Effects of Tepary Bean (Phaseolus acutifolius) Protease Inhibitor and Semipure Lectin Fractions on Cancer Cells

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INTRODUCTION

Cancer is a complex process in which changes in proliferation rate, adhesion patterns, and migration play important roles in tumorogenesis and metastasis. Proteolysis of extracellular matrix (ECM) and cell surface proteins results in crucial changes in cell–cell and cell–ECM interactions. New signals generated from the cell surface can affect gene expression and ultimately influence critical cell behavior. Transformed cells lose their strong intercellular adhesive properties and new cell–ECM interactions are produced. As a consequence, several cell surface-associated adhesion molecules change their function and are the source of signals that promote growth, motility, ECM degradation, and metastasis (1).

Natural protease inhibitors (PIs) are proteins with an interesting potential against cancer development and dissemination, mainly because of their high selectivity to inhibit specific enzymes. Soybean Bowman-Birk protease inhibitor (SBBI) has been the most studied natural PI. Its evaluation at nanogram levels to cell cultures on in vitro and in vivo assays has indicated no secondary effects, showing a potential to differentially affect cancer cells (2–9). They have also been found to enhance cisplatin-induced cytotoxicity in human mesothelioma cells (10). Treatments for short periods of time were effective, showing an ability to protect cells in later inductions (11). In some cases the anticarcinogenic potential of SBBI has been...
specifically related to its antichymotrypsin activity (4–6,12).Presently there is at least a patent on 1 inhibitor of this type (13).

On the other hand, plant lectins have been extensively studied because of their effects on animal cells, such as induction of lymphocyte mitogenesis, immunoglobulin aggregation, induction of histamine release from basophils, and mast cells (14). Lectins can bind to the cell membrane through specific recognition to glycoconjugates, resulting in changes of cell function. Different studies have shown a strong correlation between certain lectin-binding models and their biological action in several tumors. Legume lectins inhibit cell adhesion, cell proliferation, and colony formation, causing hemagglutination and cytotoxic effects on human tumor cells. Particularly, plant lectins have anticancer properties in vitro and in vivo, by binding cancer cell membrane proteins or receptors causing cytotoxicity, apoptosis, and inhibition of tumor growth (15–17). It has been observed that lectins from different sources inhibit cancer cells growth depending on their concentration and in a differential way (18–20).

We previously characterized a 7 kDa Bowman-Birk PI from Tepary bean (Phaseolus acutifolius) seeds (TBPI) with antitrypsin and antichymotrypsin activities (21). In an earlier work, we tested the effect of a semipure sample of TBPI on cancer cells, founding an increase of cell adhesion to culture dishes, as well as a decrease in cancer cell growth (22). However, we later identified the presence of at least 1 lectin in that protein sample. In this work, we separately described the effect of a semipure lectin fraction and the effect of the pure TBPI on in vitro assays on murine transformed cells as well as in human cancer cell lines.

**MATERIALS AND METHODS**

**Plant Material**

Tepary bean seeds were obtained from a local market at Hermosillo, Sonora, Mexico and stored at −20°C until their use.

**Lectin and TBPI Purification**

TBPI was purified as described by Campos-Contreras et al. (21). Briefly, the protein present in the crude extract was precipitated with ammonium sulfate (40% to 65% saturation), centrifuged (39,200 g for 60 min); the pellet was dialyzed and lyophilized. The sample was chromatographed on a 167 × 2.25 cm Sephadex G-75 column that was equilibrated with 0.02 M ammonium bicarbonate. Two protein fractions were separated: a lectin-rich fraction (TLRF) and the semi-pure Tepary PI fraction (TPIF). Both fractions were tested for protein (23), agglutination activity (24), and protease inhibition (25).

The TLRF, which showed agglutination activity but lacked protease inhibition activity, was rechromatographed using an Econo-Pac® High Q cartridge column (1 × 5 cm) (Pharmacia Biotech; Uppsala, Suiza) in an Econo System BioRad collector, equilibrated with 0.01 M Tris-HCl, pH 8.0. Elution was carried out using a 0 to 0.4 M NaCl linear gradient in 0.01 M Tris-HCl, pH 8.0, with a flow rate of 1 mL/min, collecting 2 mL fractions, starting the collection at 140 min. Those fractions containing the lectin (TLF) were pooled, dialyzed, lyophilized, and stored at −20°C until use. The presence of lectin was corroborated by SDS-PAGE glycoprotein-staining (26).

Meanwhile, TPIF was rechromatographed as described by Campos-Contreras et al. (21). Briefly, DEAE-Sepharose column (1 cm × 15 cm) was used, eluting the protein in a linear gradient form 0.1 to 0.7 M NaCl in Tris 0.02 M pH 8.0 buffer solution (0.3 mL/min), collecting 3.0 mL fractions. All fractions with PI activity were dialyzed, lyophilized, and further purified through an RP-HPLC, using a preparative Vydas C-18 column. The fractions were then equilibrated in 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile from 0% to 60% in 0.1% trifluoroacetic acid (1 mL/min). Fractions with PI activity were collected (TBP1), lyophilized, and stored at −20°C until use. To monitor the purity of the samples, SDS-PAGE (27) was done after each step.

**Cell Culture**

The antiproliferative effects of TLRF, TLF, and TBPI were tested on 3T3/v-mos transformed murine fibroblasts (kindly provided by Dr. C. Schweinfest of the Medical University of South Carolina, Charleston, SC). Cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY) with 5% calf serum (CS; HyClone, Logan, UT), and incubated at 37°C under a 10% CO2/90% air humidified atmosphere. These cells presented high proliferation rates, high invasion capacity, and very poor adhesion to culture dishes. Nontransformed 3T3 fibroblasts were used as control.

In a separate experiment, human cancer cell lines (cervix: SiHa, HeLa and C33A; breast: MCF7 and ZR-75-1; and colon: CaCo2) were tested for TLF effect. Cells were maintained according to the American Type Culture Collection (ATCC) recommendations in each case.

**Proliferation Assay**

Bioassays were performed as described by Garcia-Gasca et al. (22). Briefly, cells were seeded (1 × 10⁴ cells per well for 3T3 fibroblasts or 3 × 10⁴ cells for human cancer cells) in 24-well plates. After 48 h, conditions were changed by adding 0.5 mL of different concentrations of TLRF, TLF, or TBPI in DMEM containing 0.5% bovine serum albumin (BSA; Serologicals, Clarkston, Georgia); control cells were maintained in 0.5% BSA-DMEM. After 72 h, cells were harvested with 0.15% trypsin in phosphate buffer solution and cell number was determined using a hemocytometer. All treatments were done in duplicate in at least 3 independent experiments.

**Adhesion Assay**

Cells were seeded (1 × 10⁴ cells per well) in 24-well plates with 0.5 mL of 5% CS-DMEM. After 24 h, 0.5 mL of 1% BSA–1.5% CS-DMEM containing 0.027 μg protein/mL (equivalent to 250 IU/mL) of TBPI was added and a control treatment
using 1% BSA–1.5% CS-DMEM was included. Cells were incubated at 37°C under a 10% CO₂/90% air humidified atmosphere until confluence was reached. The number of cells in each of the treated and nontreated wells was estimated using a carmine-based colorimetric method as described by Garcia-Gasca et al. (28).

**Invasion Assay**

Cells were harvested with 0.2% EDTA and seeded as described in the adhesion assay in 24-well plates (2×10⁴ cells/well) in a Matrigel invasion chamber (Biorad, Becton Dickinson Labware, Franklin Lakes, NJ) using 5% fetal bovine serum as a chemoattractant. After 5 days of incubation, inserts were removed and cultures were incubated for 5 more days. Cells that were attached to the bottom of the well were harvested and counted using a hemocytometer.

**ECM Degradation Assay**

Cells were seeded on conventional 35 mm cell culture dishes (Corning Inc., Corning, NY) with 2 mL of 5% CS-DMEM. The conditions were changed the day after seeding, as described in the adhesion assay. When cell cultures reached confluence, ECM from culture dishes was extracted (29) and SDS-PAGE analysis was performed, applying 20 μg of protein per well. Protein band intensities were determined by densitometry measuring the peak area using an image processing software (IPLab, Scanalytics, Billerica, MA).

**Matrix Metalloproteinases (MMP) Zymography Analysis**

3T3/v-mos fibroblasts were cultured in 35 mm dishes to confluence; 12 h prior treatment cells were rinsed with PBS and incubated in 0.5% serum media. One hour before treatment medium was removed, cells were rinsed with PBS, and serum-free medium was added. For treated cells, 0.027 μg protein/mL (equivalent to 250 IU/mL, total volume of 2 mL) of TBPI was included and cells were incubated at 37°C for 24 h. Medium was collected, cells were separated by centrifugation at 13,000 rpm for 10 min and the conditioned media (CM) was transferred to new tubes. Samples were lyophilized and stored at −80°C until use. CM for electrophoresis were concentrated by ultrafiltration using a solvent resistant stirred cell (Cat. No. XFUF 076 01-76 mm) with a 3 kDa ultrafiltration membrane (Millipore Corporation, Billerica, MA). Gelatin zymography was carried out according Hawkes et al. (2010) (30). To determine whether TBPI directly inhibits the activity of some extracellular proteases, 10 μg of the inhibitor were additionally added to the CM and incubated at 37°C for 15 min before the electrophoresis was performed. To detect a possible proteolytic activation by Triton, after SDS-PAGE one on the gels was incubated 60 min in 0.05 M Tris-HCl, pH 8; 2.5% (v/v) Triton X-100, and the second one was incubated in the absence of Triton. A negative control treated with 5 mM EDTA was included to inhibit MMPs. Then, the gels were washed twice in 0.05 M Tris-HCl, pH 8 (10 min each) and incubated 24 h in the same Tris solution in the presence of 5 mM CaCl₂, with the exception of the treatment with EDTA, which was incubated in Tris-EDTA solution. At the end, the gels were stained in 0.1% Coomassie Brilliant Blue R-250 (w/v) in methanol: acetic acid 45:10 (v/v) for 4 h and faded in the same solution without the Coomassie dye. Proteolytic activity was evident as clear bands against a background of stained gelatin.

**Statistical Analysis**

All experiments were performed in duplicate, in at least 2 independent times. SPSS Version 17 software was used for comparing different treatments against control cells (analysis of variance; Tukey or Dunnett P ≤ 0.05) or for comparing only 1 treatment against control cells (t-student P ≤ 0.05). To obtain the inhibitory concentration 50 (IC₅₀), simple linear regressions of TLRF or TLF concentration logarithms versus proliferation percentage were done.

**RESULTS**

**Protein Fractionation and TBPI Purification**

Two protein fractions were obtained after the Tepary bean extract was chromatographed on Sephadex G-75: one rich in agglutinin activity (TLRF) and the other one containing the protease inhibitor activity (TPIF) (Fig. 1A). Although only TPIF presented antitrypsin inhibition activity (11,400 IU/mg protein), both fractions showed agglutination activity [represented as agglutination units (AU) per mg of protein]: 5565 and 1024 AU/mg for TLRF and TPIF, respectively. TLRF showed a main band of approximately 30 kDa after SDS-PAGE analysis, similar to the lectin previously reported (31,32), plus few more protein bands (Fig. 1B). TLRF was rechromatographed by ionic exchange chromatography (Fig. 1C), obtaining the TLF with a specific agglutination activity of 1,170 AU/mg protein. According to its electrophoretic profile, TLF showed only the band with an apparent molecular mass of 30 kDa (Fig. 1D). However, only 20% of the initial agglutination activity was recovered by this method. Finally, the TPIF fraction was rechromatographed by both ionic exchange chromatography and HPLC to finally obtain a pure PI fraction (TBPI) (Fig. 1E), whose electrophoretic profile showed only 2 protease inhibitor isoforms (P1 and P2) (Fig. 1F). This fraction showed specific PI activities of 9381 and 5352 IU/mg protein against trypsin and chymotrypsin, respectively.

**Effects of TLRF, TLF, and TBPI on Cell Proliferation**

The antiproliferative effect of TLRF, TLF, and TBPI was evaluated on 3T3/v-mos cell cultures. TLF cytotoxic effect was also tested on breast, cervix, and colon cancer cell lines, whereas TBPI was additionally tested for adhesion and invasion assays on 3T3/v-mos cells.

TLRF, lacking PI activity, inhibited 3T3/v-mos cell proliferation as a function of concentration, and also in a differential manner with respect to nontransformed 3T3 cells (Fig. 2A). The IC₅₀ values were 1.21 AU/mL and 26.27 AU/mL for transformed and nontransformed cells, respectively. Although proliferation
FIG. 1. Protein fractionation. A: Tepary lectin-rich fraction (TLRF) and Tepary protease inhibitors fraction (TPIF) obtained after molecular weight exclusion chromatography. B: SDS-PAGE profile of the protein fractions after gel exclusion chromatography. Molecular markers (M), TLRF (1), TLRF stained for glycoprotein detection (2), TPIF (3). C: TLRF ionic exchange chromatography. D: SDS-PAGE profile of the protein fractions after ionic exchange chromatography. Molecular markers (M), Tepary lecin fraction (TLF) (1), and TLF stained for glycoprotein (2). E: Tepary bean protease inhibitor (TBPI) RP-HPLC chromatogram. Two peaks, with retention times of 43 min (P1) and 47.4 min (P2), showing protease inhibitor activity. F: SDS-PAGE profile of TBPI isoforms (P1 and P2) using 13.5% polyacrylamide gels loaded with 20 μg of protein per lane (Color figure available online).
FIG. 2. Effects of Tepary lectin-rich fraction (TLRF), Tepary lectin fraction (TLF), and Tepary bean protease inhibitor (TBPI) on cell proliferation. Non-transformed or transformed fibroblasts were seeded \((1 \times 10^4\text{cells/well})\) in 24-well plates with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% calf serum. After 48 h, the medium was removed and different concentrations of (A) TLRF, (B) TLF, or (C) TBPI were added in 0.5% bovine serum albumin (BSA) serum-free medium. Treatment with DMEM containing 0.5% BSA was included as a control. After 72 h, cells were counted using a hemocytometer. Asterisks show statistically significant differences (Dunnett, \(P \leq 0.05\)) with respect to controls, (*) for transformed cells and (**) for non-transformed cells.

To determine the effect of TLF on human cancer cells, dose-response curves were performed on breast cancer cells (MCF-7 and ZR-75-1), cervix cancer cells (HeLa, SiHa, and C33-A) and colon cancer cells (CaCo2). Fig. 3 shows that TLF exhibited a differential effect on cell proliferation as a function of concentration and cell type. Cervix cancer cells were the least sensible to TLF (HeLa < SiHa < C33-A), followed by breast cancer cells. Colon cancer cells were the most sensible to the antiproliferative effect of TLF with the lowest IC50.

**Effect of TBPI on Cell Adhesion and Cell Invasion**

Considering that wild-type 3T3/v-mos fibroblasts exhibit poor adhesion to cell culture dishes, they can be easily detached with a simple washing of the plate, representing a good model to study cell attachment. The number of detached cells was determined using a colorimetric carmine-staining-based assay. As we previously showed, TPIF (250 IU/mL) increased cell adhesion of 3T3/v-mos cells after 13 days in culture (22). In this experiment, treatment with 0.027 \(\mu\)g protein/mL (250 IU/mL) of purified TBPI during 8 days increased cell attachment (Fig. 4). This result confirms that such effect was directly related to PI activity, showing an enhanced effect of 2.5 times more cell adhesion at 8 days of treatment.

Cell invasion is one of the metastatic processes highly dependent on proteolysis. To determine the effect of TBPI on ECM degradation, cell invasion was measured using reconstituted basement membrane (Matrigel) coated inserts. These membranes were used to determine the ability of cells to move in response to chemotactic signals. 3T3/v-mos cells were able to pass through Matrigel, while nontransformed 3T3 fibroblasts were not. Treatment with TBPI negatively affected the cells ability to pass through Matrigel, decreasing 70% with respect to untreated cells \((P \leq 0.05)\) (Fig. 5).

**Effect of TBPI on ECM Degradation**

Considering that TBPI is a serine PI (21) that could affect the ECM degradation process and that cultured fibroblasts can produce some ECM proteins as collagen, glycoproteins, and proteoglycans (29), we examined by SDS-PAGE the effect of treatment on ECM degradation from culture dishes. The ECM extracted from both TBPI-treated and -untreated cells showed 2 main protein bands with apparent molecular mass of 112 and 60 kDa (Fig. 6A), suggesting that they were produced by cultured fibroblasts, because no such proteins were present in of nontransformed cells was also affected by the lectin fraction, this effect was 21 times lower than the corresponding effect on transformed cells. This cytotoxic effect was confirmed using the more purified lectin, TLF, on 3T3/v-mos transformed cells observing that cytotoxicity was effectively related to the presence of this lectin, with an IC50 of 0.27 AU/mL (Fig. 2B). In the case of TBPI, none of the different concentrations assayed presented a negative effect on 3T3/v-mos cell proliferation (Fig. 2C), on the contrary, cell proliferation increased with the highest concentration tested.
FIG. 3. Effect of Tepary lectin fraction (TLF) on cancer cells proliferation. A: Dose-response curve for 72 h TLF treatment on human cancer cells. Cells were seeded (3 x 10^4 cells/well) in 24-well plates with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% calf serum. After 48 h, the medium was removed and different concentrations of TLF were added in 0.5% bovine serum albumin (BSA) serum-free medium. Treatment with DMEM containing 0.5% BSA was included as a control. After 72 h, cells were counted using a hemocytometer. Small letters show statistically significant differences (Tukey, P ≤ 0.05) with respect to controls in each case. B: IC₅₀ for human cancer cells treated with TLF. IC₅₀ was calculated by linear regression of the logarithm for TLF (AU/mL) vs proliferation percentage (Color figure available online).

DISCUSSION

Different research groups have worked on legume lectins. In the case of Tepary bean lectins purification, different chromatographic methods have been used, particularly based on affinity
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FIG. 4. Effect of Tepary bean protease inhibitor (TBPI) on cell adhesion. NIH 3T3/v-mos fibroblasts were seeded (1 × 10⁴ cells/well) with Dulbecco’s modified Eagle’s medium with 5% calf serum. After 24 h, the medium was changed and supplemented with 1% bovine serum albumin (BSA)/1.5% calf serum as a control, while adding 0.027 μg protein/mL (equivalent to 250 IU/mL) of TBPI to the experimental group. Every third day, 2 wells of each treatment were fixed with absolute ethanol until cells reached confluence. Fixed wells were stained with carmine/ethanol-HCl, and absorbance of the extracted colorant was determined at 531 nm. The asterisk shows a statistically significant difference with respect to control cells for each day (t student, P ≤ 0.05).

chromatography (31,33–35), where they reported a lectin with an apparent molecular mass ranging from 21 to 40 kDa for the single subunits. Pratt et al. (32) reported 4 isolecitins with apparent molecular mass of 30 kDa each. We obtained an enriched fraction of Tepary bean lectin by gel filtration chromatography followed by ion exchange chromatography. The positive glycoprotein staining suggested that this band with an apparent molecular mass of 30 kDa could correspond to the previously described Tepary bean lectin (31,36). However, despite the fact that ion exchange chromatography allowed the separation of the semipurified lectin, 80% of the original agglutination activity was lost. We first tried unsuccessfully to purify the lectin through fetuin-agarose affinity chromatography as previously described (31,34,36). Similarly, when HPLC was used, no positive results were obtained. Lectins are not single proteins, but families of homo- or hetero-oligomers with different biological activities (37,38); therefore, the characterization of the specific isolated glycoprotein becomes important when a correlation of certain structure to a specific function is to be established.

Lectins from different sources have shown antiproliferative activities, as well as apoptotic induction effects on cancer cells. There is evidence that the apoptotic effects of some lectins, such as Viscum album L. coloratum agglutinin, involve ROS production (39). Lectins exert their effects by binding the cell surface in a noncovalent way by recognizing specific glycoproteins on the cell membranes, which are frequently altered in cancer cells.

This ability allows lectins to selectively interact with cancer cells, affecting their proliferation and survival (16,40). Here we report that the Tepary bean protein fractions containing lectin activity exhibit differential antiproliferative effect on nontransformed and transformed murine 3T3 fibroblasts. The observed IC₅₀ values for TLRF (1.21 AU/mL) and TLF (0.27 AU/mL) on 3T3/v-mos transformed fibroblasts, suggests that TLF retained its biological activity.

Tepary bean lectin fractions have been tested on animal cells. Lectins obtained by affinity chromatography have showed mitogenic activity on human peripheral lymphocytes (33,35), as well as on cultured human lymphocytes and a differential cytotoxic effect was also observed on different clones of murine 3T3 fibroblasts (41). We show now that TLF presented specific cytotoxic effect on human cancer cells, and CaCo2 cells exhibited the highest sensitivity as indicated by its lowest IC₅₀ (0.15 AU/mL). Similar cytotoxic effects were observed by Valadez-Vega et al. (42) when a Tepary bean lectin obtained by affinity chromatography was tested on Sw480 (colon cancer) and C33-A (cervix cancer) cells lines, although no IC₅₀ were calculated in that work. In this report, we found an IC₅₀ of 8.08 AU/mL for C33-A cells.

We previously reported that a semi-pure fraction of Tepary bean PI presented cytotoxic effects on transformed cells (22); however, such effect was due to the presence of the lectin here
reported, whereas the pure protease inhibitor TBPI had no
effect on cell proliferation. Even more, proliferation rate in-
creased at the highest TBPI concentration tested (0.1 µg pro-
tein/mL, equivalent to 1000 IU/mL), effect that has previously
been observed for other anticarcinogenic protease inhibitors
(7).

One of the important features of some serine PIs for their
anticancer effect is their ability to inhibit chymotrypsin-like
activity (6). In the case of TBPI, high inhibitory activities against
both trypsin and chymotrypsin-like enzymes have been reported
(21–22). Tumor development is a process heavily dependent
on proteolysis that affects ECM degradation, changes in cell
adhesion, migration, invasion, and chemical modification of
the environment, including the growth factors production (1).

Serine proteases are considered key enzymes for cancer de-
velopment, mainly because of their role in the degradation of
some components of ECM, such as laminin and its participa-
tion in the activation of several MMPs, involved in the cleavage
of most ECM proteins (52,55–57). The effect of MMP activity
has also been supported by in vitro studies measuring inva-
sion through Matrigel (58). Kennedy and Wan (59) observed
that SBBI decreased the ability of LNCaP cells to invade across
Matrigel. Our results showed that cells treated with TBPI dimin-
ished their invasion ability most probably due to the suppression
of MMP2 and MMP9.

Here, we presented evidence that 2 proteins from Tepary
bean seeds have negative effects on different steps of cancer
progression and promotion stages in vitro. Current studies in
our group are focusing on the mechanism of action of both
proteins on murine and human cancer cells as well as in vivo
systems.
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