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Suppressive Activity of Protease Inhibitors From Buckwheat Seeds Against Human T-Acute Lymphoblastic Leukemia Cell Lines

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Abstract

The buckwheat protease inhibitor designated BWI-1, a member of the potato inhibitor I family, inhibits trypsin, chymotrypsin, and subtilisin, whereas the buckwheat protease inhibitor designated BWI-2a, a novel protease inhibitor homologous to the vicilin family, inhibits only trypsin. We examined the suppressive activity of BWI-1 and BWI-2a against T-acute lymphoblastic leukemia (T-ALL) cells, such as JURKAT and CCRF-CEM, and human normal blood lymphocytes. Both inhibitors significantly suppressed the growth of T-ALL cells as judged by the soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (tetrazolium/formazan assay). JURKAT cells showed slightly higher susceptibility to buckwheat inhibitors than CCRF-CEM cells. Modification of Arg residue(s) in inhibitors by 1,2-cyclohexandione inactivated their trypsin inhibitory activity, considerably abolishing their suppressive activity. This suggests that the trypsin inhibitory activity is involved in the suppression of growth of human T-ALL cell lines. It was further found that both inhibitors triggered programmed cell death (apoptosis) of these cell strains with DNA fragmentation.

Index Entries: Apoptosis; buckwheat; protease inhibitor; tumor hematopoietic stem cell; T-acute lymphoblastic leukemia.

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Introduction

A large number of protease inhibitors with different specificities have been isolated from a variety of plant seeds and investigated extensively from a structural point of view. In particular, the Kunitz and Bowman-Birk families of serine protease inhibitors from soybeans are well understood as a model system of protein–protein interaction (for a review, *see* ref. *1*).

Apart from a structural interest in protease inhibitors, the current interest is in their applications to chemotherapy as antitumor agents. It has been reported that Bowman-Birk family inhibitors from soybeans and other legumes are potentially nutritionally relevant anticarcinogens, especially with respect to colon cancer *(2,3)*. A subsequent study showed that nanomole levels of Bowman-Birk family inhibitors suppress in vitro malignant transformation *(4)*. Furthermore, Lin et al. *(5)* reported on the chimeric protein that can be selectively targeted to specific cell types, such as sarcoma cell and Hela cell cultures, by linking trypsin inhibitor from *Acacia confusa* to *Abrus precatorius* toxic lectin, abrin B-chain. Our group recently found that trypsin and chymotrypsin inhibitor EBI from *Erythrina variegata* seeds had a suppressive effect on tumor hematopoietic stem cell lines, whereas Kunitz family inhibitors ETIa and ECI exhibited no effects on these cells *(6)*. These observations provide the hope that Bowman-Birk family protease inhibitors can be used clinically as anticancer agents in the future.

In a series of studies on protease inhibitors from plants, we previously isolated two distinct trypsin inhibitors, BWI-1 and BWI-2a, from buckwheat seeds *(7)*. BWI-1 is classified, on the basis of sequence similarity, with the potato inhibitor I family *(8)*, whereas BWI-2a is a novel inhibitor with a sequence similarity to a plant storage protein vicilin *(7)*. Further, we found that both proteins are minor allergenic proteins in buckwheat seeds *(7)*. To explore the application of BWI-1 and BWI-2a as antitumor agents, we examined their suppressive effects on tumor hematopoietic stem cell lines. In this article, we report on their capacity to suppress the growth of T-acute lymphoblastic leukemia (T-ALL) cells, such as JURKAT and CCRF-CEM. Furthermore, we show that the buckwheat protease inhibitors induced apoptosis with DNA fragmentation of T-ALL cell lines.

Materials and Methods

Inhibitors and Chemicals

Buckwheat trypsin inhibitors BWI-1 and BWI-2a were purified from the seeds of *Fagopyrum esculentum* Moench, as described previously *(7)*. Trypsin inhibitor ETIa from *E. variegata* seeds was purified, as described previously *(9)*. Glutamine and antibiotics were obtained from Gibco (Paisly, Scotland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and fetal calf serum (FCS) were purchased from Wako (Osaka, Japan). Lymphosepar I was purchased from Immuno-Biological Institute (Gunma, Japan). The substrates benzoyl-L-arginine-*p*-nitroanilide hydro-

chloride (L-BAPA), benzoyl-L-tyrosine-*p*-nitroanilide (L-BTPA), and carbobenzoxy-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide (Z-Ala-Ala-Leu-*p*NA) for trypsin, chymotrypsin, and subtilisin, respectively, were purchased from Peptide Institute (Osaka, Japan). 1,2-Cyclohexandione was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals used were of analytical grade.

Cell Culture

The human tumor T-ALL cell lines JURKAT and CCRF-CEM were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL of streptomycin, and 100 U/mL of penicillin in a humidified atmosphere with 5% CO_2 at 37°C. The cells used for the assay were in logarithmic phase. These cells were generously provided by Dr. J. Minowada (Harashibara Biochemical Laboratories, Okayama, Japan).

Preparation of Lymphocytes

Human peripheral blood lymphocytes were purified from heparinized peripheral blood obtained from normal adults by Lymphosepar I as described by Sallay et al. *(10)*.

Determination of Suppressive Activity of Protease Inhibitors

The suppressive activity of inhibitors was evaluated according to the soluble MTT tetrazolium/formazan assay *(11)* by measuring the cellular dehydrogenase activity to convert MTT into a formazan product. Briefly, 90 µL of a cell suspension containing 2.22×10^5 cells/mL was added to each well in a 96-well microtiter plate and was cultured in RPMI-1640 medium as previously stipulated. After 24 h, cells were treated with 10 µL of varying concentrations of inhibitors for 24 h under the same conditions, and then incubated with MTT for 4 h. Dimethyl sulfoxide was added to dissolve the MTT formazan reaction product, and the optical density at a wavelength of 570 nm was measured using an Inter Med Immunomini NJ-2300 multiwell scanning spectrophotometer.

Assay for Apoptosis

Apoptosis was evaluated by detecting DNA fragmentation using a Wako Apoptosis Ladder Detection kit. Briefly, after 24 h of cultivation, cells were incubated with 10 µL of 0.5 µg/µL of inhibitors for 24 h under the same conditions as those described above, and DNA was extracted according to the supplier's instructions. The DNA was dissolved in 50 µL of TE buffer (10 m*M* Tris-HCl; 1 m*M* EDTA, pH 8.0) and electrophoresed in a 1.5% agarose gel.

Chemical Modification of Inhibitors

Chemical modification of Arg residues was done using 1,2-cyclohexandione as described by Patty and Smith *(12)*. Briefly, 3 mg of protease

inhibitors was dissolved in 4 mL of borate buffer, pH 9.0, and 50 m*M* 1,2-cyclohexandione was added. After incubation for 2 h at 37°C, the sample was chilled on ice, dialyzed against 0.1% acetic acid, and then lyophilized.

Assay for Inhibitory Activity Toward Trypsin

Trypsin and chymotrypsin inhibitory activities were assayed using L-BAPA and L-BTPA, respectively, as substrates as described by Ikeda and Kusano *(13)*. Inhibition of subtilisin by the buckwheat protease inhibitors was assayed using Z-Ala-Ala-Leu-*p*NA as a substrate as described by Shibata et al. *(14)*.

Results

Characterization of BWI-1 and BWI-2a

In a previous study *(7)*, we examined the inhibitory activity of the buckwheat protease inhibitors BWI-1 and BWI-2a toward trypsin. In this study, their inhibitory potencies against other serine proteases, including chymotrypsin and subtilisin, were investigated. Figure 1 shows the titration patterns of serine proteases with both buckwheat inhibitors. BWI-1 effectively blocked the activities of chymotrypsin and subtilisin, although trypsin was more strongly inhibited than these two serine proteases (Fig. 1A). BWI-2a scarcely affected chymotrypsin and subtilisin (Fig. 1B).

Suppressive Activity of BWI-1 and BWI-2a

The suppressive effects of BWI-1 and BWI-2a on the human tumor T-ALL cell lines JURKAT and CCRF-CEM and human normal blood lymphocytes were measured by the MTT tetrazolium/formazan assay. JURKAT cells were incubated with increasing amounts of buckwheat protease inhibitors. As shown in Fig. 2A, BWI-1 and BWI-2a showed significant suppression, reducing cellular dehydrogenase activity to 30 and 10%, respectively, with concentrations of inhibitors up to 5 µg/mL. The suppressive effect of BWI-2a seemed to be slightly stronger than that of BWI-1 except in the case of 50 µg/mL. By contrast, the Kunitz family trypsin inhibitor ETIa isolated from *E. variegata* seeds exhibited no suppression against JURKAT cells up to 50 µg/mL, as described previously *(6)*. Similar results were obtained when CCRF-CEM cells were incubated with protease inhibitors from the buckwheat and *E. variegata* seeds, although CCRF-CEM cells appeared to be slightly more resistant to buckwheat protease inhibitors than JURKAT cells (Fig. 2B). In contrast to the suppressive effects on T-ALL cells, the suppressive effects of BWI-1 and BWI-2a against human normal blood lymphocytes were not observed (data not shown). These results clearly show that two protease inhibitors from the buckwheat seeds, as is the case for EBI from *E. variegata*, can suppress growth of the human tumor T-ALL cell lines JURKAT and CCRF-CEM.

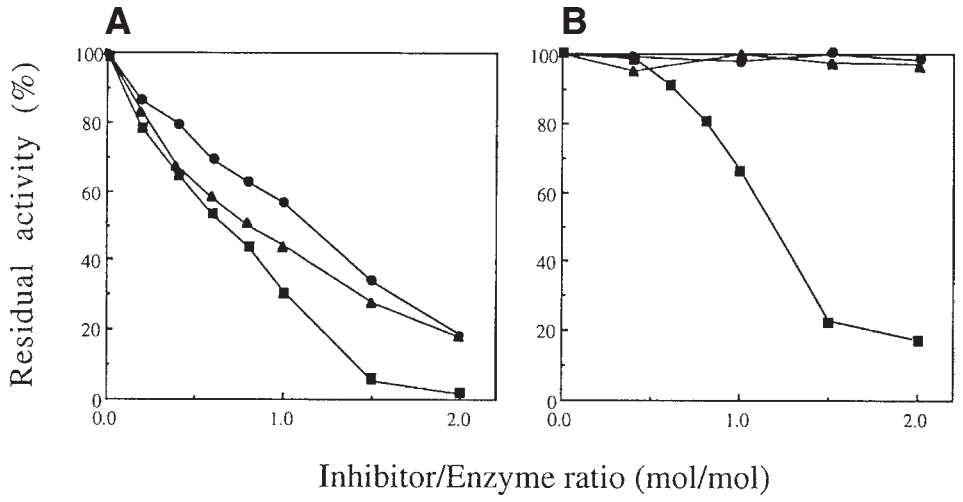

Fig. 1. Inhibitory activity of **(A)** BWI-1 and **(B)** BWI-2a toward trypsin, chymotrypsin, and subtilisin. A fixed amount of the enzymes trypsin (■), chymotrypsin (●), and subtilisin (▲) was mixed with increasing amounts of the inhibitors, and the residual enzyme activities were assayed as described under Materials and Methods.

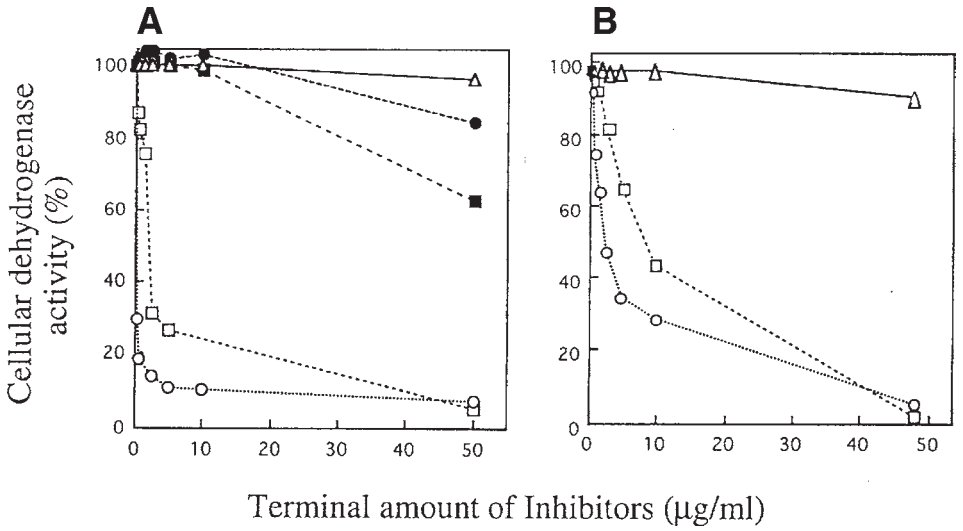

Fig. 2. Suppressive activity of protease inhibitors from buckwheat seeds toward human tumor hematopoietic stem cell lines: **(A)** JURKAT cells; **(B)** CCRF-CEM cells. Cells grown in 96-well microtiter plates were cultured in RPMI-1640 medium containing 10% heat-inactivated FCS with varying concentrations of inhibitors or their derivatives for 24 h at 37°C. Suppressive activity was evaluated according to the soluble MTT tetrazolium/formazan assay as described under Materials and Methods. Each point represents an average of three separate experiments. (□) BWI-1; (○) BWI-2a; (△) ETIa; (■) mBWI-1; (●) mBWI-2a.

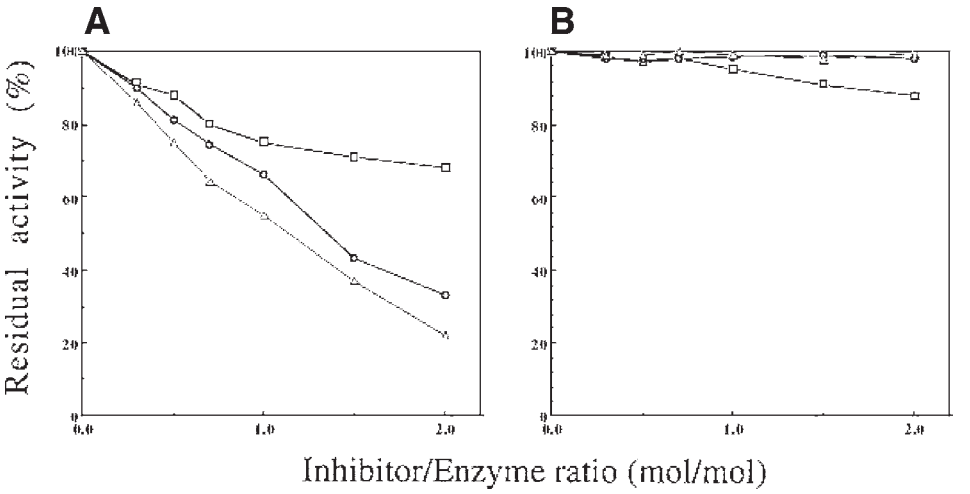

Fig. 3. Effects of chemical modification on inhibitory activity of BWI-1 and BWI-2a. **(A)** mBWI-1; **(B)** mBWI-2a. Chemical modification was conducted using 1,2-cyclohexandione according to Patty and Smith's *(12)* method. Symbols indicate residual inhibitory activity toward trypsin (□), chymotrypsin (○), and subtilisin (△).

Involvement of Trypsin Inhibitory Activity in Suppressive Activity

Sequencing studies of buckwheat protease inhibitors BWI-1 *(8)* and BWI-2a *(7)* identified the reactive-site residues for trypsin as Arg45 and Arg19, respectively. To examine a correlation between trypsin inhibitory activity and suppressive activity of these inhibitors, the influence of chemical modification of Arg residues in the two inhibitors on the suppressive activity was investigated. Chemical modification of Arg residue(s) on BWI-1 and BWI-2a by 1,2-cyclohexandione reduced their trypsin inhibitory activity up to 15% as compared with the native BWI-1 and BWI-2a (Fig. 3). When JURKAT cells were incubated with these derivatives (mBWI-1 and mBWI-2a), we found a significant reduction in suppressive activities of both derivatives. However, compared with unmodified BWI-1 and BWI-2a, the suppressive activity of mBWI-1 was less decreased than mBWI-2a at a relatively high concentration such as 50 μg/mL (Fig. 2A). This result suggests that the trypsin inhibitory activity of the two inhibitors might be involved in the suppressive activity against human tumor T-ALL cell lines.

Apoptosis Induced by BWI-1

Some plant lectins, such as phytohemagglutinin, concanavalin A, and wheat germ agglutinin, are cytotoxic against normal or malignant cells *(15)*, and it is assumed that these lectins lyse the cells by triggering a programmed cell death *(16)*. Thus, we examined whether the suppressive activity induced by buckwheat protease inhibitors involves apoptosis of JURKAT cells because JURKAT cells are more sensitive to the two inhibi-

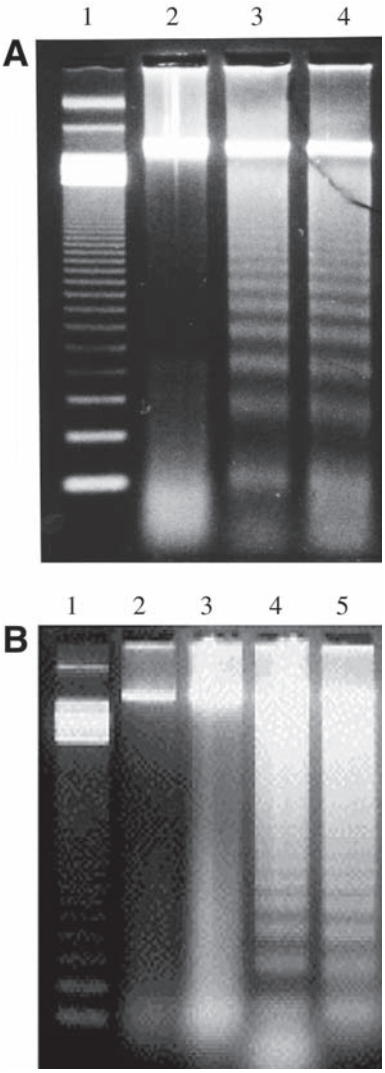

Fig. 4. Agarose gel electrophoresis of DNA extracted from JURKAT cells treated with BWI-1, BWI-2, and modified inhibitors by 1,2-cyclohexandione. Cells grown in 96-well microtiter plates were cultured in RPMI-1640 medium containing 10% heat-inactivated FCS with 50 µg/mL of BWI-1 for 24 and 48 h at 37 °C. DNA was extracted and analyzed on 1.5% agarose gel. **(A)** *Lane 1*, molecular weight marker; *lane 2*, DNA extracted from untreated cells; *lane 3*, cells treated with BWI-1 for 24 h; *lane 4*, cells treated with BWI-1 for 48 h. **(B)** *Lane 1*, marker; *lane 2*, mBWI-1; *lane 3*, mBWI-2a; *lane 4*, BWI-1; *lane 5*, BWI-2a.

tors than CCRF-CEM cells. Cells were incubated with 50 µg/mL of buckwheat protease inhibitor BWI-1, and then DNA fragmentation was examined. As shown in Fig. 4A, BWI-1 induced apoptosis of JURKAT cells, showing a typical ladder-like pattern of the fragmented DNA. A similar result was also obtained with BWI-2a; however, apoptosis was not induced by mBWI-1 and mBWI-2a (*see* Fig. 4B, *lanes 2* and *3*, respectively).

Discussion

Previously, our group showed that trypsin and chymotrypsin inhibitor EBI from *E. variegata* suppresses the growth of ALL cell lines *(6)*. In the present study, we extended this analysis to the distinct serine protease inhibitors BWI-1 and BWI-2a from buckwheat seeds; BWI-1 and BWI-2a are classified with the potato inhibitor I family and vicilin-like family, respectively, and EBI belongs to the Kunitz inhibitor family. BWI-1 has wide protease specificity, being able to block the activities of trypsin, chymotrypsin, and subtilisin, whereas BWI-2a exhibits inhibitory activity only toward trypsin (Fig. 1). The present result shows that both BWI-1 and BWI-2a are able to suppress the growth of two human tumor T-ALL cell lines (Fig. 2), whereas the suppressive effects of inhibitors on human normal blood lymphocytes were not observed. Two cell lines, JURKAT and CCRF-CEM, are representative of human tumor ALL cell lines and differ from each other in differentiation stages. The differentiation stage of CCRF-CEM cells is II and that of JURKAT cells is III *(17)*. We found that JURKAT cells were slightly more sensitive to both BWI-1 and BWI-2a than CCRF-CEM cells (Fig. 2). Thus, BWI-1 and BWI-2a might have the capability to distinguish the differentiation stages in T-ALL cell lines, similar to lectin Abrin A *(17)*.

Yavelow et al. *(18,19)* described that protease inhibitors from soybeans are anticarcinogens that inhibit the membrane-associated proteases of transformed cells. In addition, their anticarcinogenic action involves receptor-mediated endocytosis, resulting in internalization of the complex consisting of the protease inhibitor and membrane-associated protease *(18,19)*. Ohba et al. *(6)* showed that Bowman-Birk family inhibitor EBI from *E. variegata* exhibits suppressive activity toward ALL cells, whereas Kunitz protease inhibitors ETIa and ECI do not. Since Kunitz family proteins (20-kDa proteins) are larger than EBI (7-kDa protein), it is thus assumed that the difference observed in their suppressive activities is probably owing to their distinct molecular sizes. In the present study, we found that BWI-2a (6 kDa), which is smaller than BWI-1 (8 kDa), is slightly more suppressive against both JURKAT and CCRF-CEM cells than BWI-1. Although it is premature to conclude only on the basis of this observation, we assume that BWI-1, BWI-2a, as well as EBI might penetrate through the T-ALL cells with fluid-phase pinocytosis, rather than the receptor-mediated endocytosis.

It has been reported that the chymotrypsin inhibitory domain of Bowman-Birk soybean inhibitor is responsible for its suppressive effect on in vitro malignant transformation *(4)*. In the present study, we first discovered that the trypsin inhibitory activities of two proteins, BWI-1 and BWI-2a, from the potato inhibitor I family seem to be essential to their suppressive activities against ALL cells, because the chemical modification of the reactive Arg residues decreased their suppressive activities. Furthermore, at an excess amount of protease inhibitors, >10 μg/mL, a similar trend of suppressive activity in which BWI-2a had more suppressive activity than

BWI-1 in contrast to the case at <10 µg/mL was maintained before and after the inhibitors were modified (Fig. 2). This result, together with a possible different mechanism of the penetration, suggests that BWI-1 and BWI-2a might suppress growth of ALL cells by a mechanism different from that for the action of Bowman-Birk soybean inhibitor on the transformed C3H/10T1/2 cells. This observation is supported by the finding that the succinylated EBI lost their suppressive activity toward T-ALL cells *(6)*. Further studies on the interaction of BWI-1 and BWI-2a with the T-ALL and B-ALL cells from various stages should elucidate the mechanism by which they suppress the growth of ALL cells.

Kim et al. *(16)* suggested that some plant lectins such as phytohemagglutinin, concanavalin A, and wheat germ agglutinin show cytotoxic effects on certain cell lines by triggering apoptosis through activation of endogenous endonucleases, leading to DNA fragmentation. In the present study, it has become apparent that the buckwheat protease inhibitors BWI-1 and BWI-2a can induce apoptosis in T-ALL cells, although the mechanism of their mediated apoptosis is at present unclear. Thus, buckwheat protease inhibitors, like some plant lectins, might be suitable molecular probes for investigating the mechanism of apoptosis of cells. Our results presented here provide the hope that protease inhibitors from the various plant seeds can be used clinically as anticancer agents in the future. We will continue research to find optimal protease inhibitors with a strong suppressive activity of cancer cells.

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