Expression of a Buckwheat Trypsin Inhibitor Gene in *Escherichia coli* and its Effect on Multiple Myeloma IM-9 Cell Proliferation

Zheng ZHANG, Yuying LI, Chen LI, Jingming YUAN, and Zhuanhua WANG*

Key Laboratory for Chemical Biology and Molecular Engineering of the Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan 030006, China

Abstract The gene of buckwheat trypsin inhibitor (BTI) has been cloned and expressed in *Escherichia coli*. The yield of this recombinant inhibitor was over 12 mg/L by using one-step purification on a Ni^{2+}-NTA Sepharose column. Its molecular weight was 9322.1 Da, determined by mass spectrum analysis. The MTT and cytometry analyses showed that recombinant BTI could specifically inhibit the proliferation of IM-9 human B lymphoblastoid cells (from patient with multiple myeloma) in a dose-dependent manner. The test of recombinant BTI-induced apoptosis in IM-9 cells implied that the inhibitor might have potential application in the treatment of cancer.

Keywords buckwheat trypsin inhibitor; IM-9 cell; inhibitory activity; apoptosis

Protease inhibitors are widely distributed in all types of life forms and those from the cowpea, arrowhead, and peanut have been studied [1–3]. In particular, serine protease inhibitors from plants are well-known defense compounds [4,5] that also regulate endogenous proteases [6]. These proteins that are expressed in developing seeds are assumed to play an important role in inhibiting trypsin and chymotrypsin of external origin [7–9]. Two major serine protease inhibitors, Kunitz inhibitors and Bowman-Birk inhibitors (BBIs), have been extensively studied in plants [10]. BBIs, encoded by a family of related genes, are Cys-rich proteins of approximately 8–16 kDa. To date, the 3-D structures, amino acid sequences, and physiochemical properties of small molecular weight BBIs have been explored [11–14]. Interestingly, these inhibitors displayed anticarcinogenic activity and acted as a cancer preventive or anti-inflammatory agent [15–17]. In the last two decades, many studies on gene cloning and the expression of protease inhibitors have been reported with the rapid development of biotechnology [18,19]. However, the gene cloning and expression of buckwheat trypsin inhibitor (BTI) has not been reported.

Buckwheat (*Fagopyrum esculentum* Moench.) is a dicotyledonous crop with a short growing period and can tolerate poor soil conditions. It is cultivated widely in Asia, eastern Europe, North America, and Australia. Even though it is an underutilized crop, it remains important for food supply in the temperate and hilly regions of countries in east Asia, east Europe, and the Himalayan area. Buckwheat contains a rich supply of amino acids, abundant vitamins B_1 and B_2, dietary fiber, proteins, minerals, and vitamin P [20]. Traditionally, buckwheat has been used as a nutritional food and leafy vegetable, as well as for fodder and medicine in the countries of east and south Asia. Nowadays, buckwheat is an important health food, and has been widely investigated in terms of planting, breeding, and estimation of nutritional qualities. Several protease inhibitors from buckwheat seeds have been reported [21,22]. Among them, a buckwheat inhibitor (BWI)-1 protein extracted from common buckwheat seeds with a molecular weight of 7.7 kDa is a potato inhibitor I family member [23]. It was found that BWI-1 and BWI-2a extracted from buckwheat seeds could inhibit T-acute lymphoblastic leukemia cells [24].

Previously, we obtained a new gene encoding buckwheat trypsin inhibitor and the nucleotide sequence data

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*Corresponding author: Tel, 86-351-7017022; Fax, 86-351-7011499; E-mail, zhwang@sxu.edu.cn

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have been submitted to the GenBank database under accession No. AY335158. A homology analysis showed that the amino acid sequence of buckwheat trypsin inhibitor obtained in our laboratory is totally identical to that of BWI-1, and its molecular size and inhibitory activity are similar to those of BBIs. To reveal the inhibitory activities towards proteases and tumor cells, the open-reading frame of the buckwheat trypsin inhibitor gene was chemically synthesized and expressed in *Escherichia coli* M15. The product was subjected to one-step purification and the bioactivity was analyzed.

Materials and Methods

Materials

Bacterial strains *E. coli* M15 (pREP4), *E. coli* DH5α, and pQE-31 (Qiagen, Carlsbad, USA) were stored in our laboratory at –70 ºC. A Ni^{2+}-NTA agarose column (HiTrap chelating HP) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Restriction enzymes for gene manipulation and expression were purchased from Promega (Madison, USA). RPMI 1640 was purchased from Gibco Life Technologies (Carlsbad, USA). Fetal bovine serum was purchased from the Institute of Hematology (Hangzhou, China). MTT assay kit was purchased from Sigma-Aldrich (St. Louis, USA). The Annexin V-FITC apoptosis detection kit was obtained from Pharmingen-Becton Dickinson (San Diego, USA). The plasmid mini kit for plasmid recovery and purification and the DNA gel extraction mini kit were from HuaShun Company (Shanghai, China). Trypsin was from Sigma-Aldrich. All chemicals and reagents used were of analytical grade.

Cloning and construction of recombinant plasmids

The buckwheat trypsin inhibitor cDNA was synthesized using total RNA isolated from buckwheat leaves as the template for amplification of the target gene by polymerase chain reaction (PCR). The oligonucleotide primers used were 5′-ATGGATCCTCTGCGTCAGTGCTCCGGTAA-ACAAGAATGGCCAGAGCTCG-3′ and 5′-ATAAGCTTT-TATCACATAACAACAGGAGTATCAACAA-3′. The PCR thermal profile consisted of 30 cycles of 94 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min, followed by a final extension at 72 ºC for 10 min. The PCR product was purified and digested with *Bam*HI and *Hin*dIII, and the DNA fragment encoding buckwheat trypsin inhibitor was then ligated into the pGEM-T easy vector and transformed into *E. coli* DH5α. The sequence of the inserted fragment was confirmed by using an automatic DNA sequencer (ABI Prism, Foster city, USA); the gene fragment was then digested with *Bam*HI and *Hin*dIII and separated by agarose gel electrophoresis. Then BTI cDNA was inserted into the pQE-31 expression vector to form the recombinant plasmid pQE-31-BTI.

Expression of the recombinant protein

The recombinant plasmid pQE-31-BTI was transformed into competent *E. coli* M15(pREP4) cells [25]. The transformed *E. coli* cells were grown in 1 liter of LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37 ºC until the optical density (OD) at 600 nm reached 0.6–0.7. Then isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture was further incubated at 37 ºC for 4 h. The cells were harvested by centrifugation at 6000 *g* for 30 min at 4 °C and then lysed by ultrasonication. The cell lysate was then centrifuged at 15,000 *g* for 30 min at 4 ºC. Both the supernatant and the pellet were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide gel [26]. The gel was stained by Coomassie brilliant blue R-250 and scanned using a densitometer (GeneGenius 8000; Syngene, Upland, USA) to determine the percentage of the fusion protein in the total proteins from the cultured cells.

One-step purification of the fusion protein

The supernatant was applied to a Ni^{2+}-NTA agarose column pre-equilibrated with buffer I (20 mM Tris-HCl, 500 mM NaCl, and 10 mM imidazole, pH 7.4). The column was thoroughly washed with the same buffer until the OD at 280 nm was returned to the baseline. Unbound proteins were eluted with 20 mM imidazole. The bound His_6-tagged fusion protein was eluted with buffer II (20 mM Tris-HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.4) and the fractions were checked by SDS-PAGE, and the fractions containing the fusion protein were collected and dialyzed overnight against 20 mM phosphate buffer (pH 7.4) at 4 ºC.

Western blot analysis and molecular weight determination

The purified fusion protein was subject to SDS-PAGE and transferred onto nitrocellulose membrane using the TE-22 blotter (Amersham Pharmacia Biotech) at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methyl alcohol, pH 8.3) according to the

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manufacturer's instructions. After being blocked with defatted milk, the membrane was incubated with mouse anti-His antibody (1:1000) (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h and then with goat anti-mouse IgG-AP (1:10,000) (Sino-American Biotechnology, Beijing, China) for 30 min at room temperature. The membrane was stained for 1 h in AP color development solution containing 1 ml of 1×AP reaction buffer with 50 µl of NBT solution and 50 µl of BCIP solution (Tiangen, Beijing, China). The molecular weight of the fusion protein was determined by MALDI-TOF-MS (Ciphergen, Fremont, USA) analysis.

Determination of inhibitory activity

The trypsin inhibitory activity was determined by the method of Erlanger *et al*. [27]. Briefly, in 3 ml of 0.01 M Tris (pH 8.0), 1 mM $CaCl_2$, 3 µg of trypsin, and suitable amounts of rBTI were incubated with 0.15 mM BApNA. The residual trypsin activity was measured at 410 nm. One unit of inhibitory activity (U_I) was defined as the amount of the inhibitor that led to a decrease of 0.01 in absorbance at 410 nm in 1 ml of reaction solution per minute [28]. The protein concentration was determined by the method of Bradford [29] using bovine serum albumin as standard.

Bioactivity of rBTI for inducing apoptosis

The bioactivity of rBTI was determined using human B lymphoblastoid cells with multiple myeloma (IM-9 cells). The inhibition of IM-9 cell proliferation was measured by using a colorimetric method [30] with the reagent MTT. Briefly, 1×10^5 IM-9 cells or Bcl-2 over-expressing IM-9 cells (IM-9/Bcl-2 cells) were treated with rBTI (12.5–100 µg/ml) and transferred to quadruplicate wells of 96-well microtiter plates at the indicated time points, respectively. After incubating at 37 ºC for 24 h, 20 µl of MTT was added and incubated for 4 h; following this, 80 µl of DMSO was added. The color intensity was measured using a microtiter plate reader (Bio-Rad model 550, Hercules, USA) at 570 nm. Medium alone was taken as a blank control.

Flow cytometry analysis of apoptosis

A flow cytometry analysis of Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI)-stained cells was performed using an apoptosis detection kit according to the manufacturer's instructions. Briefly, IM-9 cells and IM-9/Bcl-2 cells (1×10^5) treated with or without rBTI were washed with 50 mM cold phosphate buffer (pH 7.6) and centrifuged at 12,000 *g* for 5 min. The cells were suspended in 100 µl of binding buffer. A mixture of 5 µl of fluorescence-conjugated Annexin V (a Ca^{2+}-dependent and phospholipid-binding protein) and 2 µl of PI was added to the cell suspension and then incubated for 15 min at room temperature. The samples were analyzed for Annexin V binding within 1 h by flow cytometry.

Results

Expression of the recombinant protein

The coding region of BTI amplified by PCR could be detected after double-enzymatic digestion, and the DNA sequence analysis of the pQE-31-BTI plasmid also confirmed that the *BTI* gene was correctly inserted into the pQE-31 vector (data not shown). The optimal conditions chosen for expression were OD_{600} 0.6 for induction and 0.5 mM IPTG at 37 ºC for 4 h. In SDS-PAGE analysis (**Fig. 1**), a clearly expressed band of the IPTG-inducible target protein (**Fig. 1**, lane 3) was observed in the cell lysates. The expressed product was mainly in the supernatant, and the amount of fusion protein reached approximately 30% of the soluble protein.

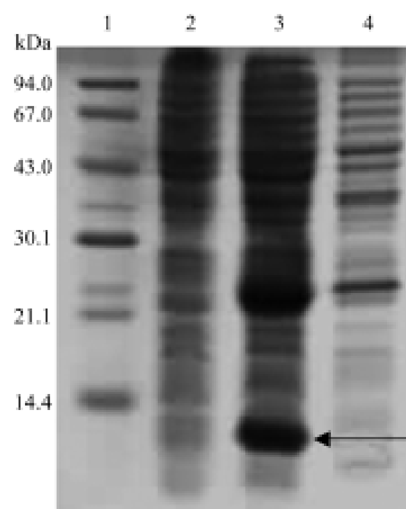

Fig. 1 Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of expression products
Lane 1, standard proteins marker; lane 2, uninduced cell lysate of M15/pQE-31; lane 3, total soluble protein with isopropyl-D-thiogalactopyranoside induction for 4 h (M15/pQE-31-BTI); lane 4, insoluble protein fraction obtained from the pellet. Arrow indicates the target protein. rBTI, recombinant buckwheat trypsin inhibitor.

Purification and analysis of rBTI

The SDS-PAGE analysis showed that a highly purified

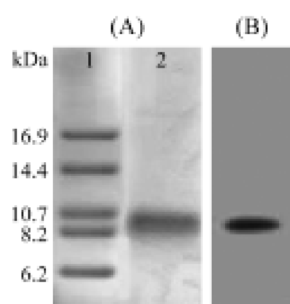

Fig. 2 Affinity chromatography and Western blot analysis
(A) Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of affinity chromatography fraction. Lane 1, standard proteins marker; lane 2, 20 μg of purified recombinant buckwheat trypsin inhibitor. (B) Western blot analysis of the purified recombinant protein.

target protein was obtained as a single band [**Fig. 2(A)**] and the purity was >95%. The Western blot analysis indicated that the target protein appeared at the expected position [**Fig. 2(B)**]. The molecular weight of the recombinant inhibitor with His_6 tagged was 9322.1 Da, determined by mass spectrum analysis. This was in good agreement with its theoretical value (9315.5 Da) deduced from the DNA sequence. The amino acid sequence deduced according to the nucleotide sequence of the *BTI* gene showed that rBTI is totally identical with that of BWI-1 [22] extracted from buckwheat seeds and had 85% homology with the potato inhibitor I family of serine protease inhibitors from other plants [31]. The yield of the purified rBTI was 12 mg per liter (**Table 1**), showing that the purification of rBTI by affinity chromatography was mainly achieved by elution with imidazole at a concentration of 500 mM in 20 mM Tris-HCl buffer, with only one step required for 10-fold purity of the target protein.

Identification of the inhibitory activity of rBTI

The inhibitory activity of the target protein against trypsin was assayed with BApNA as substrates, and the inhibitory activity unit (U_I) was calculated based on that described in “Materials and Methods”. The result showed that rBTI strongly inhibits the activity of trypsin, and the specific activity reached 13.0 U_I/mg (**Table 1**).

Inducing cancer cell apoptosis by rBTI

The results showed that rBTI could obviously inhibit the proliferation of multiple myeloma IM-9 and IM-9/Bcl-2 cells in a dose-dependent manner at all concentration levels, and the inhibitory effects showed statistical significance at 50 μg/ml ($P<0.05$), 100 μg/ml ($P<0.01$), and 200 μg/ml ($P<0.01$) (**Fig. 3**). In addition, the target protein at 100 μg/ml could inhibit the proliferation of IM-9 cells in a time-dependent manner within 6–72 h of exposure (data not shown).

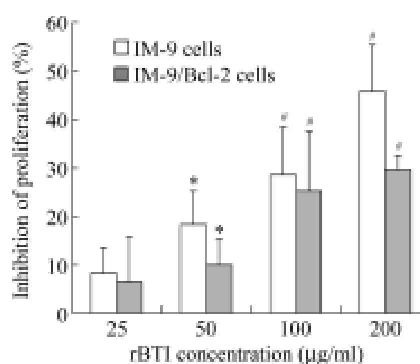

Fig. 3 Effects of recombinant buckwheat trypsin inhibitor (rBTI) on cell proliferation
IM-9 (1×10^5) and IM-9/Bcl-2 cells (1×10^5) were respectively transferred to quadruplicate wells of a 96-well microtiter plate in 0.25 ml of culture medium (80 μl of rBTI, 50 μl of DMSO, and 20 μl of MTT). After incubating for 24 h, the growth inhibitions of the IM-9 and IM-9/Bcl-2 cells by rBTI were measured. $^*P<0.05$, $^\#P<0.01$.

Furthermore, apoptosis was induced by adding rBTI to the IM-9 cells in the culture. The IM-9 cells and the IM-9/Bcl-2 cells treated with or without rBTI (25–200 μg/ml) for 24 h were double-labeled with the Annexin V-FITC conjugate and PI. The cell suspension was subsequently analyzed by flow cytometry. As shown in **Fig. 4**,

Table 1 Purification of recombinant buckwheat trypsin inhibitor expressed in *Escherichia coli* cells

Purification step	Total protein (mg)	Inhibitory activity[a] (U_I)	Specific activity (U_I/mg)	Yield (%)	Purity (fold)
Cell lysate	300	406	1.35	100	1
Ni^{2+}-NTA agarose	12	156	13.00	38	10

[a] One unit of inhibitory activity (U_I) is defined as the amount of inhibitor that decreases the absorbance at 410 nm by 0.01 in 1 ml of the reaction solution per minute. Experimental data for the inhibitory activity of recombinant buckwheat trypsin inhibitor are the average of three measurements.

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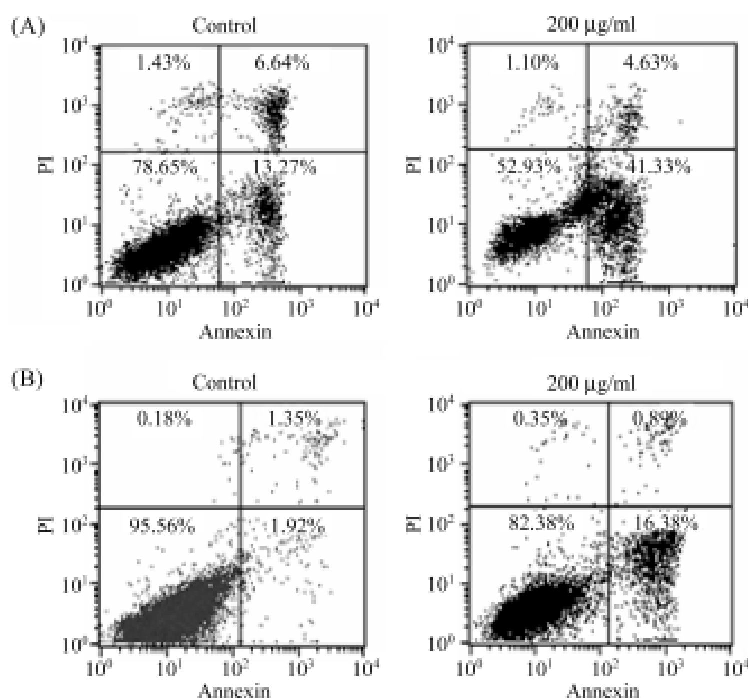

Fig. 4 Flow cytometric analysis of tumor cells treated with recombinant buckwheat trypsin inhibitor (rBTI)
IM-9 and IM-9/Bcl2 cells were cultured with or without 200 μg/ml rBTI. After 24 h of culture, the cells were double-labeled with Annexin V-fluorescein isothiocyanate conjugate and propidium iodide and were subjected to flow cytometry. Apoptosis of IM-9 (A) and IM-9/Bcl2 (B) cells were induced by rBTI.

13.27% of untreated and 41.33% of rBTI (200 μg/ml) treated IM-9 cells were stained positive with Annexin V and negative with PI [**Fig. 4(A)**]. In addition, approximately 1.92% of untreated and 16.38% of rBTI (200 μg/ml) treated IM-9/Bcl-2 cells were undergoing apoptosis after 24 h of incubation [**Fig. 4(B)**].

Discussion

The expression and purification of a buckwheat trypsin inhibitor and its effect on the proliferation of cancer cells has not been previously reported, although there have been some studies on the isolation and purification of natural trypsin inhibitors from buckwheat seeds [32–34]. In this study, the rBTI was highly expressed in a soluble state in *E. coli* M15 cells. The target protein could be easily purified by one-step affinity chromatography, with approximately 12 mg of pure rBTI obtained from 1 liter of bacterial culture. The molecular weight of rBTI was 9322.1 Da, and the purity was >95%. Furthermore, inhibitory activity assays suggested that the recombinant protein strongly inhibited the activity of trypsin. The purified protein also specifically inhibited the proliferation of multiple myeloma IM-9 cell lines in a dose-dependent manner.

It is known that protease inhibitors have multiple functions, including the regulation of endogenous proteases during germination and protection of the plant from insects and microorganisms. Recent interest has focused on pharmaceutical applications of protease inhibitors as anti-apoptotic [14] and targeted antitumor polymeric agents [15,17]. In recent years, the incidence of various tumors and hematopathies has remained high, whereas the number of antitumor drugs is still comparatively low. In this paper, the results of the apoptosis induction tests suggest that rBTI is bioactive to multiple myeloma IM-9 cells; not only were IM-9 cells undergoing apoptosis, but IM-9/Bcl-2 cells [35] were also inhibited after treatment with the rBTI. How rBTI can inhibit the proliferation of IM-9 cells and whether other plant trypsin inhibitors have an induction of apoptosis in cancer cells are not fully understood. Trypsin inhibitors may effect the expression of one or more promoting tumor factors. Because some tumor cells have a specific receptor for trypsin inhibitors on their surface [36], when binding occurs, tumor cell invasion and metastasis can be prevented and the pericellular matrix can also be restabilized.

In conclusion, rBTI was first expressed and easily purified with a one-step method. The novel function of rBTI in inhibiting the proliferation of myeloma IM-9 cells might be valuable for further studies on inducing the

apoptosis pathway by the recombinant buckwheat trypsin inhibitor.

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