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Cationic Inhibitors of Serine Proteinases from Buckwheat Seeds: Study of Their Interaction with Exogenous Proteinases

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Abstract—The inhibition of exogenous serine proteinases of different origin by cationic protease inhibitors BWI-1c, -2c, -3c, and -4c from buckwheat (*Fagopyrum esculentum* Moench) seeds has been studied. High efficiency of the inhibitors in binding bovine trypsin and chymotrypsin as well as their broad antiprotease effect, including inhibition of proteinases secreted by fungi and bacteria, has been demonstrated. According to the data obtained, it is proposed that cationic inhibitors from buckwheat seeds may participate in the defense of plants against fungal and bacterial infection.

Key words: inhibitors of serine proteinases, inhibition constant, trypsin, subtilisin, chymotrypsin

The search for and study of new low molecular weight protein inhibitors is now important since it may reveal inhibitors with new specificity of action and participating in metabolic processes of plant cells. Protein serine proteinase inhibitors are widely distributed in plants and now are obtained from many sources. These inhibitors can function as regulatory and/or defense proteins against insects and pathogenic microflora depending on their localization and specificity of inhibition of endogenous and/or exogenous enzymes [1].

Most of inhibitors studied at present are inactive towards endogenous proteinases but are active towards exogenous proteolytic enzymes, including digestive enzymes of insects, proteinases of phytopathogens, and bacterial subtilisins.

This work presents results on the study of inhibition of exogenous proteinases of various origin by cationic inhibitors BWI-1c, -2c, -3c, and -4c from buckwheat seeds in order to elucidate their physiological role, namely, possible participation in plant defense, and their possible use in bioengineering to increase plant tolerance to pests and phytopathogens.

Abbreviations: Bz) benzoyl; Glp) pyroglutamyl; Z) N-carbobenzoxy; pNA) *p*-nitroanilide; BWI) serine proteinase inhibitors from buckwheat seeds.

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MATERIALS AND METHODS

Chemicals. Trypsin was purchased from Merck (Germany). The content of active molecules in the sample was determined by titration with *p*-nitrophenyl ester of *p'*-guanidine benzoic acid [2].

α-Chymotrypsin was purchased from Merck. The content of active molecules in the sample was determined by titration with N-trans-cinnamoylimidazole from Sigma (USA) [3].

Preparation of duodenase was obtained from bovine duodenum according to Zamolodchikova et al. [4]. The concentration of the active enzyme was determined by standard rate of hydrolysis of substrate Suc-Ala-Ala-Pro-Phe-pNA [5].

Preparation of cathepsin G was obtained from human polymorphous nuclear leukocytes according to Neshkova et al. [6]. The concentration of the active enzyme was determined by titration with soybean Bowman–Birk inhibitor [7].

Preparation of subtilisin-72 from *Bacillus subtilis* was a gift from colleagues from the Faculty of Chemistry of Lomonosov Moscow State University.

Secreted proteinases from culture fluid of fungi *Botrytis cinerea*, *Ulokladium botrytis*, *Penicillium terlicowskii*, and *Trichoderma harzianum* were isolated by a similar scheme including $(NH_4)_2SO_4$ fractionation, affin-

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ity chromatography on bacitracin-silochrome, and FPLC ion-exchange chromatography on Mono-Q [8].

Ethyl ester of N-benzoyl-L-arginine hydrochloride (BAEE) and ethyl ester of N-benzoyl-L-tyrosine (BTEE) were purchased from Merck; Glp-Ala-Ala-Leu-pNA was a gift from colleagues from the Faculty of Chemistry of Lomonosov Moscow State University. Suc-Ala-Ala-Pro-Phe-pNA and Bz-Arg-pNA were purchased from Sigma. Other reagents were of especially pure and chemically pure grades.

Plant material. Dry buckwheat (*Fagopyrum esculentum* Moench cv. Shatilovskaya 5) seeds were used.

Isolation and purification of trypsin inhibitors (BWI) from dry buckwheat seeds. Proteins were extracted from buckwheat seed meal with 0.1 M K,Na-phosphate, pH 6.8 (1 : 4 w/v) at 4°C for 18 h and precipitated with 80% $(NH_4)_2SO_4$. Further total fraction of inhibitors was isolated by affinity chromatography on a trypsin-Sepharose 4B column. Inhibitors BWI-1c, -2c, -3c, and -4c were purified to homogeneity by ion-exchange FPLC chromatography on Mono Q at pH 6.8 and Mono S at pH 6.8 and 4.0. All of the studied inhibitors were electrophoretically homogenous [9].

Protein concentration in inhibitor solutions was assayed according to Lowry et al. [10]. Protein solutions were concentrated by ultrafiltration in Amicon (The Netherlands) cells with YM-5 membranes.

Isoelectric points of inhibitors were determined by column chromatofocusing with PBI-96. The column was equilibrated with 0.025 M Tris-HCl, pH 9.3, a sample of electrophoretically pure inhibitor preparation in starting buffer was applied to the column, and protein was eluted with Polybuffer-96, pH 6.0, prepared according to instruction of the manufacturer. The values of isoelectric points corresponded to the pH values at which activities of the inhibitors were maximal.

All spectrophotometric measurements were carried out using a Shimadzu UV-265FW (Japan) spectrophotometer.

Determination of K_i of trypsin and subtilisin. Solutions (0.05-0.2 ml, 11 μM) of inhibitor (BWI-1c, -2c, -3c) were placed into a cuvette, solution volume brought to 0.8 ml with 0.05 M Tris-HCl, pH 8.0, containing 0.02 M $CaCl_2$ (for trypsin), then 0.1 ml of substrate (for trypsin, 1.5 mM BAEE solution in the same buffer; for subtilisin, 1.9 mM Glp-Ala-Ala-Leu-pNA solution in DMFA) was added, and after thorough mixing 0.1 ml of enzyme solution was introduced (concentration of active trypsin in the cuvette was 33 nM; active subtilisin, 1.1 μM). The rate of substrate hydrolysis was monitored spectrophotometrically at 253 nm (BAEE) or 410 nm (Glp-Ala-Ala-Leu-pNA). Inhibition constants were calculated according to Levilliers [11].

Determination of K_i of chymotrypsin and duodenase. Solution of 0.05 M Tris-HCl, pH 8.0, 0.1 ml of chymotrypsin solution (227 nM) in 1 mM HCl, pH 3.0, or 0.015 ml of duodenase solution (5 μM) in water and 0.55 μM inhibitor solution (BWI-4c, 1.5 μM) were placed into a cuvette. The total volume of the mixture was 0.8 ml (for chymotrypsin) or 0.465 ml (for duodenase). The mixture was incubated at 25°C for 10 min. Then the substrate (for chymotrypsin, 0.1 ml of 1.27 mM BTEE solution in methanol; for duodenase, 0.02 ml of 10 mM Suc-Ala-Ala-Pro-Phe-pNA solution in dimethylsulfoxide (DMSO)) was placed into the cuvette and the change in optical density was registered at 256 or 410 nm, respectively. Inhibition constants were calculated according to Bieth [12].

To determine activity of BWI-4c inhibitor towards trypsin, 0.05 M Tris-HCl, pH 8.0, containing 0.02 M $CaCl_2$, 0.1 ml of 2.3 μM trypsin solution in 1 mM HCl, pH 3.0, and 15 μM of BWI-4c solution were placed into a cuvette. The total volume of the mixture was 0.7 ml. The mixture was incubated at 25°C for 15 min. Then 0.2 ml of 1.15 mM Bz-Arg-pNA solution in 2% DMSO was placed into the cuvette, and the rate of *p*-nitroaniline formation was registered spectrophotometrically at 410 nm.

To determine activity of BWI-4c inhibitor towards cathepsin G, 0.1 M HEPES, pH 7.4, containing 0.5 M NaCl and 0.005% Triton X-100, 0.01 ml of 0.5 μM cathepsin G solution in 0.05 M Na-acetate, pH 5.5, and 15 μM of BWI-4c solution were placed into a cuvette. The total volume of the mixture was 0.7 ml. The mixture was incubated at 25°C for 15 min. Then 0.05 ml of 10 mM Suc-Ala-Ala-Pro-Phe-pNA solution in DMSO was placed into the cuvette, and the rate of *p*-nitroaniline formation was registered spectrophotometrically at 410 nm.

To determine inhibitor activity towards proteinases isolated from culture fluid of fungi *Botrytis cinerea*, *Ulokladium botrytis*, *Penicillium terlicowskii*, and *Trichoderma harzianum*, 0.05 M Tris-HCl, pH 8.0, 0.1 ml of proteinase solution in the same buffer, and 11-μM inhibitor solution were placed into a cuvette. The total volume of the mixture was 0.78 ml. The mixture was incubated at 25°C for 15 min. Then 0.02 ml of 20 mM of Bz-Arg-pNA solution in 2% DMSO were added into the cuvette. The rate of *p*-nitroaniline formation was registered spectrophotometrically at 410 nm.

Table 1. Main properties of cationic protease inhibitors (BWI) from buckwheat seeds

Inhibitor	Molecular mass, daltons [9]	Isoelectric point	Reactive center [9]
BWI-1c	5203	8.17	Arg-X
BWI-2c	5347	8.25	Arg-X
BWI-3c	7760	8.48	Lys-X
BWI-4c	6031	8.07	Lys-X

To determine I_{50}, the curves of rate of substrate hydrolysis with enzyme in the presence of different inhibitor concentrations were plotted, and inhibitor concentration necessary for 50% inhibition (I_{50}) was determined. In case of fungal proteinases 1 mol of enzyme corresponded to the amount that digested the substrate with the same rate as 1 mol of pure trypsin or subtilisin-72 from *Bacillus subtilis* preparations.

RESULTS AND DISCUSSION

Preparations of low molecular weight serine proteinase inhibitors BWI-1c, -2c, -3c, and -4c were obtained electrophoretically homogenous from buckwheat seed extract. Their main properties are presented in Table 1.

Analysis of the N-terminal amino acid sequences of the cationic inhibitors revealed that BWI-3c and BWI-4c

Table 2. Constants of inhibition of serine proteinases with inhibitors from buckwheat seeds and other plant sources

Inhibitor (family)	Plant source	K_i, nM	
		trypsin	chymotrypsin
BWI-1c (not established)	buckwheat seeds	0.38 ± 0.11	2.0 ± 0.7
BWI-2c (not established)	buckwheat seeds	0.47 ± 0.12	48 ± 14
BWI-3c (potato inhibitor I)	buckwheat seeds	0.41 ± 0.1	8.4 ± 2.5
BWI-4c (potato inhibitor I)	buckwheat seeds	1000*	0.20 ± 0.04
BWI-1a (potato inhibitor I) [13]	buckwheat seeds	1.5	n.i.
PSI-1.1 (potato inhibitor II) [14]	paprika seeds	0.48	47
ATSI (potato inhibitor I) [15]	*Amaranthus caudatus* L. seeds	0.34	0.41
MTI-2 (not established) [16]	mustard *Sinapsis alba* L. seeds	0.16	500
TcTI (Bowman–Birk) [17]	*Torresea cearensis* seeds	1.0	50
PDTI (Kunitz) [18]	*Peltophorum dubium* (Spreng.) seeds	0.4	260

Note: n.i., no inhibition.
* Value calculated from the inhibitor/enzyme ratio at 50% inhibition = 15.

Table 3. Effect of cationic protease inhibitors from buckwheat seeds on the activity of bacterial and fungal proteolytic enzymes

Protease	Inhibitor/enzyme ratio (mol/mol) at 50% inhibition			
	BWI-1c	BWI-2c	BWI-3c	BWI-4c
Subtilisin-72 from *Bacillus subtilis*	n.i.	n.i.	0.72	0.81
Thiol-dependent subtilase from *Bacillus intermedia*	n.i.	n.i.	4.2	5.2
Trypsin-like protease from *Botrytis cinerea*	1.6	1.8	n.d.	n.d.
Trypsin-like protease from *Ulokladium botrytis*	0.34	1.15	n.i.	n.d.
Subtilase from *Penicillium terlicowskii*	n.i.	n.i.	2.56	2.64
Subtilase from *Botrytis cinerea*	n.i.	n.i.	8.4	3.6
Subtilase from *Trichoderma harzianum*	n.i.	n.i.	n.i.	n.i.

Note: n.i., no inhibition; n.d., not determined.

inhibitors appeared to belong to potato proteinase inhibitor I family, whereas BWI-1c and BWI-2c inhibitors could not be classified according to their N-terminal amino acid sequences [9].

The measured values of inhibition constants of trypsin and chymotrypsin by preparations of the cationic inhibitors from buckwheat seeds as well as the inhibition constants of these enzymes by earlier studied inhibitors of plant origin are given in Table 2.

As seen from Table 2, the studied proteins inhibit trypsin and chymotrypsin no less effectively than those studied earlier. While the high affinities of buckwheat seed inhibitors to trypsin are almost similar (excluding BWI-4c), their affinities to chymotrypsin are significantly different: BWI-2c binds with chymotrypsin 250-fold worse than BWI-4c, which in contrast the first three inhibitors inhibit only chymotrypsin. In this regard, it was of interest to study the effect of BWI-4c on proteases with so-called "double" trypsin- and chymotrypsin substrate specificity—duodenase and cathepsin G. Only a limited number of natural inhibitors from plants, mainly from the Bowman–Birk and Kunitz families, were found for these enzymes [5, 19, 20]. It was demonstrated that BWI-4c efficiently inhibited (K_i = 65 nM) and weakly bound cathepsin G (ratio of inhibitor/enzyme concentrations was 1500 at 50% inhibition, which corresponds to K_i value of 0.1 mM).

It is noteworthy that besides trypsin and chymotrypsin BWI-3c is an efficient inhibitor of subtilisin (K_i = 6.4 nM). This is a rare case of efficient inhibition of three different enzymes. Similar properties towards these three enzymes were described for ATSI inhibitor from amaranthus (*Amaranthus caudatus* L.) seeds (for subtilisin BPN′ K_i = 0.37 nM [15]). Endogenous BAPAse and pepsin were found to be stable to the effect of all studied inhibitors [21].

In view of possible participation of cationic inhibitors from buckwheat seeds in defense of the plants against pathogens, their effect on the activity of secreted proteases of some bacteria and filamentous fungi has been studied. The results are summarized in Table 3.

The presented data indicate that while BWI-1c and BWI-2c inhibited only secreted trypsin-like fungal proteases, BWI-3c and BWI-4c suppressed the activity of bacterial subtilisins and secreted fungal subtilases. Besides proteases mentioned in Table 3, BWI-3c and BWI-4c also efficiently suppressed the activity of subtilisin-like enzymes, thermitase and savinase, which are used in production of washing materials (data not shown).

Thus, cationic serine proteinase inhibitors from buckwheat seeds (BWI) demonstrated a high spectrum of anti-protease activity, but had no effect on endogenous enzymes. According to these data, it can be proposed that the studied inhibitors are part of the defense system of buckwheat seeds and plants; BWI-1c and BWI-2c inhibitors perform defense from fungal infection and BWI-3c and BWI-4c inhibitors defend also from bacterial infection.

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