypsin or chymotrypsin had been inhibited.

2.3 Chemical inactivation of soybean BBI

To abolish the trypsin and chymotrypsin inhibitory activities of soybean BBI, alkylation of the sulfhydryl groups was carried out. Ten milligrams of soybean BBI were reduced with DTT, and alkylated with 400 μL of 0.25 M iodoacetamide for 15 min at 50°C under dark conditions. In order to remove residual DTT and iodoacetamide, samples were dialysed extensively against distilled water and freeze-dried. To confirm their inactivation, soybean BBI were tested for loss of activity against trypsin and chymotrypsin enzymes, and were stored at −20°C.

2.4 Purification of major soybean BBI isoinhibitors

The major BBI isoinhibitors, IBB1 and IBBD2, were purified from soybean BBI, using a reverse-phase HPLC column (Ace® 300 Å, C_4, 250 × 4.6 mm id, 5 μm particle size, Advanced Chromatography Technologies, Aberdeen, Scotland) attached to a Beckman System Gold HPLC equipped with System Gold Software data acquisition system version 711 (Beckman Instruments, Fullerton, CA, USA). Soybean BBI were dissolved in solvent A (0.1% v/v TFA in MilliQ water) at a concentration of 5 mg/mL. The elution was performed at room temperature using a linear gradient by increasing the concentration of solvent B (0.1% v/v TFA in ACN/MilliQ water (90:10, v/v)) from 15–35% v/v for 20 min. The flow rate and volume injection were 1 mL/min and 100 μL, respectively, and the absorbance was recorded at 214 nm using a Beckman 166 UV detector. Eluted proteins were collected manually, concentrated in a vacuum centrifuge (SpeedVac Concentrator A 160, Savant Instruments, Farmingdale, NY, USA) and stored at −20°C, before further analyses. The purity of the BBI isoinhibitors was determined by IEF; 10 μg of each isoinhibitor were dissolved in Novex® IEF, pH 3–7, sample buffer and loaded on Novex® gels in the pH range 3–7, according to the manufacturer's protocol (Invitrogen). Gels were stained using the Colloidal Blue staining kit (Invitrogen).

2.5 Peptide mass fingerprinting of BBI isoinhibitors

Proteins (10 μg) were dissolved in NuPAGE® lithium dodecyl sulphate sample buffer (Invitrogen) and separated by electrophoresis on Novex 12% Bis-Tris pre-cast gels using NuPAGE® 2-N-morpholine-ethane sulphonic acid as running buffer (Invitrogen). Immediately before use, samples were reduced with DTT and NuPAGE antioxidant added to the upper buffer chamber to prevent reduced proteins from re-oxidation during electrophoresis. Bands corresponding to individual isoinhibitors were excised from Colloidal Blue (Invitrogen)-stained gels and subjected to in-gel trypsin digestion. Peptide fragments from digested proteins were desalted and concentrated using C-18 ZipTip columns (Millipore, Madrid, Spain) and then directly loaded onto the MALDI plate, using α-cyanohydroxycinnamic acid as the matrix for MALDI-MS analysis. MS spectra were obtained automatically in a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) operating in reflectron mode with delayed extraction. Peptide mass data were used for protein identification against the MS protein sequence database (www.matrixscience.com).

2.6 Cell viability assays

Human colorectal adenocarcinoma HT29 and normal colon fibroblastic CCD-18Co cells were maintained by serial

passage in 75 cm^2 plastic culture flasks. HT29 cells were cultured in DMEM, supplemented with fetal bovine serum (5%), 2 mM glutamine and 1% antibiotic-antimicotic solution (Sigma, A5955). In the case of CCD-18Co fibroblastic cells, media were additionally supplemented with 1% non-essential amino acids solution (Sigma, M7145). Optimal assay conditions for colonic cells were reported previously [20]. Briefly, 96-well microtitre plates were inoculated at a density of 2000 cells *per* well in 200 μL of growth media, and were incubated under 5% CO_2 in humidified air for 24 h to allow the cells to adhere to the wells. Soybean BBI, native or chemically inactivated (see Section 2.3), or the purified major isoforms (IBB1 and IBBD2, individually or in combination in order to investigate a potential synergistic effect), were dissolved in growth media at a range of concentrations (0–125 μM) and added to the cells under sterile conditions. Control cells received no BBI. At the end of the growth period (24–96 h), the viability of HT29 and CCD-Co18 cells was assessed by the NR (3-amino-7-dimethylamino-2-methyl-phenazine hydrochoride) cytotoxicity assay, based on the ability of viable uninjured cells to incorporate and actively bind NR, a supravital dye, into lysosomes. Cells were stained with NR solution (2 h at 37°C), followed by cell fixation (0.5% formaldehyde, 0.1% $CaCl_2$ for 30 s) at room temperature. Plates were washed by two brief immersions in PBS and the dye extracted from the viable cells using an acidified ethanol solution (50% ethanol, 1% acetic acid) at 4°C overnight. The absorbance of the solubilised dye was quantified at 550 nm using a BioRad Model 550 microplate reader (BioRad, CA, USA). Cell viability data, expressed as a percentage of the values determined for control cells grown in the absence of BBI, were obtained from at least three independent experiments (with $n \geq 4$ replicates *per* experiment). The concentration of BBI and individual isoinhibitors that reduced cell viability by 50% (IC_{50}), as compared with untreated controls, was calculated by non-linear regression fit using the GraFit software. Statistical analysis was performed using Statgraphics Plus 5.1 software (StatPoint, Herndon, VA, USA). Bonferroni's test was used to compare means and statistical significance was set at $p < 0.05$.

2.7 Cell cycle distribution analysis

To assess whether or not the effects of soybean BBI on cell growth are mediated *via* alterations in the cell cycle, cell cycle distribution patterns were analysed. HT29 cells were seeded at a density of 10^5 cells *per* millilitre of growth media and incubated, under 5% CO_2 in humidified air, for 24 h to allow the cells to adhere to 25 cm^2 cell culture flasks. Soybean BBI were dissolved in growth media at concentrations of 31 or 62 μM and immediately added, under sterile conditions, to the HT29 colon cancer cells. Control samples received no BBI. After 24 h exposure, cells were harvested by centrifugation, washed with cold PBS (200 μL) and fixed in ice-cold 70% ethanol (2 mL) for 30 min at 4°C, before addition of 100 μL RNase (1 mg/mL) and 100 μL of propidium iodide (400 μg/mL). After incubation for 30 min at 37°C in the dark, the fluorescence of stained cells was analysed by fluorescence activated cell sorting flow cytometry (Becton Dickinson Immunocytometry System, San Jose, CA, USA). Data acquisition and analysis were performed using ModFit LT (Verity Software House Topsham, ME, USA) and CellQuest software (Becton Dickinson Immunocytometry Systems), respectively.

3 Results

3.1 Isolation and functional characterisation of soybean BBI isoinhibitors

Analysis of commercial preparations of soybean BBI by SDS-PAGE showed a single band of appropriate molecular mass (8 kDa) (Fig. 1A). Nevertheless, IEF analysis demonstrated the presence of up to 11 components within the p*I* range 4.1–5.2, with two major peptides showing p*I* values of 4.7 and 4.1, respectively (Fig. 1B). Comparison of amino acid sequences of soybean BBI from the UniProt KB-Swiss-Prot database predicted an overall difference in charge, reflecting differences in the content of both negatively and positively charged amino acids. In addition, soybean BBI differed in their predicted overall mass and hydrophobicity, features that are likely to contribute to their separation by

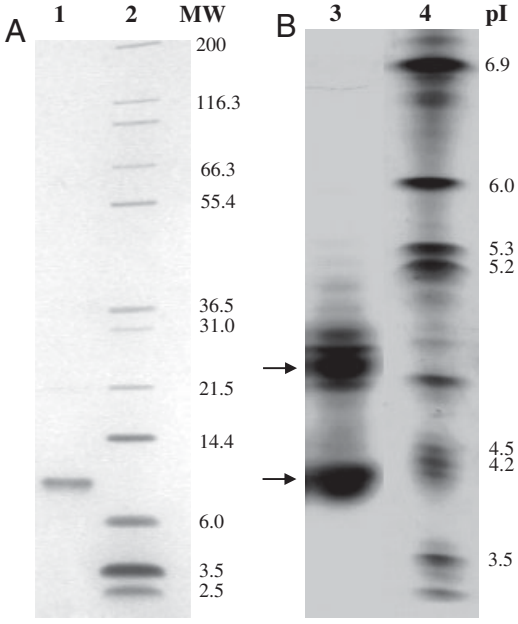

Figure 1. (A) SDS-PAGE under reducing conditions and (B) IEF of unfractionated BBI from soybean (lanes 1 and 3, respectively). Molecular weight and p*I* markers are in lanes 2 and 4, respectively. Arrows indicate the two major isoinhibitors of the mixture.

RP-HPLC. In agreement with this, soybean BBI were resolved as two major chromatographic peaks by RP-HPLC (Fig. 2A). These peaks, representing approximately 33 and 41% of the total BBI content as estimated from their relative peak areas, were collected manually and shown to correspond to the main IEF bands present in the starting material (Fig. 2B). Additional minor chromatographic peaks were also detected as either separate or unresolved peaks (Fig. 2A) that were not collected to maximise the purity of the major isoinhibitors. In order to identify the purified isoinhibitors, in-gel tryptic digestion of excised bands was performed, followed by separation of the peptides generated and MS-based analysis. A search of peptide mass data against the MS protein sequence database enabled the unambiguous identification of both BBI isoinhibitors. The purified proteins, corresponding to the chromatographic peaks 1 and 2 (see Fig. 2A), were identified by mass peptide fingerprinting as Bowman–Birk type proteinase inhibitor D-II (Swiss-Prot entry: IBBD2_SOYBN) and Bowman–Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN), showing 96 and 56% sequence coverage, respectively (Table 1). An amino acid sequence comparison of IBBD2 and IBB1 proteins is shown in Table 2, where the peptide sequences that contributed to protein identification by MS are indicated. The 14 cysteine residues are in the conserved positions, as previously described for other BBI proteins [10]. Following the nomenclature of Schechter and Berger [23], IBBD2 showed two almost identical inhibitory domains, except for positions P'_2 and P'_4. The residue Arg was present at position P_1 in both inhibitory domains, conferring specificity for inhibition of trypsin-like proteases. In the case of IBB1, variation at several positions within the two inhibitory domains was observed, and the presence of Lys or Leu in position P_1 confers a different specificity for inhibition of trypsin- or chymotrypsin-like proteases, respectively.

The specific TIA and CIA of the starting material (commercially available BBI, Fig. 1) were 3075 ± 59 and 2190 ± 27 units *per* milligram of protein, respectively. Following reduction and alkylation of disulphide bonds, these activities were reduced by greater than 95% (data not shown). Of the two purified BBI isoinhibitors, IBBD2 showed TIA but no detectable CIA, whereas IBB1 showed both TIA and CIA (Table 3). IBBD2 showed a higher specific TIA than IBB1 (3710 ± 257 and 2572 ± 122 trypsin inhibitor units *per* milligram of protein, respectively, Table 3) in agreement with the nature of the two P'_1 residues. IBB1 showed a high specific CIA (5691 ± 365 chymotrypsin inhibitor units *per* milligram of protein), in contrast to IBBD2, where CIA was not detected. These significant differences in specific inhibitory activities are likely to reflect variation in the amino acid sequences of the inhibitory domains (Table 3) that play an essential role in determining specificity and potency [4]. Based on IC_{50} and K_i calculations, IBBD2 was demonstrated to be a stronger inhibitor of trypsin (K_i of 14.8 nM) when compared with IBB1 (K_i of 29.8 nM), where the latter was a potent inhibitor of chymotrypsin (K_i of 3.3 nM) (Table 3). Such values fall within the nanomolar range reported previously for various members of the BBI family, including those from pea [19, 24], lentil (*Lens culinaris*) [25] and lupin (*Lupinus albus*) [26]. As shown in Fig. 3, zymography under non-denaturing conditions allowed the separation of soybean BBI isoinhibitors as well as the detection of specific inhibitory activities against the digestive enzymes trypsin and chymotrypsin. In good agreement with the kinetic data (Table 3), IBBD2 showed inhibition against trypsin only (Fig. 3, track 3), whereas IBB1 clearly inhibited both trypsin and chymotrypsin enzymes (Fig. 3, track 4). Chemically inactivated BBI failed to inhibit the activity of either enzyme (Fig. 3, track 1) in contrast to unfractionated proteins showing inhibition of both enzymes (Fig. 3, track 2).

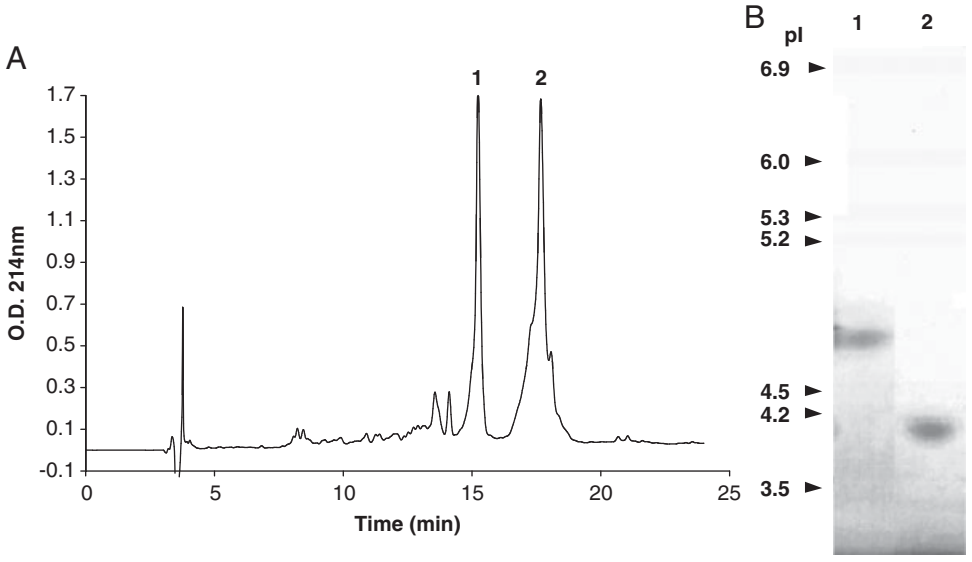

Figure 2. (A) Fractionation of BBI from soybean by reverse-phase HPLC. (B) IEF of peaks 1 (lane 1) and 2 (lane 2) that contain purified isoinhibitors.

Table 1. Identification of two major soybean BBI separated by reverse-phase HPLC

Chromatographic ID	Protein name	Swiss-Prot accession number	Entry name	Sequence coverage (%)	Matched peptides	Protein score
1	Bowman–Birk type proteinase inhibitor D-II	P01064	IBBD2_SOYBN	96	9	205
2	Bowman-Birk proteinase inhibitor	P01055	IBB1_SOYBN	56	5	147

Database searching was performed using the MASCOT database (http://www.matrixscience.com).

Table 2. Amino acid sequence alignment of IBB1 and IBBD2 proteins

IBB1_SOYBN ---------*DDESSKPCCDQCACT**KS**NPPQCRCSDMRLNSCHSACKS*CICA**LS**YPAQCFCVDITDFCYEPCKPSEDDKEN
IBBD2_SOYBN *SDQSSSYDDDEYSKPCCDLCMCT**RS**MPPQCSCEDIRLNSCHSDCKSCMCT**RS**QPGQCRCLDTNDFCYKPCK*SRDD- - - -
1 - - - - - - - - 10 - - - - - - - - - 20 - - - - - - - - -30 - - - - - - - - - 40- - - - - - - - -50- - - - - - - - -60- - - - - - - - - 70 - - - - - - - - - 80

Sequences are from the Swiss-Prot database (P01055, P01064). Amino acid sequences of inhibitory domains are underlined. P_1-P_1' are the reactive peptide bond sites, in bold text. Either K or R at position P_1 determines specificity for trypsin, whereas L determines specificity against chymotrypsin. The peptides that contributed to protein identification are indicated in italics.

Table 3. K_i and specific inhibitory activity for trypsin (T) and chymotrypsin (C) of soybean Bowman–Birk isoinhibitors

Protein	Amino acid sequence of inhibitory domains		K_i (nM)		Specific inhibitory activity (IU/mg protein)	
	Domain 1	Domain 2	T	C	T	C
IBBD2	CMCT**RS**MPPQC	CMCT**RS**QPGQC	14.8 ± 3.2	ND	3710 ± 257	ND
IBB1	CA CT**KS**N PPQC	C I CA**LS**YPA QC	29.8 ± 4.0	3.3 ± 1.0	2572 ± 122	5691 ± 365

Specific activities and K_i values represent means ± SD from at least three independent determinations. P_1-P_1', the reactive peptide bond sites, are marked in bold text. ND, not detected.

3.2 Effect of soybean BBI on the proliferation of human colon cells

The effects of BBI on the growth of human colon adenocarcinoma HT29 cells were determined by comparing the growth of cells cultured in the absence or presence of BBI (0–125 μM), monitored by the cytotoxic NR cell assay. At concentrations greater than 31 μM, soybean BBI inhibited the *in vitro* cell growth of HT29 human colon adenocarcinoma cells in a concentration-dependent manner (Fig. 4). The growth of HT29 cells was much less significantly reduced when treated with BBI that had been chemically inactivated (Fig. 5A). Our results clearly suggest that the antiproliferative effects of soybean BBI on HT29 cells are associated with their intrinsic ability to inhibit serine proteases. In contrast, the growth of colonic fibroblast CCD-18Co cells was unaffected by soybean BBI, in either active or inactivated form, even at the highest concentration tested (125 μM) **(**Fig. 5B**)**.

Given the contrasting specific activities (Table 3) of the two purified isoinhibitors, IBBD2 and IBB1, the effects of these two on the growth of HT29 cells were examined. A statistically significant ($p < 0.05$) and dose-dependent decrease of the growth of HT29 colon cells was observed after treatment with either IBBD2 or IBB1 (Fig. 6). At 31 and 62 μM, a larger effect was observed for IBBD2, compared with IBB1. Despite showing significant differences in their functional properties (Table 3), the IC_{50} values for the individual isoinhibitors, IBBD2 and IBB1, on HT29 cell growth were not significantly different (39.9 ± 2.3 and 48.3 ± 3.5 μM, respectively). These data were in agreement with the IC_{50} values obtained for the commercial BBI preparation (Fig. 1) (46 ± 2.4 μM), suggesting a non-synergistic effect of individual inhibitors. This was confirmed when the two isoinhibitors were used individually or in combination at a final concentration of 62 μM (data not shown).

To investigate whether the effects of soybean BBI on cell growth were due to cell cycle arrest, the cell cycle distribution pattern of HT29 cells was evaluated in the presence or absence of soybean BBI, using different concentrations of BBI (31 and 62 μM). After 24 h exposure to soybean BBI, the accumulation of HT29 cells in the G0–G1 stage was revealed, compared with control cells grown without BBI, and this effect was shown to be dose-dependent (Fig. 7). The histogram of DNA content in HT29 cells treated with 62 μM soybean BBI showed a significant increase in the G0–G1 peak from 62.7 ± 0.2% to 89.1 ± 1.6%, whereas the cell population in G2-M and S stage decreased significantly from 28.2 ± 1.2% to 4.3 ± 1% and 9.2 ± 1% to 6.6 ± 0.6%, respectively, as compared with untreated cells.

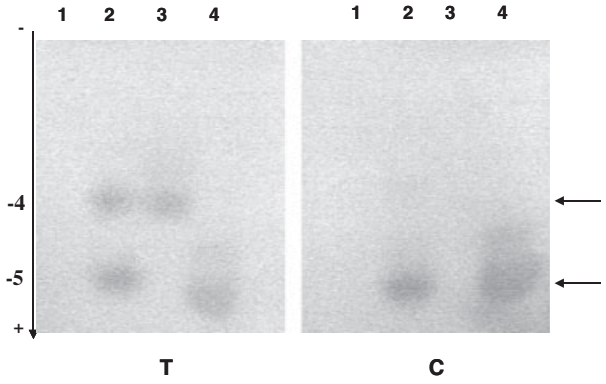

Figure 3. In-gel protease inhibitory activity analyses of soybean BBI. Zymogram Blue casein gels were treated with digestive enzymes, trypsin (T) or chymotrypsin (C); dark areas indicate where the enzyme has been inhibited (horizontal arrows). Lane 1: chemically inactivated soybean BBI; Lane 2: unfractionated BBI; Lane 3: purified IBBD2; Lane 4: purified IBB1. Lanes 1 and 2 contained 16 μg of protein, whereas lanes 3 and 4 contained 32 μg of protein. The direction of electrophoresis on non-denaturing gels is indicated, alongside the overall charge of the two isoinhibitors (vertical arrow).

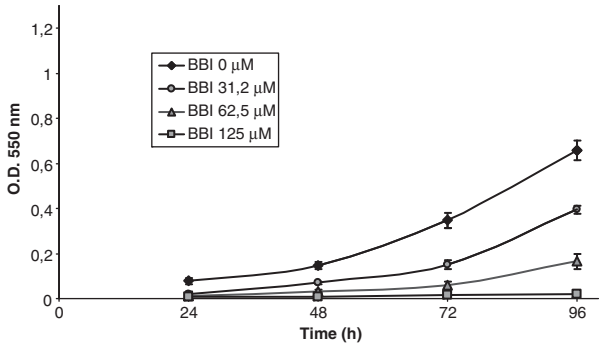

Figure 4. Dose-response effects of unfractionated soybean BBI on the growth of HT29 colon adenocarcinoma cells. Cells were treated with native soybean BBI (0–125 μM) for up to 96 h. Every point represents the mean of two independent experiments, each having four technical replicates; bars represent standard deviations.

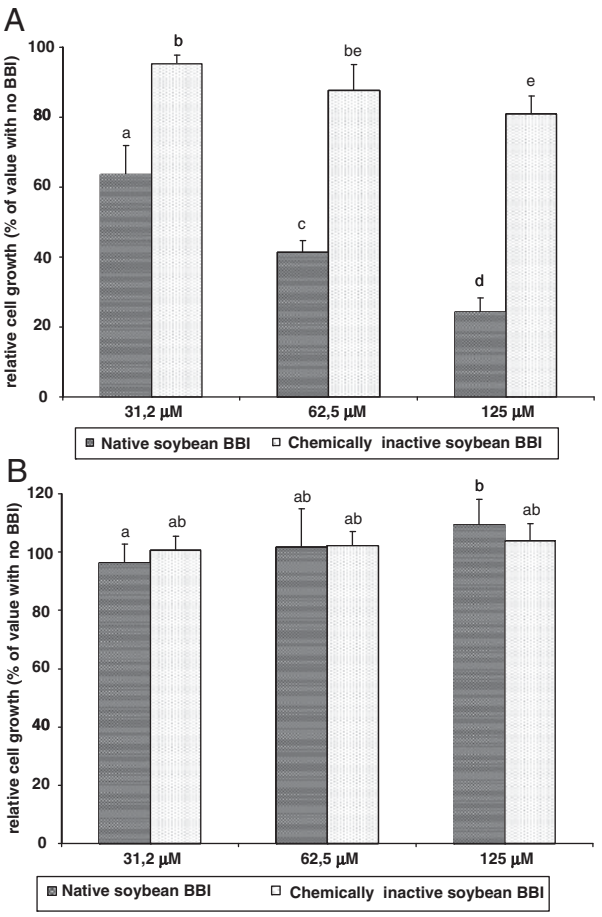

Figure 5. Effects of native and chemically inactive BBI from soybean on the *in vitro* growth of (A) HT29 human colorectal adenocarcinoma cells, and (B) normal colon fibroblastic CCD-18Co cells. Growth media were supplemented with concentrations of BBI in the range 0–125 μM and cells harvested after a period of 96 h. Data are means of at least three independent experiments, each having four technical replicates; bars represent standard deviations. Means not sharing superscript letters differ significantly ($p < 0.05$; Bonferroni's test).

4 Discussion

The use of naturally occurring compounds as chemopreventive agents in order to block, inhibit, reverse or retard the process of carcinogenesis is a novel and promising approach to prevent cancer [27]. In CRC, one of the leading causes of cancer-related mortality in western countries, nutritional intervention offers great potential to delay or prevent the development of malignant processes; such an interventional strategy might result in a positive impact on the incidence of disease and mortality [28]. In this context, soybean BBI and related proteins have recently emerged as highly promising chemotherapeutic compounds within the GIT [3–5]. The effectiveness of soybean BBI in preventing or suppressing cancer development in dimethylhydrazine-induced colon tumours has been demonstrated in rodents [3, 29]. In this work, we demonstrate that soybean BBI exert an anti-proliferative effect on HT29 colon adenocarcinoma cells in a dose-dependent manner, whereas non-malignant colonic fibroblast CCD-18Co cells were unaffected. Interestingly, chemically inactivated BBI had a weak but non-significant effect on the proliferation of HT29 colon cancer cells; such a weak effect could be a result of the residual inhibitory activity ($\leq 5\%$ of the original activity). These data clearly suggest that the anti-proliferative activity of BBI on HT29 cells is mediated *via* protease inhibition. These findings reveal the need to evaluate the amounts of active BBI present in soybean foods that could potentially exert a protective function in the large intestine. Recent studies have demonstrated the presence of BBI in a large number of

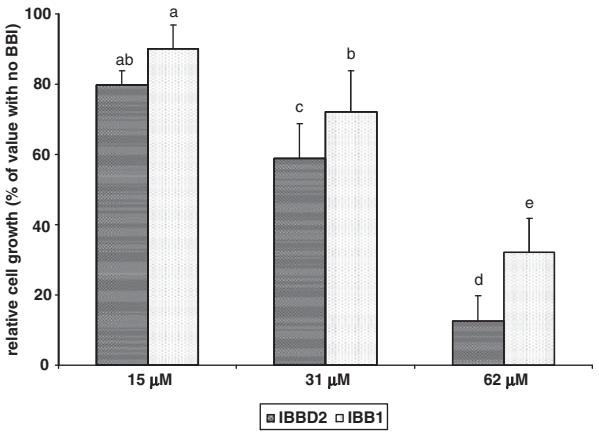

Figure 6. Effects of the major soybean BBI isoinhibitors, IBBD2 and IBB1, on the *in vitro* growth of HT29 human colorectal adenocarcinoma cells. Growth media were supplemented with concentrations of BBI in the range 0–125 μM and cells harvested after a period of 96 h. Data are means of at least three independent experiments, each having four technical replicates; bars represent standard deviations. Means not sharing superscript letters differ significantly ($p < 0.05$; Bonferroni's test).

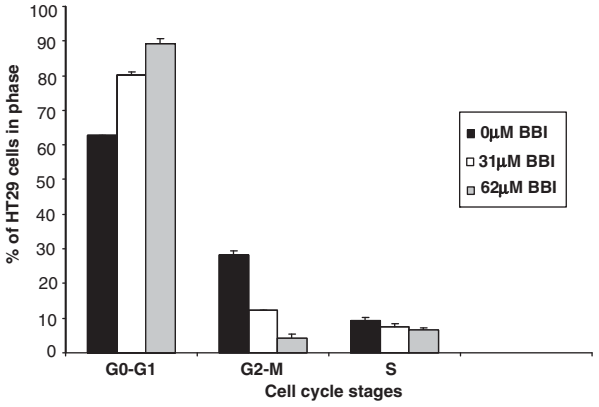

Figure 7. Cell cycle distribution pattern of HT29 cells after 24 h culture in the presence (31 and 62 μM) or absence of soybean BBI. Cells were stained with propidium iodide and analysed by flow cytometry to measure fluorescence. The values given are the percentages of cells in every phase. Data are means of three independent experiments; bars represent standard deviations.

commercial soybean foods [30]. In soymilk samples, BBI were present at between 7.2 and 55 mg *per* 100 mL of product; in the case of other soybean foods like soybean cake and bean curd, up to 19.2 mg of BBI *per* 100 g of product was found. The reported amounts seem to be physiologically relevant in order to exert anticancer effects in humans [31]; however, these data are based on immunoreactive forms of BBI that could be functionally inactive. It is worth noting that BBI are extremely resistant to denaturation by heat treatment [32, 33]. The chemical denaturation that was performed in this study is a harsh treatment that is quite removed from any process performed during food manufacture, but it did not completely abolish activity. Understanding the relationships between protease inhibitory activities of BBI, specifically linked to their chemopreventive properties, and food manufacturing would provide a valuable insight into the likely beneficial effects of BBI-containing foods on gastrointestinal health.

Previous studies have suggested an involvement of the chymotrypsin-binding site of BBI in the anti-carcinogenic properties of these proteins, leading to the hypothesis that chymotrypsin-like proteases might play a relevant role in carcinogenesis [18, 34]. Yavelow *et al.* [17] reported that an enzymatically modified BBI from soybean having chymotrypsin inhibitory activity only was still fully effective as an inhibitor of radiation-induced transformation *in vitro*. We have demonstrated previously the effect of sequence variation within the chymotrypsin inhibitory site, on the anti-proliferative properties of BBI from pea [20]. In the current work, we show that both trypsin and chymotrypsin inhibitory activities of BBI proteins are likely to be involved in the anti-proliferative properties of BBI on colon cancer cells. A purified soybean isoinhibitor, IBBD2, having trypsin inhibitory activity only, exerted a significant inhibitory effect on the growth of HT29 cells. To our knowledge, data regarding the positive contribution of the trypsin inhibitory domain of BBI on their anti-proliferative properties have not been reported previously. Our results suggest clearly that trypsin-like proteases involved in carcinogenesis should be considered also as potential targets of BBI and related proteins.

The homeostatic control between proteolytic enzymes and their cognate inhibitors plays a pivotal role in a number of physiological as well as pathological processes, including cancer and inflammatory disorders. An understanding of the role played by proteases in the biological processes associated with disease offers novel opportunities for therapeutic intervention [35]. Several serine proteases have been linked to tumour cell invasion and metastasis and, more recently, to angiogenesis and tumour growth [36, 37]. One such candidate is matriptase (MT-SP1), an epithelial-derived type II transmembrane serine protease, which exhibits trypsin-like protease activity and has been described in a variety of epithelial colon cancer cell lines [38]. Recent studies support the hypothesis that MT-SP1 acts as an upstream activator in metastasis and cancer invasion through the selective degradation of various elements of the cell-surrounding extracellular matrix and its inhibition could potentially modulate tumorigenesis and metastasis *in vivo* [39]. Although the ability of soybean BBI to inhibit the trypsin-like activity of MT-SP1 has been demonstrated [40], the clinical relevance of such inhibition has not been proven yet. Chymase, a key mediator in inflammatory cell signalling pathways, is a chymotrypsin-like serine protease, which is stored primarily in mast cell granules and released upon degranulation, and has been reported to be susceptible to inhibition by soybean BBI [18]. It has also been suggested that BBI internalisation by epithelial cells could facilitate the inhibition of intracellular target proteases associated with

the transformation of normal to malignant cells [12]. Proteasomes are involved in control of the cell cycle by proteolytic degradation of several cell cycle regulatory proteins such as cyclins and cyclin-dependent kinases and thus represent a promising target structure for early anticancer strategies in combination with cytotoxic drugs [41]. Recently, it has been demonstrated that soybean BBI can inhibit *in vitro* and *in vivo* the proteasomal chymotrypsin-like activity in MCF7 breast cancer cells, accompanied by down-regulation of cyclin D1 and cyclin E [42]; these recent findings suggest a novel mechanism for BBI in controlling cell proliferation processes and cell death. Elucidation of the mechanism(s) by which these dietary proteins can block cell cycle progression and exert antiproliferative activity will provide insights into the effect of BBI and related proteins as chemopreventive agents, and support the characterisation of variants as described in this work. In combination, these data contribute to the development of new strategies for inhibitor design in cancer prevention programmes and potentially for further medical applications.

This work was carried out with the financial support of grants AGR2006-00706 and AGL2007-60007/ALI from Junta de Andalucía and Spanish CICYT, respectively. Alfonso Clemente acknowledges receipt of a Ramon and Cajal contract. The JIC is supported by competitive grants from the Biotechnology and Biological Sciences Research Council (BBSRC, U.K.), as well as from the Department for Environment Food and Rural Affairs (Defra, U.K.). We are very grateful to Dr. S. Ogueta from the Proteomics Facility of University of Cordoba for carrying out peptide mass fingerprinting. Thanks are due to Dr. J. Lazuen from the Scientific Instrumentation Centre at the University of Granada (CIC-UGR, Granada, Spain) for carrying out the flow cytometry analysis on HT29 and CCD-18Co cells.

The authors have declared no conflict of interest.

5 References

[1] Cummings, J. H., Bingham, S. A., Fortnightly review - diet and the prevention of cancer. *Br. Med. J.* 1998, *317*, 1636–1640.

[2] Reddy, B. S., Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention. *Cancer Epidemiol. Biomark. Prev.* 2000, *9*, 239–247.

[3] Kennedy, A. R., Billings, P. C., Wan, X. S., Newberne, P. M., Effects of Bowman-Birk in-hibitor on rat colon carcinogenesis. *Nutr. Cancer* 2002, *43*, 174–186.

[4] Clemente, A., Domoney, C., Biological significance of polymorphism in plant protease inhibitors from the Bowman-Birk class. *Curr. Prot. Pept. Sci.* 2006, *7*, 201–216.

[5] Clemente, A., Domoney, C., in: Govil, J. N., Singh, V. K., Sharma, R. K. (Ed.), *Recent Progress in Medicinal Plants (Vol. 20) - Therapeutic properties of legume protease inhibitors from the Bowman-Birk class*, SCI Tech Publishing LLC, Houston 2007, pp. 397–417.

[6] Clemente, A., Jiménez, E., Marín-Manzano, M. C., Rubio, L. A., Functional Bowman-Birk inhibitors survive gastrointestinal digestion at the terminal ileum of cannulated pigs fed chickpea-based diets. *J. Sci. Food Agric.* 2008, *88*, 523–531.

[7] Marín-Manzano, M. C., Ruiz, R., Jimenez, E., Rubio, L. A., Clemente, A., Anti-carcinogenic Bowman-Birk inhibitors from soybean survive fermentation in active form using faecal inoculum and do not affect the microbiota composition. *Br. J. Nutr.* 2009, *101*, 967–971.

[8] Chen, P., Rose, J., Love, R., Wei, C. H., Wang, B. C., Reactive sites of an anticarcinogenic Bowman-Birk proteinase-inhibitor are similar to other trypsin-inhibitors. *J. Biol. Chem.* 1992, *267*, 1990–1994.

[9] Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J. *et al*. Factors affecting the *in vitro* digestibility of chickpea albumins. *J. Sci. Food Agric.* 2000, *80*, 79–84.

[10] Qi, R. F., Song, Z. W., Chi, C. W., Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application. *Acta Biochim. Biophys. Sin.* 2005, *37*, 283–292.

[11] St Clair, W., Billings, P., Carew, J., Keller-McGandy, C. *et al.*, Suppression of dimethylhydrazine-induced carcinogenesis in mice by dietary addition of the Bowman-Birk protease inhibitor. *Cancer Res.* 1990, *50*, 580–586.

[12] Billings, P. C., Brandon, D. L., Habres, J. M., Internalization of the Bowman-Birk protease inhibitor by intestinal epithelial cells. *Eur. J. Cancer.* 1991, *27*, 903–908.

[13] Fernandes, A. O., Banerji, A. P., Inhibition of benzopyrene-induced forestomach tumors by field bean protease inhibitor(s). *Carcinogenesis* 1995, *16*, 1843–1846.

[14] Ware, H. W., Wan, S., Newberne, P., Kennedy, A. R., Bowman-Birk concentrate reduces colon inflammation in mice with dextran sulphate sodium-induced ulcerative colitis. *Digest. Dis. Sci.* 1999, *44*, 986–990.

[15] Domoney, C., Welham, T., Ellis, N., Mozzanega, P., Turner, L., Three classes of proteinase inhibitor gene have distinct but overlapping patterns of expression in *Pisum sativum* plants. *Plant Mol. Biol.* 2002, *48*, 319–329.

[16] Sonnante, G., De Paolis, A., Pignone, D., Bowman-Birk inhibitors in Lens: identification and characterization of two paralogous gene classes in cultivated lentil and wild relatives. *Theor. Appl. Genet.* 2005, *110*, 596–604.

[17] Yavelow, J., Collins, M., Birk, Y., Troll, W., Kennedy, A. R., Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress X-ray induced transformation *in vitro*. *Proc. Natl. Acad. Sci. USA* 1985, *82*, 5395–5399.

[18] Ware, J. H., Wan, X. S., Rubin, H., Schechter, N. M., Kennedy, A.-R., Soybean Bowman-Birk protease inhibitor is a highly effective inhibitor of human mast cell chymase. *Arch. Biochem. Biophys.* 1997, *344*, 133–138.

[19] Clemente, A., MacKenzie, D. A., Jeenes, D. J., Domoney, C., The effect of variation within inhibitory domains on the activity of pea protease inhibitors from the Bowman-Birk class. *Protein Express. Purif.* 2004, *36*, 106–114.

[20] Clemente, A., Gee, J. M., Johnson, I. T., MacKenzie, D. A., Domoney, C., Pea (*Pisum sativum* L.) protease inhibitors from the Bowman-Birk class influence the growth of human colorectal adenocarcinoma HT29 cells *in vitro*. *J. Agric. Food Chem.* 2005, *53*, 8979–8986.

[21] Domoney, C., Welham, T., Trypsin inhibitors in *Pisum*: variation in amount and pattern of accumulation in developing seed. *Seed Sci. Res.* 1992, *2*, 147–154.

[22] Copeland, R. A., Lombardo, D., Giannaras, J., Dedicco, C. P., Estimating K_i values for tight-binding inhibitors from dose-response plots. *Bioorg. Med. Chem. Lett.* 1995, *5*, 1947–1952.

[23] Schechter, I., Berger, A., On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* 1967, *27*, 157–162.

[24] Ferrasson, E., Quillien, L., Gueguen, J., Proteinase inhibitors from pea seeds: purification and characterization. *J. Agric. Food Chem.* 1997, *45*, 127–131.

[25] Ragg, E. M., Galbusera, V., Scarafoni, A., Negri, A. *et al.*, Inhibitory properties and solution structure of a potent Bowman-Birk protease inhibitor from lentil (*Lens culinaris* L.) seeds. *FEBS J.* 2006, *273*, 4024–4039.

[26] Scarafoni, A., Consonni, A., Galbusera, V., Negri, A. *et al.*, Identification and characterization of a Bowman-Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds. *Phytochemistry* 2008, *69*, 1820–1825.

[27] Pan, M. H., Ho, C. T., Chemopreventive effects of natural dietary compounds on cancer development. *Chem. Soc. Rev.* 2008, *37*, 2558–2574.

[28] Lao, C. D., Brenner, D. E., Strategies for prevention of colorectal cancer: pharmaceutical and nutritional interventions. *Curr. Treat. Options Oncol.* 2004, *5*, 417–426.

[29] Billings, P. C., Newberne, P., Kennedy, A. R., Protease inhibitor suppression of colon and anal gland carcinogenesis induced by dimethylhydrazine. *Carcinogenesis* 1990, *11*, 1083–1086.

[30] Hernandez-Ledesma, B., Hsieh, C. C., de Lumen, B. O., Lunasin and Bowman-Birk protease inhibitor (BBI) in US commercial soy foods. *Food Chem.* 2009, *115*, 574–580.

[31] Kennedy, A. R., The Bowman-Birk inhibitor from soybean as an anticancer agent. *J. Clin. Am. Nutr.* 1998, *68*, 1406S–1412S.

[32] Rayas-Duarte, P., Bergeron, D., Nielsen, S. S., Screening of heat-stable trypsin inhibitors in dry beans and their partial purification from great northern beans (*Phaseolus vulgaris*) using anhydrotrypsin-Sepharose affinity chromatography. *J. Agric. Food Chem.* 1992, *40*, 32–42.

[33] Osman, M. A., Reid, P. M., Weber, C. W., Thermal inactivation of tepary bean (*Phaseolus acutifolius*), soybean and lima bean protease inhibitors: effect of acidic and basic pH. *Food Chem.* 2002, *78*, 419–423.

[34] Kennedy, A. R., Szuhaj, B. F., Newberne, P. M., Billings, P. C., Preparation and production of a cancer chemopreventive agent. Bowman-Birk inhibitor concentrate. *Nutr. Cancer* 1993, *19*, 281–302.

[35] Turk, B., Targeting proteases: successes, failures and future prospects. *Nat. Rev. Drug Discov.* 2006, *5*, 785–799.

[36] Darmoul, D., Gratio, V., Devaud, H., Lehy, T., Laburthe, M., Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. *Am. J. Pathol.* 2003, *162*, 1503–1513.

[37] Lee, S. L., Dickson, R. B., Lin, C. Y., Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J. Biol. Chem.* 2000, *275*, 36720–36725.

[38] Bhatt, A. S., Takeuchi, T., Ylstra, B., Ginzinger, D. *et al.*, Quantitation of membrane type serine protease 1 (MT-SP1) in transformed and normal cells. *Biol. Chem.* 2006, *384*, 257–266.

[39] Bugge, T. H., List, K., Szabo, R., Matriptase-dependent cell surface proteolysis in epithelial development and pathogenesis. *Front. Biosci.* 2007, *12*, 5060–5070.

[40] Yamasaki, Y., Satomi, S., Murai, N., Tsuzuki, S., Fushiki, T., Inhibition of membrane-type serine protease 1/matriptase by natural and synthetic protease inhibitors. *J. Nutr. Sci. Vitaminol.* 2003, *49*, 27–32.

[41] Kisselev, A. F., Callard, A., Goldberg, A. L., Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J. Biol. Chem.* 2006, *281*, 8582–8590.

[42] Chen, Y. W., Huang, S. C., Lin-Shiau, S. Y., Lin, J. K., Bowman-Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1. *Carcinogenesis* 2005, *26*, 1296–1306.

