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Pea (*Pisum sativum* L.) Protease Inhibitors from the Bowman−Birk Class Influence the Growth of Human Colorectal Adenocarcinoma HT29 Cells *in Vitro*

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The Bowman−Birk trypsin−chymotrypsin inhibitor (BBI) from soybean has been described as a potential cancer chemopreventive agent. We have compared the effects of BBI with those of two variant recombinant pea (*Pisum sativum* L.) seed protease inhibitors, rTI1B and rTI2B, homologous to BBI but differing in inhibitory activity, on the growth of human colorectal adenocarcinoma HT29 cells *in vitro*. A significant and dose-dependent decrease in the growth of HT29 cells was observed using all protease inhibitors, with rTI1B showing the largest decrease (IC_{50} = 46 μM). Inclusion of the pan−caspase inhibitor, Boc-D-FMK, did not negate the effects of rTI1B or rTI2B in the cell assays. The relative effectiveness of rTI1B and rTI2B may correlate with a variant amino acid sequence within their respective chymotrypsin inhibitory domain, in agreement with a chymotrypsin-like protease as a potential target.

KEYWORDS: Bowman−Birk inhibitor; cell growth; chymotrypsin inhibitory domain; colon cancer cells; pea; *Pisum sativum*

1. INTRODUCTION

Experimental data suggest that plant protease inhibitors (PI) of the Bowman−Birk class may be highly promising as cancer chemopreventive agents. The Bowman−Birk inhibitor (BBI) from soybean has been shown to be capable of preventing or suppressing carcinogenic processes in a wide variety of *in vitro* and *in vivo* animal model systems (*1*). As a result of this evidence, BBI acquired the status of an "investigational new drug" from the FDA in 1992 and is being evaluated currently in large-scale human trials as an anticarcinogenic agent in the form of BBI concentrate (BBIC) (*2*). The results of phase I and II clinical trials have shown that BBI has a substantial positive clinical effect in patients with oral leukoplakia (*3*) and no significant adverse effects on surrounding normal tissue (*4*). No clinical toxicity was apparent when BBI was administered as a single oral dose to 24 patients (*5*).

The exploitation of plant PI in human health-promotion programs depends upon their relative survival rates in the gastrointestinal tract. Several studies have demonstrated that BBI has a relatively high thermal stability and resistance to the acidic conditions and digestive enzymes of the upper gastrointestinal tract (*6*, *7*). When given orally to mice, more than 90% of the ingested BBI reached the colon in an active form (*8*) and/or could be transported as biologically active peptides by intestinal epithelial cells (*9*). A recent report has shown the effectiveness of purified BBI, using the dimethylhydrazine (DMH) rat model, in reducing the incidence or frequency of colorectal tumors when compared with animals treated with DMH alone (*10*). PI from field beans have proven to be biologically active, under acidic conditions, in suppressing benzopyrene-induced forestomach carcinogenesis in mice following oral treatment (*11*). These characteristics make plant PI attractive proteins for evaluation as chemopreventive agents, in modulating cell viability and tumor progression within the gastrointestinal tract.

Several possible mechanisms for the anticarcinogenic activity of BBI-like PI have been postulated (*1*); however, the precise targets of PI remain unknown. Serine proteases are key players in a wide range of biological processes, and aberrant functioning of certain proteases has been linked to tumor cell invasion and metastasis and, more recently, angiogenesis and tumor growth (*12*, *13*). BBI has two active sites involved in the independent inhibition of the serine proteases, trypsin and chymotrypsin. It has been suggested that the ability of BBI to inhibit transformation *in vitro* is related to the presence of an active chymotrypsin inhibitory domain. Yavelow et al. (*14*) reported that an

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enzymatically modified BBI having only chymotrypsin inhibitory activity (CIA) was still fully effective as an inhibitor of radiation-induced transformation *in vitro*, whereas the PI with trypsin inhibitory activity (TIA) only was ineffective. Recently, Gueven et al. (*15*) found a similar protective effect that was associated with the antichymotrypsin domain of the protein. However, the target protease was not identified in these studies.

Distinct PI classes have been described in many plant species (*16*). There is considerable sequence variation within different inhibitors of the PI class that includes BBI, which has not been investigated systematically to date for effects on the inhibition of digestive and nondigestive mammalian serine proteases. In a previous paper, we reported the expression, purification, and characterization of pea protease inhibitor (PPI) isoforms as recombinant proteins in a system capable of efficiently folding and secreting disulfide-bonded proteins (*17*). The effect of variation within the inhibitory domains on the protease inhibitory activity of PPI was investigated. Significant differences in specific CIA [chymotrypsin inhibitor units (CIU)/mg of protein] and in the inhibition constant (K_i) between two recombinant PPI, rTI1B and rTI2B, homologous to BBI but differing in amino acids at the two sites of enzyme inhibition were observed; rTI1B showed a much higher specific CIA than either rTI2B or BBI (4973, compared with 3260 or 3827 CIU/mg of protein, respectively) (*17*). In this study, we have screened PI for their potential effectiveness as cancer chemopreventive agents, using a modification of a protocol previously described for assessing cell viability (*18*), following an *in vitro* period of growth with or without PPI. First, we compared BBI with a pea seed fraction highly enriched in PPI and then studied the effects of two individual PPI isoforms, TI1B and TI2B, purified as recombinant proteins, on the growth of human colorectal adenocarcinoma HT29 cells.

2. MATERIALS AND METHODS

2.1. Materials. Bowman−Birk inhibitor (BBI) from soybean, 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), Dulbecco's modified Eagles medium, essential minimum Eagle's medium, Ham's F12 medium, nonessential amino acids, and fetal bovine serum (FBS) were obtained from Sigma (Dorset, U.K.). Flat-bottom 96-well microtiter plates were purchased from Nunc (Wiesbaden, Germany). Sephacryl S-100 HR, MonoQ, and MonoS HR 5/5 columns were supplied by Pharmacia Biotech (Uppsala, Sweden). Microtiter plates were assessed on automatic plate readers (Dynatech 5000, Dynatech Lab Inc., VA, and THERMOmax, Molecular Devices, Wokingham, U.K.). Human colorectal (HT29, LoVo) and breast (MCF7) adenocarcinoma cell lines were obtained from the European Collection of Cell Cultures (Salisbury, U.K.). Caspase inhibitor (Boc-D-FMK) was obtained from Calbiochem−Novabiochem (Nottingham, U.K.). All other chemicals were of analytical grade.

Genotypes of pea were from the John Innes Centre germplasm collection with the exception of D106 and D317, which were a gift from Dr. G. Duc, INRA-URGAP, Dijon, France.

2.2. Preparation of BBI and Pea Protease Inhibitor Concentrate (PPIC). BBI (Sigma) was dissolved in distilled water, dialyzed extensively against distilled water, and freeze-dried. This removed salts and low-molecular-weight compounds (≤3500 mass), yielding a protein preparation that was pure on native and SDS gel analyses. Analysis of three different preparations of BBI gave similar protease-inhibitor-specific activities (see section 3).

A protease inhibitor concentrate was prepared from seeds of the pea cultivar (cv.) Birte. Albumin proteins were prepared and fractionated by ammonium sulfate precipitation (*19*). Proteins corresponding to the 0−50% albumin fraction were dissolved in 50 mM Tris-HCl buffer at pH 7.5 and 50−100 mg fractionated on a Sephacryl S-100 HR gel-filtration column (100 × 2 cm) at a flow rate of 36 mL/h using the same buffer. The elution of PPI was monitored by analysis of TIA (see 2.4), using flat-bottom microtiter plates scanned at 405 nm (*20*). Fractions containing TIA were pooled, dialyzed against a 100-fold excess of distilled water, and freeze-dried. All purification steps were carried out at 4 °C.

2.3. Expression and Purification of Recombinant Pea Protease Inhibitors (PPI). Two PPI variants, TI1B and TI2B, were expressed as recombinant proteins using *Aspergillus niger* as an expression system, as described previously (*17*). This system allows the efficient folding and recovery of disulfide-linked proteins in the active form. Culture media were processed in bulk as described (*17*), and protein samples were fractionated by FPLC on a MonoQ HR 5/5 anion-exchange column using a linear gradient of 0−0.5 M NaCl in 50 mM Tris-HCl buffer at pH 7.8 and a flow rate of 1 mL min^{-1}. Column fractions containing TIA were pooled, dialyzed extensively against distilled water, and freeze-dried. Samples were dissolved in 25 mM sodium acetate buffer at pH 4.4 and fractionated by FPLC on a MonoS HR 5/5 cation-exchange column, using a linear gradient of 0−0.5 M NaCl in 25 mM sodium acetate buffer at pH 4.4 and a flow rate of 1 mL min^{-1}. The purified PPI, detected by TIA assays, as above, and gel electrophoresis, were dialyzed against distilled water and freeze-dried.

2.4. Measurement of Protease Inhibitor Activities. Several pea genotypes were screened for their relative TIA and CIA. Finely ground meal from samples of 5−8 pooled seeds of every genotype was assessed for TIA, using a modified small-scale quantitative assay with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as a specific substrate (*20*). CIA was measured in the same seed samples, using assays with *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as a specific substrate, as described previously (*17*). The trypsin and chymotrypsin inhibitor isoform patterns of BBI and PPIC (400 CIU) were analyzed on 12% nondenaturing polyacrylamide gels. After electrophoresis, gels were equilibrated in 1 M phosphate buffer at pH 7.4 for 1 h at room temperature, incubated with trypsin (0.1 mg/mL phosphate buffer) or chymotrypsin solution (0.05 mg/mL phosphate buffer) at 37 °C for 1 h and washed with distilled water prior to staining for protease activity as described by Domoney et al. (*19*).

2.5. Viability Assays Using Human Colon Adenocarcinoma Cells. Following a period of growth *in vitro*, the viability of human colorectal (HT29, LoVo) and breast (MCF7) adenocarcinoma cells was assessed by a neutral red (NR) cytotoxicity assay procedure, on the basis of the ability of viable uninjured cells to incorporate actively NR, a supravital dye, into lysosomes. Optimal screening conditions (exposure period, growth media, and number of cells/well) were established using BBI (600 CIU). The 96-well microtiter plates were inoculated at a density of 2000 cells/well in 200 μL of media, which gave optimal cell growth. These media were Dulbecco's modified Eagles medium with 5% FBS and 2 mM glutamine for HT29 cells, Ham's F12 medium with 10% FBS and 2 mM glutamine for LoVo cells, essential minimum Eagle's medium with 10% FBS and 1% nonessential amino acids for MCF7 cells, as determined by viable cell density measurements based on NR staining, at the end of a growth period. For all assays, cells were incubated under 5% CO_2 in humidified air for 24 h, to allow them to adhere to the wells. BBI, PPIC, or the recombinant PPI (rTI1B or rTI2B) were then dissolved in growth media to give a defined range of CIU and added to the cells under sterile conditions. Control wells received no PI. Cells were harvested after 96 h of exposure, during which time the growth media were not changed, except in one instance where this was done after 24 h to limit the exposure period of the cells to the test proteins. At the end of the growth period, cell viability was assessed by staining with NR (2 h at 37 °C), followed by cell fixation (0.5% formaldehyde and 0.1% $CaCl_2$ for 30 s at room temperature). Plates were washed by two brief immersions in phosphate-buffered saline (PBS), and cells were lysed (50% ethanol containing 1% acetic acid overnight at 4 °C). The optical densities of the resulting solutions were measured at 550 nm using a Dynatech 5000 automatic plate reader. Cell viability data are presented as means ± standard deviation (SD) for at least two independent experiments ($n \geq 10$/experiment). The concentration of purified PI that reduced cell viability by 50% (IC_{50}), as compared with untreated controls, was calculated by the nonlinear regression fit of the data obtained in three independent experiments ($n \geq 10$/experiment). The data were analyzed statistically by the

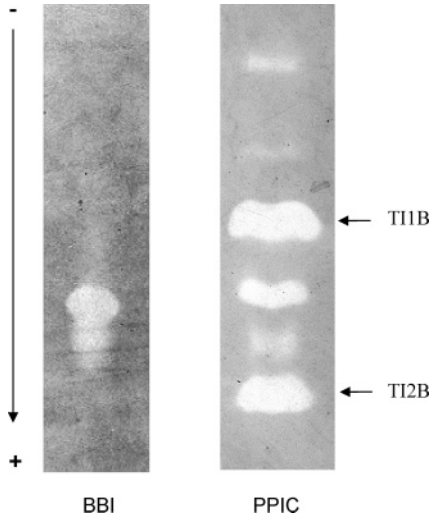

Figure 1. Analysis of BBI and PPIC (cv. Birte), separated by electrophoresis on nondenaturing gels and stained for chymotrypsin activity. Clear zones show where chymotrypsin is inhibited. Two variants among the isoforms present in cv. Birte represent the primary mature products of the *TI1B* and *TI2B* genes (horizontal arrows). The direction of electrophoresis is shown (vertical arrow).

Table 1. Trypsin (TIA) and Chymotrypsin Inhibitory Activity (CIA) Expressed as TIU and CIU per Milligram of Dry Seed Meal from Five Pea Genotypes[a]

pea genotype	TIU	CIU	TIU/CIU
HA7	10.76 ± 0.52	11.06 ± 0.40	1:1.0
Victor	8.20 ± 0.38	9.04 ± 0.33	1:1.1
D317	7.64 ± 0.41	10.13 ± 0.42	1:1.3
Birte	5.29 ± 0.14	9.29 ± 0.58	1:1.8
D106	4.13 ± 0.35	5.30 ± 0.10	1:1.3

[a] Data are the means ± SD for at least two independent experiments ($n \geq 6$).

Student's *t* test, using the Minitab statistical software (State College, PA).

2.6. Caspase Inhibition. To determine if the PPI (rTI1B or rTI2B) induced caspase-dependent apoptosis in HT29 cells, a cell-permeable, irreversible, broad-spectrum caspase inhibitor (Boc-D-FMK) was incorporated at a final concentration of 75 μM, with or without 300 CIU of PPI, into the assay described above for HT29 cells. The effect of the caspase inhibitor on cell viability in the presence of PI was compared with the response to PI alone.

3. RESULTS

3.1. Characterization of the PI Used in Cell Assays. Analysis of BBI preparations by SDS−PAGE showed only a single band of appropriate mass (8 kDa) (not shown). Previous data have shown that BBI has a ratio of TIU/CIU close to 1:1 with specific TIA and CIA of 4396 ± 159 and 3827 ± 336 units/mg of protein, respectively (*17*). Analysis on nondenaturing gels stained for chymotrypsin activity showed a major isoform (**Figure 1**). Differences in either purity or functional properties (specific inhibitor activity and effects in cell assays) were not observed when BBI from three different batches was used (data not shown).

Several pea genotypes were screened for their content of TIA and CIA. Significant differences in both TIU and CIU/mg of seed meal, as well as differences in the TIU/CIU ratios, were found among pea genotypes (**Table 1**). Among these, the cv. Birte was selected for the preparation of PPI because of its relatively low specific TIA (5.29 ± 0.14 TIU/mg of dry weight), relatively high specific CIA (9.29 ± 0.58 CIU/mg of dry weight), and, as a result, a ratio of 1:1.8 for TIU/CIU. The preparation procedure for PPIC (section 2.2) allowed the recovery of 80% of the TIA present in the starting material as well as the removal of both trypsin-like endogenous proteases and lectins (*19*), which could mask any effects of PPI on the cell growth. PPIC had a ratio of TIU/CIU (1:1.9) that was similar to that of the starting material.

Earlier studies established that PPIC from cv. Birte is composed of several PPI isoforms, with two being the mature full-length TI1B and TI2B proteins (*21*, *22*). Other isoforms result from both amino- and carboxy-terminal processing of the primary mature proteins. In this study, analysis of PPIC on nondenaturing gels stained for chymotrypsin activity revealed four major PPI isoforms (**Figure 1**), in agreement with the trypsin inhibitor pattern (*17*) of albumins from cv. Birte. Any effects of total PPI in cell assays represent, therefore, the combined effects of the individual PPI variants and other low-molecular-weight proteins; the specific activities (expressed as inhibitor units/mg of dry weight) of PPIC against trypsin and chymotrypsin, were 354 ± 23 and 680 ± 45, respectively, consistent with impure PPI. For this reason, we have expressed individual PPI isoforms as recombinant proteins and purified two of these (rTI1B and rTI2B) to evaluate their specific effects in cell assays (section 3.3). The functional properties of rTI1B and rTI2B have been reported previously in a study where the cv. Birte was the source of the gene sequences for the expression of recombinant PPI variants (*17*). The sequences deduced for the recombinant PPI, in comparison with BBI, are shown in **Table 2**; clearly, the recombinant proteins are processed forms as a result of both amino- and carboxy-terminal processing.

3.2. Comparison of the Effect of Pea Protease Inhibitor Concentrate (PPIC) and BBI on the Growth of Human Colon Adenocarcinoma HT29 Cells. The effect of PI on human adenocarcinoma cells was determined, following the establishment of optimal culture conditions to promote and maintain growth of a number of cell lines. Under these conditions, a preliminary screening of colon (HT29, LoVo) and breast (MCF7) carcinoma cell lines was carried out; differences in the responses of the three cell lines, following exposure to BBI (600 CIU), were observed. The greatest effect was seen with the HT29 cells, where values of 44.2 ± 3.5, 88.9 ± 4.8, and 88.1 ± 5.3% (expressed as a percentage of the data obtained with control media, ±SD) were obtained for viability measurements at the end of the growth period, for HT29, LoVo, and MCF7 cells, respectively. Additional studies indicated that such differences were not due to variation in the concentrations of FBS (5 and 10%) in the growth media for the different cell lines (not shown). Because the HT29 cell line was preferentially affected by inclusion of BBI, in comparison with other cell lines, it was selected therefore for further comparative experiments to screen PPIC and recombinant PPI variants.

The effects of PPIC from cv. Birte and BBI on the growth of HT29 colon cancer cells were compared. Because the ability of BBI to inhibit carcinogenic processes has been reported to be linked to its CIA (*14*), the addition of PI to the cell assays was expressed in terms of CIU. A statistically significant ($p < 0.001$) decrease in the growth of HT29 cells was observed after exposure to either BBI or PPIC, compared with control cells, and the latter was significantly more effective in this *in vitro* system (**Figure 2**). The effects of both PI were dose-dependent, with BBI showing an IC_{50} of 100 μM after 96 h of exposure. Estimation of an IC_{50} value for PPIC is complex, because of the presence of several PI isoforms with significant differences

Table 2. Amino Acid Sequences of the Two Recombinant PPI, rTI1B and rTI2B, and BBI from Soybean[a]

rTI1B	- - - VKSACCD TCLCTKSNPP TCRCVDVRET CHSACDSCIC AYSNPPKCQC FDTHKFCYKA CHNS - - - - - - - -
	- - - - - - ACCD TCLCTKSNPP TCRCVDVRET CHSACDSCIC AYSNPPKCQC FDTHKFCYKA CHN - - - - - - - - -
rTI2B	- - - VKSACCD TCLCTKSDPP TCRCVDVGET CHSACDSCIC ALSYPPQCQC FDTHKFCYKA CHNS - - - - - - - -
BBI	DDESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N

[a] Underlined sequences are trypsin and chymotrypsin inhibitory domains, with KS and YS/LS (bold) being the reactive peptide bonds (termed P_1–P_1' residues for each site) for trypsin and chymotrypsin inhibition sites, respectively. Dashed lines indicate where post-translational processing of recombinant PPI occurred, relative to predicted amino acid sequences.

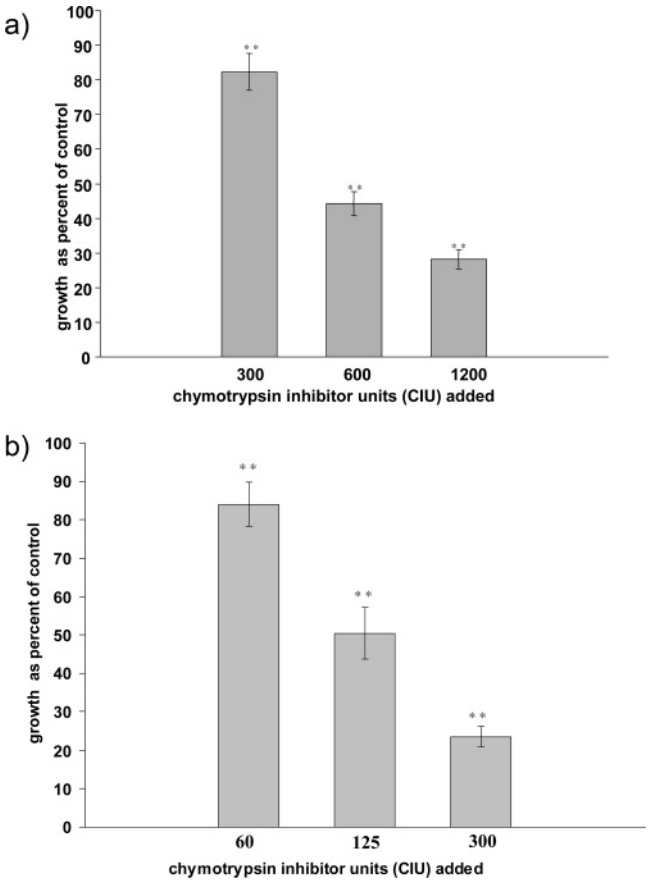

Figure 2. Effect of (a) BBI from soybean or (b) PPIC (cv. Birte) on the *in vitro* growth of HT29 human colorectal adenocarcinoma cells. Growth media were supplemented with protease inhibitors, expressed in terms of CIU: 300–1200 CIU of BBI (a) and 60–300 CIU of PPIC (b). Controls received no inhibitor. Bars represent standard deviations ($n = 10$ samples). *t* test, (**) $p < 0.001$.

in both specific inhibitory activities and molecular weights (*17*, *21*); this complexity together with the presence of other protein constituents of low molecular mass with potential biological activities incur a large error in the determination of an IC_{50} for this fraction. Purified BBI, when present at concentrations as low as 0.01% in the diet, has been reported to have suppressive effects on carcinogenic processes, reducing the incidence and frequency of colon tumors in DMH-treated rats compared with animals treated with DMH alone (*10*). Pharmacokinetic studies carried out with radiolabeled BBI suggest that approximately 40−50% of the labeled BBI reached the colon and was excreted (*8*). These combined data suggest, therefore, that the amounts of BBI used in our study are well within the range of concentrations shown to prevent/suppress tumorigenesis *in vivo* (*1*, *10*). The higher effectiveness of PPIC in the *in vitro* comparisons with BBI suggested, therefore, the possible existence of individual PPI with a greater ability to inhibit the growth of HT29 cells than BBI. These preliminary experiments established conditions for an investigation of individual PPI variants.

3.3. Effect of the Recombinant PPI, rTI1B and rTI2B, on the Growth of HT29 Human Colon Adenocarcinoma Cells. Parts **a** and **b** of **Figure 3** show the data obtained when the purified rTI1B and rTI2B proteins, respectively, were included in assays of HT29 colon cancer cells. A statistically significant ($p < 0.001$) and dose-dependent decrease in the growth of HT29 colon cells was observed after treatment with either rTI1B or rTI2B (parts **a** and **b** of **Figure 3**). Using equivalent CIU in these assays showed that rTI1B and rTI2B gave similar results. Taking into account the specific CIA of the three PI **(**see earlier, in the Introduction), the recombinant proteins were more effective than BBI in inhibiting cell growth, with an IC_{50} value lower than 50 μM in the case of rTI1B (**Figure 3c** and **Table 3**). At concentrations lower than 20 μM, the ability of rTI1B or rTI2B to decrease the growth of HT29 cells was lost; in the case of BBI, concentrations greater than 40 μM were necessary to suppress the growth of HT29 cells significantly (**Figure 3c**).

These effects were less marked when the media were replaced, after 24 h of exposure of cells to PPI or BBI, with fresh media containing no PI (**Figure 4**). Because an effect of PI was still observed following this minimal exposure, a reversible inhibition of a target protease could explain the partial recovery of cell growth observed when PI were removed (**Figure 4**).

To determine if the effects of PI on the cell growth were caspase-dependent, studies were performed on HT29 cells cultured in the presence or absence of the cell-permeable pan−caspase inhibitor Boc-D-FMK, together with the recombinant PPI. Boc-D-FMK blocks apoptosis by irreversibly inhibiting the activity and/or processing of a number of caspases (*23*, *24*). There was no significant effect of Boc-D-FMK alone on the assay results of HT29 cells (data not shown). As before, the addition of PPI (300 CIU) had a significant effect (50.0 ± 7.1 and 54.3 ± 2.3% for rTI1B and rTI2B, respectively, as a percentage of the cell growth of untreated cultures, ± SD). The Boc-D-FMK inhibitor did not alter the decrease in the HT29 viable cell number in the presence of rPPI (42.2 ± 2.3 and 48.08 ± 8.1% for rTI1B and rTI2B, respectively, as a percentage of the cell growth in the absence of PPI, ±SD). These data suggest

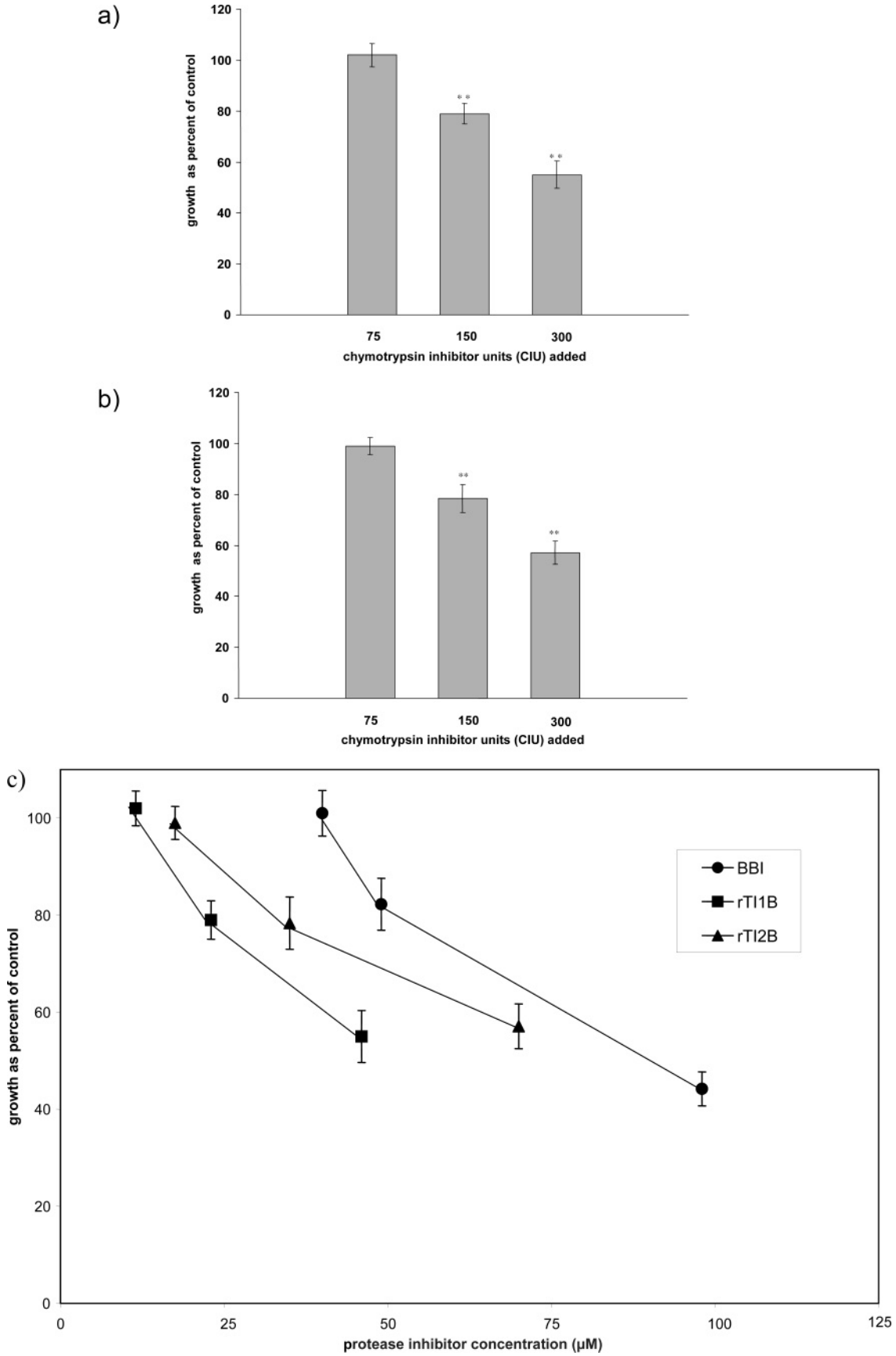

Figure 3. Effect of (a) rTI1B and (b) rTI2B on the *in vitro* growth of HT29 human adenocarcinoma colorectal cells. Growth media were supplemented with protease inhibitors, expressed in terms of CIU: 75–300 CIU. Negative controls received no inhibitor. A positive control (300 CIU of BBI) showed 72% of the cell growth achieved in control media (not plotted). Bars represent standard deviations ($n > 10$ samples). *t* test, (**) $p < 0.001$. (c) Effect of three protease inhibitors (rTI1B, rTI2B, and BBI) at different final concentrations (specific CIA = 4973, 3260, and 3827 CIU/mg, respectively) on the growth of HT29 cells; bars represent standard deviations ($n > 10$ samples).

Table 3. IC_{50} Values Determined for the Effect of the Recombinant Pea Protease Inhibitors, rTI1B and rTI2B, and BBI on the Growth of HT29 Human Adenocarcinoma Colorectal Cells[a]

inhibitor	IC_{50} (μM)
rTI1B	46 ± 7
rTI2B	73 ± 10
BBI	100 ± 9

[a] Data are the means ± SD of three independent experiments ($n \geq 10$).

that the mechanism of action of PPI does not involve an apoptotic pathway, with the PI possibly acting only as protease inhibitors, as suggested by Foehr et al. (*25*). Further studies will be necessary to elucidate the cellular processes that are likely to be affected when HT29 cells are treated with PPI.

4. DISCUSSION

In this work, we have described the effects of PPIC and two recombinant PPI, rTI1B and rTI2B, on HT29 cell growth, in comparisons with BBI. The rTI1B and rTI2B proteins share a high level of sequence identity but differ at the chymotrypsin inhibitory site (*26*) (**Table 2**) and in specific CIA and associated K_i (*17*). The differences observed with the two recombinant proteins in their effects on HT29 cell growth may reflect, therefore, the sequence differences within the chymotrypsin inhibitory domains. The data suggest that the sequence "YSN" (rTI1B) at P_1, P_1', and P_2' positions [notation of Schechter and Berger (*27*)] is a more potent inhibitor of the growth of HT29 cells, when compared with the sequence "LSY" (rTI2B and BBI) (**Figure 3c**), and are in agreement with a chymotrypsin-like protease as a target protein in HT29 cells.

In the present study, neither of the recombinant proteins mimicked the effects observed with PPIC, indicating that other PPI isoforms need to be investigated. Indeed, the existence of other proteins in PPIC that could potentiate the effects of PPI on cell growth should not be underestimated. Such is the case with a soybean protease inhibitor concentrate, where the presence of active compounds with the ability to potentiate the anticarcinogenic properties of BBI has been suggested (*2*). Differences between the activity of BBI and rTI2B cannot be explained simply in terms of sequence variation within the chymotrypsin inhibitory domain. Although both proteins have the same amino acid sequence in this domain, BBI was less effective than either rTI1B or rTI2B in decreasing the growth of HT29 cells. These results suggest that other factors, including divergence of amino acid sequences in other regions of the proteins, differences in molecular masses, and differential processing of amino- and carboxy-terminal ends (**Table 2**) that can affect the interaction of protease and PI (*26*, *28*, *29*), need to be explored further. All of this further work will demand modifications to the production and purification procedures for rPPI to facilitate a wider comparison of rPPI variants, processed and unprocessed at one of or both termini and added in a wide range of different proportions to cell assays.

Variability in the amino acid sequence of the inhibitory domains of PI from the Bowman−Birk class, within and between legume species, has been reviewed recently (*30*). In a previously reported proteinomimetic approach, amino acids at different positions of cyclic nonapeptides, designed on the basis of active-site loops, were randomized and chymotrypsin inhibition was evaluated (*31*, *32*). Amino acid substitutions significantly affected the potency and hydrolysis rates of synthetic chymotrypsin inhibitors. Although the anticarcinogenic activity of PI has been related directly to the ability to inhibit chymotrypsin-like proteases (*1*), the biological relevance of amino acid sequence variation within the chymotrypsin inhibitory domains of the holoproteins has not been explored until now. In protease−protease inhibitor interaction, molecular recognition is governed mainly by position P_1. The high affinity of chymotrypsin-like proteases for aromatic residues at position P_1 has been documented, with tyrosine (as occurred in rTI1B)

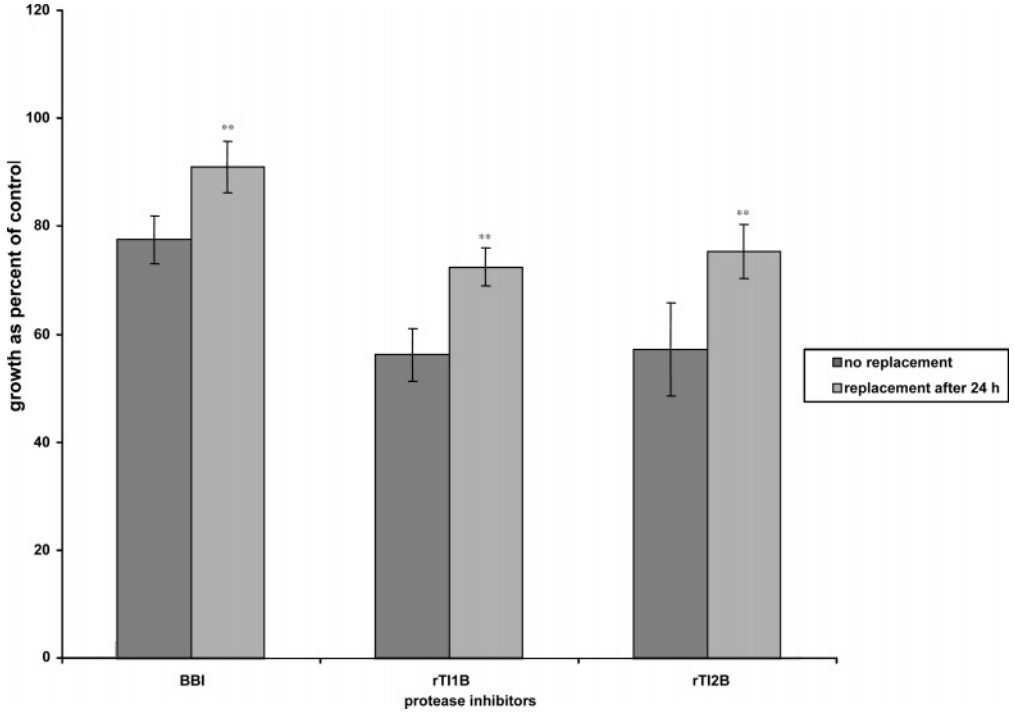

Figure 4. Effect of media replacement, after 24 h exposure to BBI, rTI1B, or rTI2B, on the growth of HT29 human colorectal adenocarcinoma cells. Growth media were supplemented with protease inhibitors (300 CIU). Controls received no inhibitor. Bars represent standard deviations ($n = 10$ samples). *t* test, (**) $p < 0.01$.

showing the highest binding strength (*33*, *34*). These data have been confirmed in a study where a combinatorial library of chymotrypsin inhibitor cyclic peptides has been screened (*31*). On the other hand, the presence of leucine at P_1, as occurred in rTI2B and BBI, has been shown to give relatively high K_i values in chymotrypsin inhibitor cyclic peptides (*32*) and to cause a decrease in the specificity for chymotrypsin, with the peptides showing additionally antitrypsin activity (*35*).

Recently, other workers have reported the use of cultured malignant cells to examine the antiproliferative effects of a protein fraction having protease inhibitor activity from tepary beans, but a target protease was not identified (*36*). Other studies have implicated serine protease activities in cancer progression. In a functional proteomic approach, serine protease activities from a set of colon carcinoma biopsies were attributed to mast cell tryptase and chymase (*37*). Recently, food-derived inhibitors, such as BBI from soybean and lima bean trypsin inhibitor, have been reported to inhibit the activity *in vitro* of matriptase (*38*), a member of the class of type II transmembrane serine proteases, which exhibits trypsin-like activity. This serine protease, which is implicated in the selective proteolysis of key extracellular substrates that activate urokinase−plasminogen activator, hepatocyte-growth factor/scatter factor, and protease-activated receptor-2 appears to be expressed differentially in different cell lines and may contribute to cell migration (*12*, *39*). Further studies will be necessary to evaluate the interaction of PPI with these candidate serine proteases that could provide opportunities for therapeutic intervention. The cell-culture-based screening procedure employed in the present study will facilitate such work.

In summary, our data show that the recombinant PPI, rTI1B and rTI2B, reduced the growth of HT29 cells *in vitro*, to a greater extent than BBI from soybean. The relative importance of the sequence of the chymotrypsin inhibitory site was indicated by comparisons of the two recombinant pea proteins that differed primarily in their chymotrypsin inhibitory domain, with the variant "YSN" (rTI1B) being the more effective. To our knowledge, this is the first indication of the importance of variation within the chymotrypsin inhibitory sites of plant PI for their anticarcinogenic properties. These data address the need for systematic evaluation of the biological activities of the vast number of natural sequence variants that exist among PI. These studies contribute to an assessment of the chemopreventive properties of plant PI toward developing new strategies for inhibitor design in clinical nutrition.

ABBREVIATIONS USED

BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; BBI, Bowman−Birk inhibitor; BBIC, Bowman−Birk inhibitor concentrate; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; CIA, chymotrypsin inhibitor activity; CIU, chymotrypsin inhibitor unit; DMH, dimethylhydrazine; FBS, fetal bovine serum; FPLC, fast-performance liquid chromatography; K_i, inhibition constant; NR, neutral red; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, protease inhibitor; (r)PPI, (recombinant) pea protease inhibitor; PPIC, pea protease inhibitor concentrate; TIA, trypsin inhibitor activity; TIU, trypsin inhibitor unit.

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