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

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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; **CIU**, chymotrypsin inhibitor unit(s); **CRC**, colorectal cancer; **DMH**, dimethylhydrazine; **GIT**, gastrointestinal tract; **IAA**, iodoacetamide; **IU**, inhibitor units; K_i, inhibition constant; **MT-SP1**, matriptase; **NR**, neutral red; **PI**, protease inhibitors; **TIA**, trypsin inhibitor activity; **TIU**, trypsin inhibitor unit(s).

Keywords: Bowman-Birk inhibitors, cell growth, cell proliferation, colorectal cancer cells, HT29 cells, protease inhibitory activity, soybean

Abstract

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 and between 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 (IC_{50}) 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, will 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.

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 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 (PI) of the Bowman-Birk family, a major PI 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 colorectal cancer 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 BBI concentrate (BBIC), an extract enriched in BBI, exerted a protective effect in dimethylhydrazine (DMH)-treated animals, reducing the incidence and frequency of colon tumours in mice [11,12] and rats [3]. In these studies, no adverse side effects of BBIC were 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 and between 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. Bowman-Birk inhibitors (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 ninety-six 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 (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 (TIU) 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 (CIU) 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 (IU) per mg 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}) was determined for each protease, using the GraFit software (GraFit Version 5, Erithacus Software Ltd., 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 analyzed 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 ºC 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 (IAA) for 15 min at 50 ºC under dark conditions. In order to remove residual DTT and IAA, samples were dialyzed 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® 300Å, C_4, 250 x 4.6mm I.D., 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 5mg/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 acetonitrile/MilliQ water (90:10, v/v)] from 15 to 35 % (v/v) in 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 11735, 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® LDS sample buffer (Invitrogen) and separated by electrophoresis on Novex 12 % Bis-Tris pre-cast gels using NuPAGE® MES 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 MSDB 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, ninety-six well microtitre plates were inoculated at a density of 2,000 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 sec) 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 ºC overnight. The absorbance of the solubilized 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 ≥ 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 Inc., 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 mL 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 ºC, before addition of 100 µL RNase (1 mg/mL) and 100µL of propidium iodide (400 µg/mL). After incubation for 30 min at 37 ºC 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), respectively.

3. Results

3.1 Isolation and functional characterization of soybean BBI isoinhibitors. Analysis of commercial preparations of soybean BBI by SDS-PAGE showed a single band of appropriate molecular mass (8 kDa) (**Figure 1a**). Nevertheless, IEF analysis demonstrated the presence of up to 11 components within the pI range 4.1-5.2, with two major peptides showing pI values of 4.7 and 4.1, respectively (**Figure 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 RP-HPLC. In agreement with this, soybean BBI were resolved as two major chromatographic peaks by RP-HPLC (**Figure 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 isoelectrofocusing bands present in the starting material (**Figure 2b**). Additional minor chromatographic peaks were also detected as either separate or unresolved peaks (**Figure 2a**), that were not collected to maximize 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 mass spectrometric-based analysis. A search of peptide mass data against the MS protein sequence database (MSDB) enabled the unambiguous identification of both BBI isoinhibitors. The purified proteins,

corresponding to the chromatographic peaks 1 and 2 (see **Figure 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 mass spectrometry 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, **Figure 1**) were 3075 ± 59 and 2190 ± 27 units per mg 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 TIU per mg of protein, respectively, **Table 3**) in agreement with the nature of the two P_1' residues. IBB1 showed a high specific CIA (5691 ± 365 CIU per mg of protein), in contrast to IBBD2, where CIA was not detected. These significant differences in specific inhibitory activities are likely to reflect the 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 **Figure 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 (**Figure 3**, track 3), whereas IBB1 clearly inhibited both trypsin and chymotrypsin enzymes (**Figure 3**, track 4). Chemically inactivated BBI failed to inhibit the activity of either enzyme (**Figure 3**, track 1) in contrast to unfractionated proteins showing inhibition of both enzymes (**Figure 3**, track 2).

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 (**Figure 4**). The growth of HT29 cells was much less significantly reduced when treated with BBI that had been chemically inactivated (**Figure 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) **(Figure 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 (**Figure 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 median inhibitory concentration (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 (**Figure 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 (**Figure 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 to untreated cells.

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 DMH-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 (≤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 commercial soybean foods [30]. In soy milk 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 yet the former 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 to 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 internalization 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 MCF-7 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.

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FIGURE LEGENDS

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

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.

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 hours. 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 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 hours. 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 percentage of cells in every phase. Data are means of three independent experiments; bars represent standard deviations.

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

Chromatographic peak identification	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

	1 - - - - - - - - 10 - - - - - - - - - 20 - - - - - - - - -30 - - - - - - - - - 40- - - - - - - - -50- - - - - - - - -60- - - - - - - - - 70 - - - - - - - - - 80
IBB1_SOYBN (P01055)	**- - - - - - - -***DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACK*SCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN
IBBD2_SOYBN (P01064)	*SDQSSSYDDDEYSKPCCDLCMCTRSMPPQCSCEDIRLNSCHSDCKSCMCTRSQPGQCRCLDTNDFCYKPCK*SRDD- - - -
	1 - - - - - - - - 10 - - - - - - - - - 20 - - - - - - - - -30 - - - - - - - - - 40- - - - - - - - -50- - - - - - - - -60- - - - - - - - - 70 - - - - - - - - - 80

Accession numbers are from Swiss_Prot database. 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.

601

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

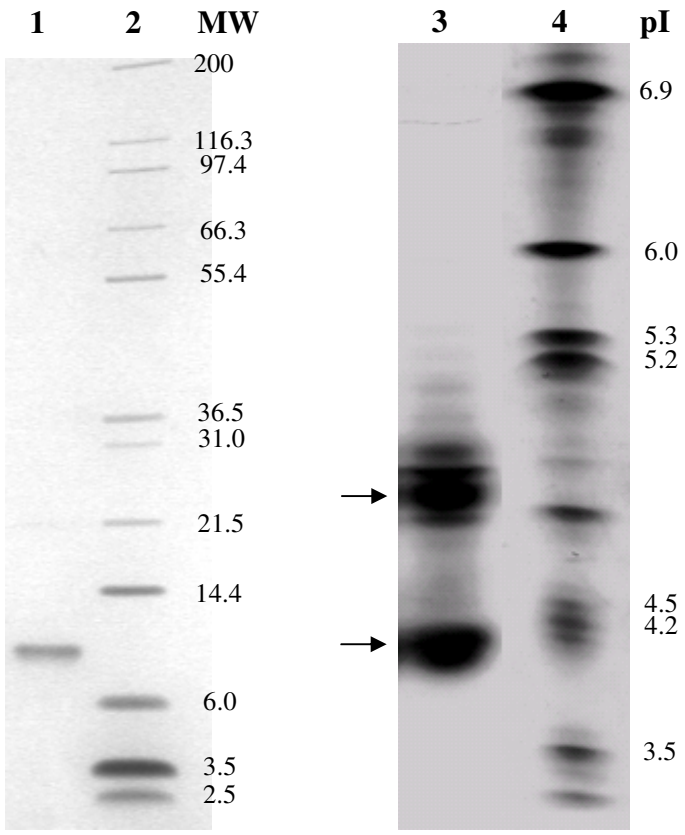
Soybean Bowman-Birk isoinhibitors	Amino acid sequence of inhibitory domains		Inhibition constant (nM)		Specific inhibitory activity (IU/mg protein)	
	Domain 1	Domain 2	T	C	T	C
IBBD2	CMCT**RS**MPPQC	CMCT**RS**QPGQC	14.8 ± 3.2	ND	3710 ± 257	ND
IBB1	CACT**KS**NPPQC	CICA**LS**YPAQC	29.8 ± 4.0	3.3 ± 1.0	2572 ± 122	5691 ± 365

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

602

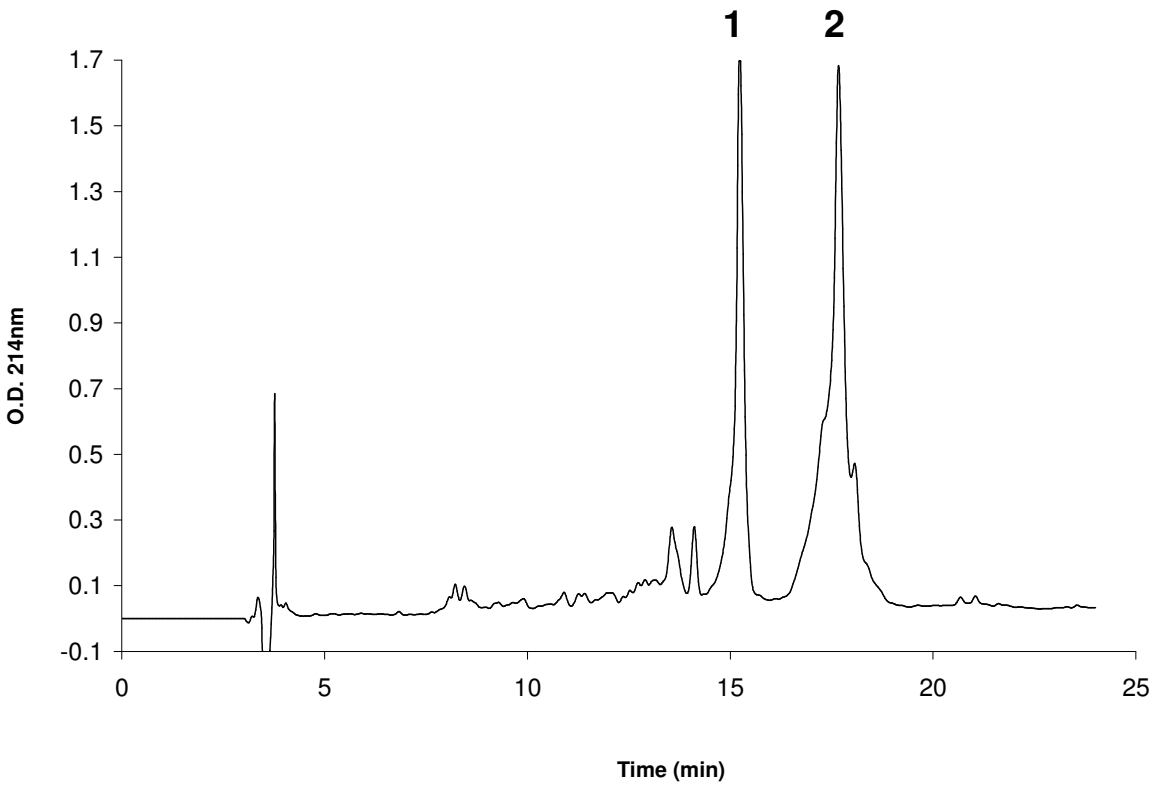
603

Figure 1

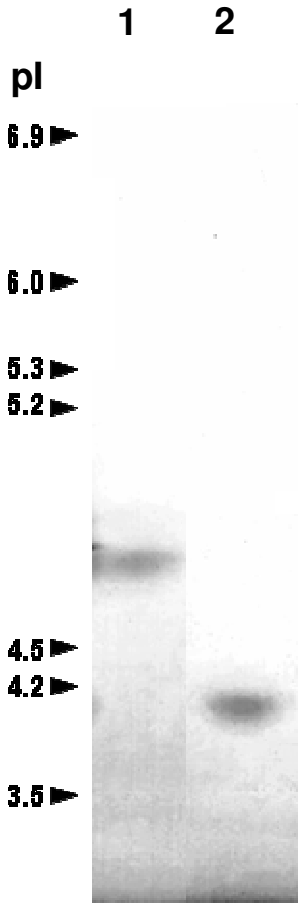


(Clemente et al.,)

Figure 2



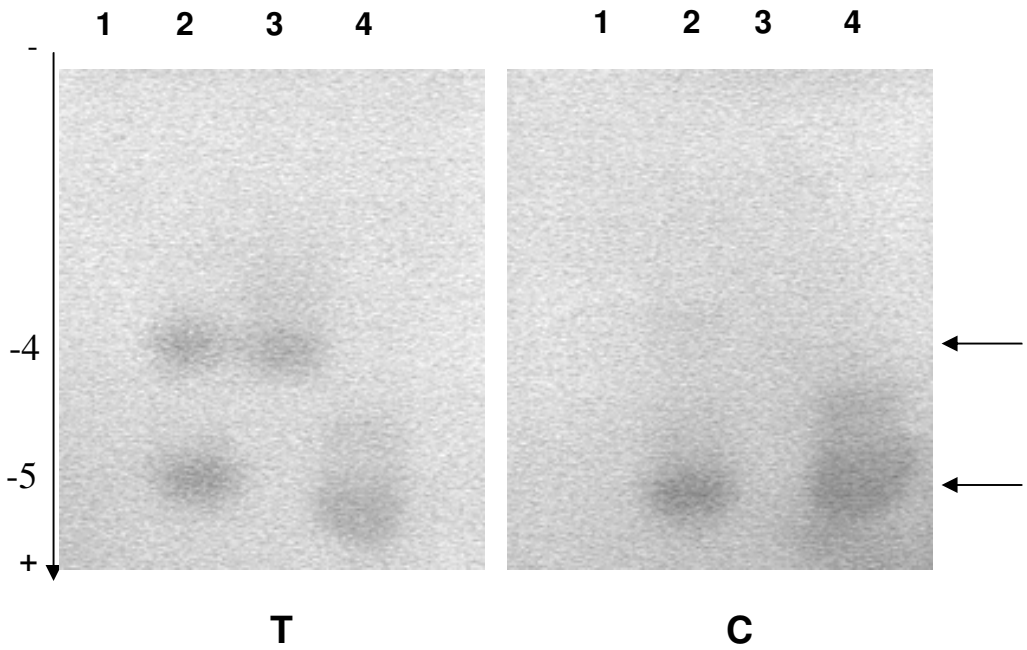
(a)



(b)

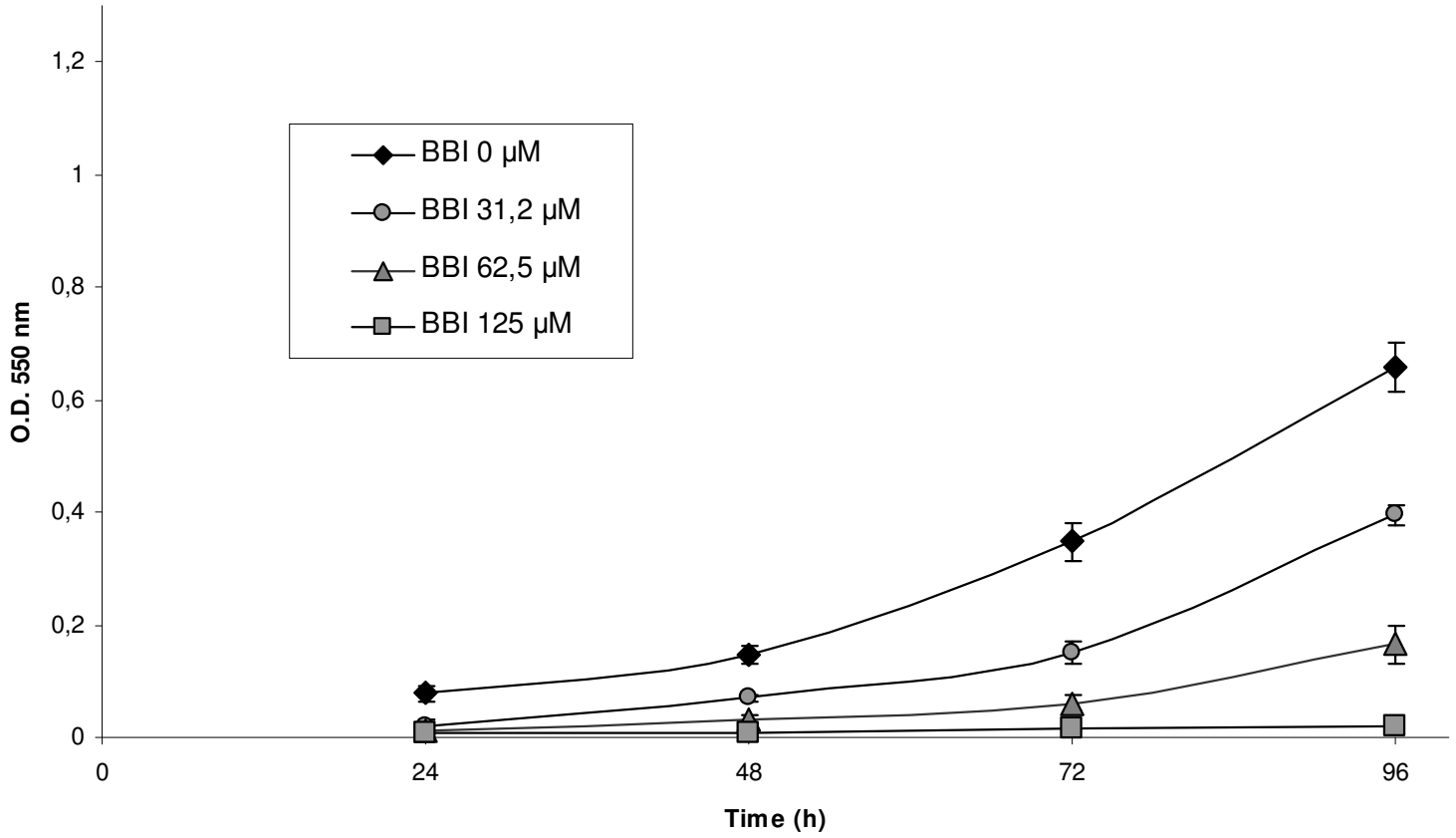
(Clemente et al.,)

Figure 3



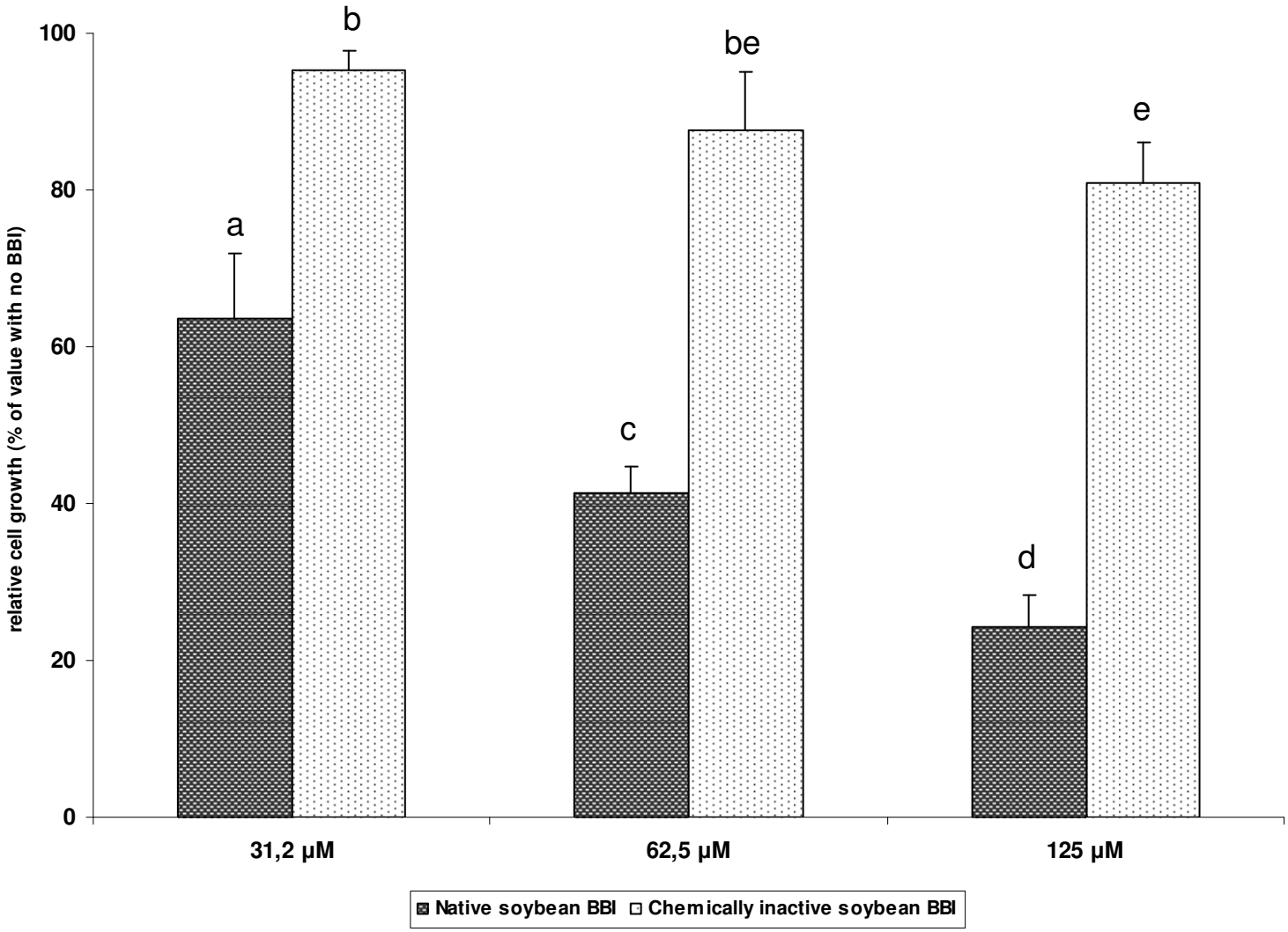
(Clemente et al.,)

Figure 4



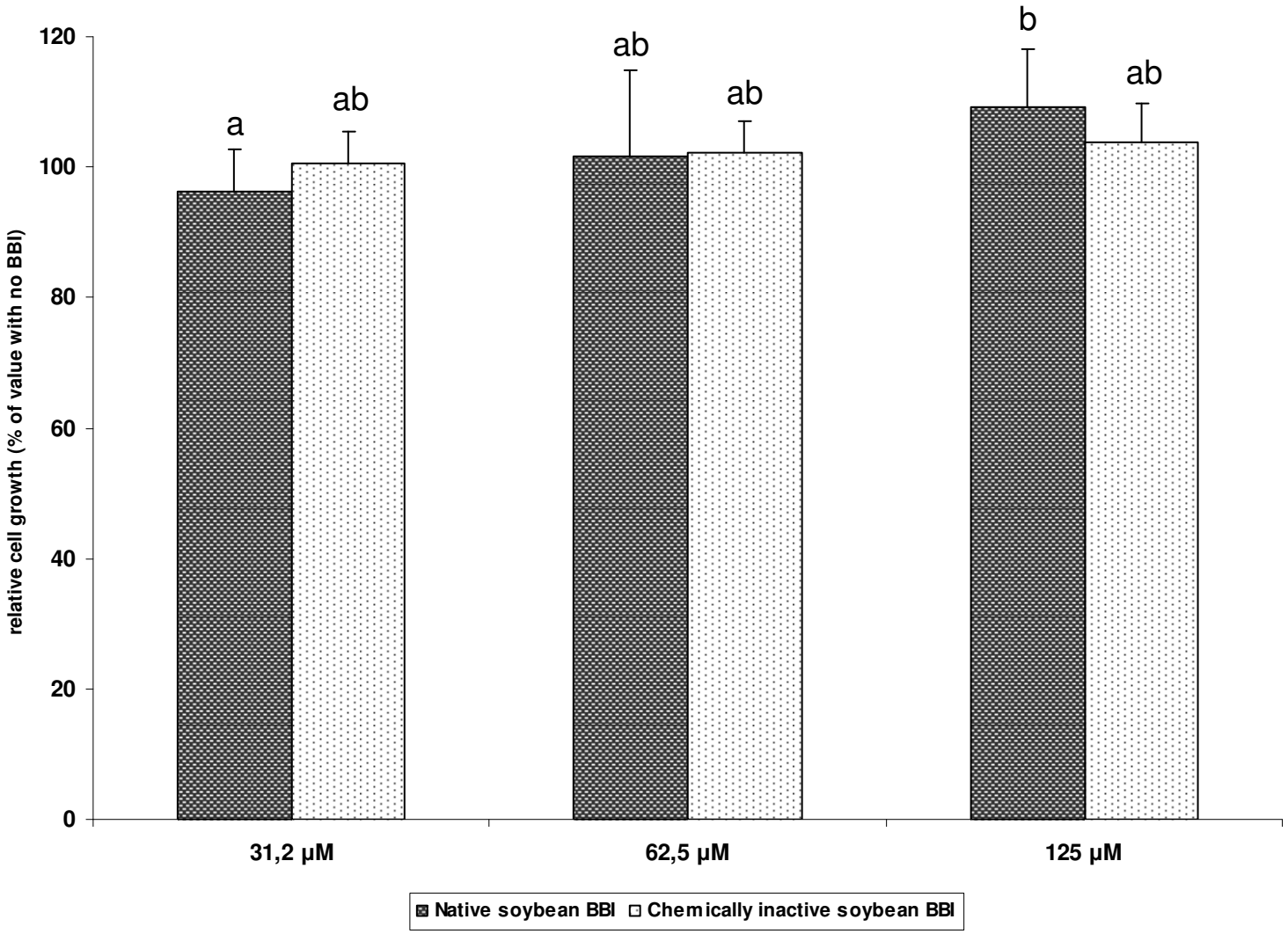
(Clemente et al.,)

Figure 5a



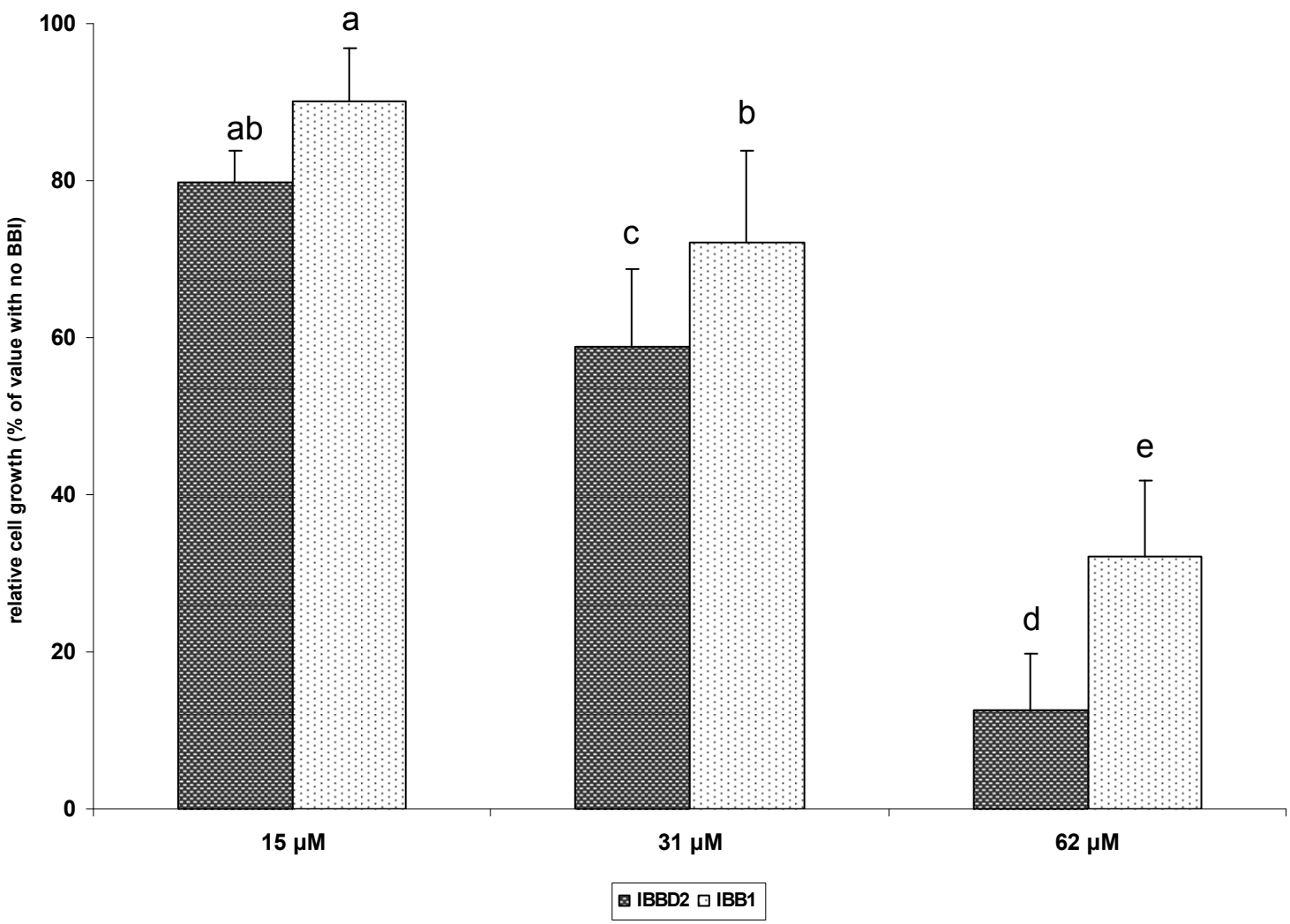
(Clemente et al.,)

Figure 5b



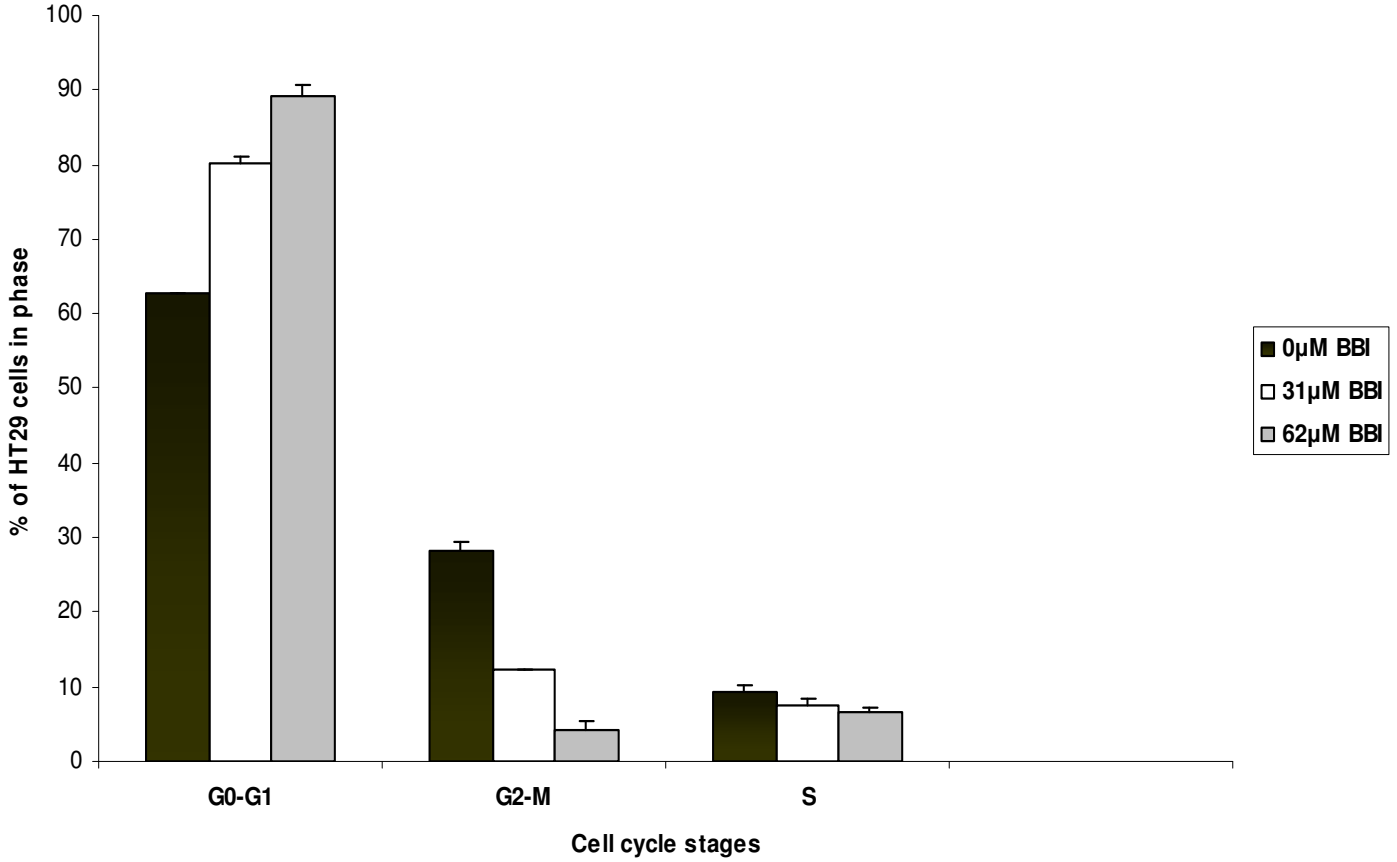
(Clemente et al.,)

Figure 6



(Clemente et al.,)

Figure 7



(Clemente et al.,)