Trypsin inhibitors in turnip (*Brassica rapa* L.) seeds

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Abstract

A method of trypsin inhibitors isolation from turnip seeds is described. Inhibitors were extracted with 0.01 N HCl, concentrated by salting out with ammonium sulfate, and purified using ion-exchange chromatography on Sp-Sephadex C-25, QAE-Sephadex A-25 and affinity chromatography on immobilized trypsin. Among the three isolated inhibitors, ITR I of molecular weight 15.9 kDa, pl. 6.4, inhibited trypsin activity only. Inhibitors ITR II and ITR III inhibited also chymotrypsin activity, they had similar molecular weight (about 10 kDa), but their pl is 7.5 and over 10, respectively. Arginine residue occurred in P₁ position of the reactive site of inhibitors ITR I and ITR III, while in ITR II this position was occupied by lysine residue. Electrophoresis on polyacrylamide gel revealed that each inhibitor possessed two protein fractions, probably a virgin and modified form, with the reactive site peptide bond broken by trypsin.

Key words: trypsin, inhibitor, Cruciferae, *Brassica rapa*

INTRODUCTION

Polypeptide inhibitors of proteinases are commonly present in plant and animal tissues. Inhibitors of serine proteinases are most frequent and best known. Most inhibitors, both of plant and animal origin (with the exception of blood serum inhibitors), are simple proteins of low molecular weight, rarely exceeding 20 kDa.

The smallest isolated inhibitors are those obtained from seeds of plants belonging to the *Cucurbitaceae* family. They are composed of 29 amino acids only (Polanowski et al. 1987). Specificity of inhibitors is determined by the presence of definite amino acid in P₁ position of the reactive site. Inhibitors in

which this position is occupied by lysine or arginine residue inhibit activity of trypsin or trypsin-like enzymes. However, most inhibitors of plant origin inhibit activity of at least two enzymes of different specificity, because they usually possess two independent reactive sites in the molecule.

Seeds of plants belonging to *Papilionaceae* and *Cucurbitaceae* families proved to be an especially rich source of inhibitors (Richardson 1977, Polanowski et al. 1987). *Cruciferae* seeds also contain trypsin and chymotrypsin inhibitors as well as inhibitors of some proteinases produced by microorganisms. The latter, however, are less known than the inhibitors from seeds of the above mentioned families, which have been very well characterized. So far, amino acid sequence of any inhibitors from *Cruciferae* seeds has not been known. Inhibitors from radish (*Raphanus sativus*) seeds have been quite well characterized by Ogawa et al. (1968, 1971a); they inhibit trypsin, chymotrypsin, pronase and subtilisin BPN' (nagarse). Menegatti et al. (1985) described a method of inhibitor isolation from mustard (*Sinapis alba*) seeds and determined some of its properties and amino acid composition.

Inhibitors from the seeds of *Brassica* genus are less known. From *Brassica oleracea* var. *sabellica* seeds one inhibitor of trypsin, chymotrypsin and subtilopeptidase A (subtilisin Carlsberg) has been isolated (Wilimowska-Pelc 1985).

This paper presents a method of isolating inhibitors from turnip (*Brassica rapa*) seeds as well as describes some of their properties.

MATERIALS AND METHODS

Turnip (*Brassica rapa* L. var. *Schneeboall*) seeds were obtained from the Plant Seed Corporation in Wrocław. They were stored in temp. of about 10°C and humidity of 40-60%.

Reagents. Sepharose 4B, Sp-Sephadex C-25, QAE-Sephadex A-25, Sephadex G-15 were from Pharmacia (Uppsala, Sweden), α -N-benzoyl-DL-arginine p-nitroanilide (BAPNA), chymotrypsin A, chymotrypsinogen, ribonuclease, myoglobin, ovalbumin, bovine blood serum albumin from Sigma Chemical Company (St. Louis, MO, USA), casein, white soluble, benzoquinone 1,2-tetrahydride (cyclohexanedione) were from Koch-Light Labs. Ltd (Colnbrook, England), p-nitrophenyl p-guanidinobenzoate (pNPGb) was from Merck (Darmstadt, FRG). Reagent for polyacrylamide gel electrophoresis were from Fluka A. G. (Buchs, S. G., Switzerland). Trypsin and Kazal inhibitor from bovine pancreas were prepared according to the methods of Wilimowska-Pelc and Mejbaum-Katzenellenbogen (1978), and Wilimowska-Pelc et al. (1973), respectively.

Other reagents were from PPH POCH Gliwice, Poland.

Protein determination. Protein was determined with microbiuret method (Goa 1953) or spectrophotometrically at 280 nm. Trypsin concentration was

determined by titration of the reactive site with pNPGb (Chase and Shaw 1970).

Determination of trypsin and antitrypsin activity. Trypsin and antitrypsin activity was determined with the method of Erlanger et al. (1961) using BAPNA as a substrate. A unit of antitrypsin activity was equal to the inhibitor amount which inhibited activity of 1 mg of trypsin.

Determination of chymotrypsin and antichymotrypsin activity. Chymotrypsin activity was determined with the method of Kunitz (1947) using casein as the substrate.

Modification of arginine and lysine residues in the inhibitor molecule. Arginine modification was carried out using 1,2-cyclohexanedione (Pathy and Smith 1975). Free amino groups were acetylated with acetic acid anhydride according to Frankel-Conrat (1957).

Modification of the inhibitors by trypsin. 0.01 to 0.02 ml of 0.3% trypsin solution (1 to 2 mol%) was added to 1 ml of 0.05 M citrate-phosphate buffer, pH 3.2, containing 1 mg of the inhibitor. Degree of modification was checked after 48 hrs incubation in room temperature, applying polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Electrophoresis on 7.5% polyacrylamide gel, at pH 8.3 was carried out according to Davis (1964), and at pH 2.4 in 15% gel according to Panyim and Chalkley (1969). Gels were stained with 1% solution of amido black in 7% acetic acid.

Determination of isoelectric points of the inhibitors. Isoelectric points were determined applying isoelectric focusing according to Karlsson et al. (1973) over the pH range 3.5 to 10.0. After electrophoresis gels were cut into 5-mm slices, homogenized in water, pH was measured, and antitrypsin activity was located.

Determination of molecular weight of the inhibitors. Molecular weight of the inhibitors was determined by polyacrylamide gel electrophoresis in the presence of SDS, at pH 7.0 (Weber and Osborn 1969). The following standards were used: Kazal inhibitor from bovine pancreas (6.1 kDa), ribonuclease from bovine pancreas (12.4 kDa), myoglobin (17.6 kDa), chymotrypsinogen (25 kDa), ovalbumin (45 kDa), bovine serum albumin (65 kDa).

Trypsin immobilization. Trypsin was immobilized on Sepharose 4B according to Kassel and Marciniszyn (1971).

RESULTS

PREPARATION OF THE INHIBITORS FROM TURNIP SEEDS

Extraction. Ground seeds were extracted with three volumes of 0.01 N HCl for an hour, at constant mechanical stirring, then centrifuged at about $3000 \times g$ for 20 min. The supernatant was decanted and the sediment was

Table 1
Isolation of trypsin inhibitors from turnip (*Brassica rapa*) seeds

Procedure steps		Protein, mg/kg of seeds	Antitrypsin activity, units/kg of seeds	Antitrypsin activity, units/mg of protein	Yield, %	Degree of purification
Extraction to 0.01 N HCl		99000.0	360.0	0.0036	100	1
Protein concentration by salting out with $(\text{NH}_4)_2\text{SO}_4$		39500.0	150.1	0.0038	41.6	1.05
Chromatography on Sp-Sephadex C-25	I	2465.0	21.2	0.0086	5.6	2.4
	II	5040.9	110.9	0.022	30.8	6.1
Rechromatography on Sp-Sephadex C-25	I	465.5	13.5	0.029	3.75	8.0
	II	3300.0	85.8	0.026	23.8	7.2
Chromatography II on QAE- Sephadex A-25	Adsorbed fraction (ITR II)	405.7	44.2	0.104	12.2	28.9
	Non-adsorbed fraction (ITR III)	1666.6	13.3	0.008	3.7	2.2
Chromatography on immobilized trypsin	ITR I	3.87	7.6	1.96	2.1	544.4
	ITR II	11.0	30.9	2.8	8.6	777.7
	ITR III	4.2	9.3	2.2	2.6	611.1
Desalting on Sephadex G-15	ITR I	2.6	3.4	1.3	0.9	361.0
	ITR II	7.7	21.5	2.8	6.0	777.7
	ITR III	2.9	6.4	2.2	1.8	611.1

extracted again with two volumes of the same acid. The two extracts were pooled. About 360 antitrypsin units from 1 kg of seeds were obtained (Table 1).

Concentration of extracted proteins by salting out with ammonium sulfate. The extract was adjusted to pH 7.6 using 1 N NaOH. Precipitating sediment was removed by centrifugation, and ammonium sulfate was added to the supernatant to reach 90% of saturation. The sediment of salted out proteins was centrifugated after 20 hrs, suspended in 0.05 M acetate buffer, pH 3.6, and dissolved by dialysis to this buffer. Considerable decrease of activity accompanying this procedure was connected with partial passage of the inhibitors through semi-permeable membrane; this being possible because of their relatively low molecular weight.

Ion-exchange chromatography on Sp-Sephadex C-25. Protein solution after dialysis was applied onto a column filled with ion exchanger, equilibrated with 0.05 M acetate buffer, pH 3.6. The adsorbed proteins which contained all antitrypsin activity were eluted from the column with NaCl concentration gradient from 0 to 1 M. Three protein peaks, not well separated were obtained after chromatography. Antitrypsin activity was localized in the second and the third peak (Fig. 1). Fractions 51 to 75 (determined as peak I of activity) and 80 to 125 (determined as peak II) were pooled separately and subjected to rechromatography in the same conditions. This procedure resulted in an almost three-fold increase of specific inhibitor activity of peak I, but inhibitor activity of peak II did not change (Table 1).

Chromatography on QAE-Sephadex A-25. Proteins of peak II (after chromatography and rechromatography on Sp-Sephadex C-25) were applied on a column equilibrated with 0.05 M carbonate buffer, pH 10.5. About 75% of antitryptic activity was adsorbed on the column. The adsorbed inhibitor, denoted as ITR II, was eluted with 0.5 M NaCl. Non-adsorbed inhibitor was denoted as ITR III.

Affinity chromatography on immobilized trypsin. Proteins of peak I (Fig. 1) after rechromatography on Sp-Sephadex C-25 (denoted as ITR I), proteins which were adsorbed on QAE-Sephadex A-25 (ITR II), as well as non-adsorbed proteins (ITR III), were applied separately onto immobilized trypsin at pH 7.6, in the presence of 0.5 M NaCl. Inactive protein material was washed out with 0.05 M tris-HCl buffer, pH 7.6, containing 0.5 M NaCl. The inhibitors were released from their complex with trypsin by means of 0.01 N HCl in temp. 0 to +5°C and then lyophilized.

Desalting on Sephadex G-15. Lyophilized inhibitors were dissolved in 0.05 M solution of ammonium carbonate, desalted on a column equilibrated with the same solution, and lyophilized again.

The results of inhibitors purification are presented in Table 1. From 1 kg of seeds about 2.6 mg of the inhibitor ITR I, 7.7 mg of ITR II, and 3 mg of ITR III were obtained.

Fig. 1. Chromatography of protein extract from turnip (*Brassica rapa*) seeds on Sp-Sephadex C-25 in 0.05 M acetate buffer, pH 3.6. Protein material obtained from 1 kg of seeds was applied to 2.5×40 cm column. Proteins were eluted with NaCl concentration gradient 0 to 1 M, 15 ml fractions were collected. 1 — A_{280} , 2 — antitrypsin activity, 3 — gradient of NaCl concentration

SOME PROPERTIES OF THE INHIBITORS FROM TURNIP SEEDS

Electrophoresis on polyacrylamide gel at pH 8.3 (ITR I and ITR II), and at pH 2.4 (ITR III) showed that each inhibitor was composed of two fractions (proteins) differing slightly in the mobility (Fig. 2). Each of these fractions, when eluted from the gel, inhibited trypsin activity. Incubation of ITR II and ITR III with catalytic amount of trypsin in slightly acidic condition, resulted in the increase of fraction of higher mobility. Most probably, fraction of higher mobility represented a modified form of the inhibitor, with broken by trypsin peptide bond in reactive site. In case of ITR I no electrophoretic changes were observed after incubation with trypsin in acidic conditions (Fig. 2). This inhibitor contained high percentage of the fraction of higher mobility even before the incubation with trypsin. Figure 3 presents inhibition of trypsin activity by the isolated inhibitors. One microgram of inhibitors ITR I, ITR II, and ITR III inhibited 1.3, 2.8 and 2.2 micrograms of trypsin, respectively. ITR II and ITR III inhibited also chymotrypsin A_x activity (Fig. 4).

Fig. 2. Scheme of electrophoresis of the inhibitors from turnip seeds on polyacrylamide gel, pH 8.3 (ITR I and ITR II) and 2.4 (ITR III), before (a) and after (b) 48 h incubation with a catalytic amount of trypsin, at pH 3.2. 1 — Fraction of lower mobility (probably virgin form), 2 — fraction of higher mobility (probably form with broken peptide bond in the reactive site)

Fig. 3. Inhibition of trypsin activity by the inhibitors from turnip seeds. Trypsin activity determined according to Erlanger et al. (1961) with BAPNA as the substrate

Fig. 4. Inhibition of chymotrypsin A_2 activity by the inhibitors from turnip seeds. Activity determined according to Kunitz (1947) with casein as the substrate

Table 2

Physico-chemical and biological properties of the inhibitors from turnip seeds

Inhibitor	pI	Molecular weight	Approximate $A_{280}^{1\text{cm}}$ value for 1% solution	Inhibited enzyme		Amino acid in pos. P_1 of the reactive site
				trypsin	chymotrypsin	
ITR I	6.4	15 900	5.6	+	—	arginine
ITR II	7.5	10 000	11.2	+	+	lysine
ITR III	10.0	9 100	7.2	+	+	arginine

The highest molecular weight (about 16 kDa) and the lowest pI (6.4) was noted for the inhibitor ITR I (Table 2). The other two inhibitors had molecular weight of about 10 kDa. ITR III was very alkaline, with pI over 10 (hence, it did not penetrate the gel during electrophoresis at pH 8.3 according to Davis (1964)).

Inhibitors ITR I and ITR III lost 81.3 and 91% of the activity respectively under the effect of cyclohexanedione, a reagent modifying guanidine residues of arginine. Hence, the two inhibitors possessed arginine residue in the reactive site. Inhibitor ITR II proved to be resistant to this reagent but it lost 90% of activity after modification of free amino groups with acetic acid anhydride. Hence, reactive site of this inhibitor contained lysine residue.

DISCUSSION

Ogawa et al. (1971b) studied seeds of various species from the genus *Brassica* in order to determine content of serine proteinase inhibitors. Separation of fresh extracts on molecular sieves resulted in two groups of the inhibitors, of molecular weights about 10 and 20 kDa. Electrofocusing revealed that each group contained two or three inhibitors, differing in their isoelectric points and the inhibition spectrum. The inhibitors were not isolated in pure form, so their physico-chemical properties were not determined.

This paper presents a method of isolating three trypsin inhibitors from turnip seeds (*Brassica rapa*). The inhibitors differ as to the biological and physico-chemical properties.

Each of the isolated inhibitors was heterogenous in electrophoresis on polyacrylamide gel. Most probably, their heterogeneity resulted from the fact that immobilized trypsin was used for the isolation. When the inhibitors were released from complex with trypsin in acidic conditions, the enzyme hydrolysed peptide bond of the reactive site in some inhibitor molecules. Inhibitor ITR I seemed to be most susceptible to trypsin because the modified form predominated in this inhibitor preparation over the virgin form, and additional incubation with trypsin did not increase content of the modified fraction.

Presence of two forms (virgin and modified) in the inhibitors isolated in course of chromatography on immobilized trypsin is very common. This type of chromatography was used to isolate inhibitors from *Brassica oleracea* var. *sabellica* seeds (Wilimowska-Pelc 1985) and from squash seeds (Polanowski et al. 1987) and in each case two forms of the inhibitors were obtained.

Antitrypsin activity of the extracts from turnip seeds is about 5 times lower than in case of *Brassica oleracea* var. *sabellica* seeds. Inhibitor isolated from this plant had molecular weight 8.1 kDa and its properties were similar to ITR III. Also inhibitor from mustard seeds (*Sinapis alba*) had high isoelectric point, similar to ITR III, and possessed arginine in the reactive site. Menegatti et al. (1985) isolated one trypsin inhibitor from the seeds of this plant. However, studies carried out in our Institute suggest that mustard seeds contained at least three inhibitors of this enzyme (unpubl. data).

Radish seeds, similarly as turnip seeds, contain three trypsin inhibitors (Ogawa et al. 1968, 1971a). Two of them have been characterized. Inhibitor R I, of molecular weight 7.2 kDa, is composed of 67 amino acids and possesses arginine residue at position P₁ of the reactive site. Inhibitor R III, of molecular weight 11 kDa, is composed of 95 amino acids and possesses lysine residue in the reactive site. Inhibitor R III, similarly as ITR II and ITR III from turnip seeds, inhibits also chymotrypsin activity.

Results of these studies as well as data from the literature suggest that seeds of plants from the family *Cruciferae* possessed inhibitors characterized by considerable variety and multitude of particular forms.

Common occurrence of proteinase inhibitors in plants and animals results in the fact that many authors become interested in their physiological functions. However, in order to study function of the inhibitors, they must first be isolated and characterized.

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REFERENCES

- Chase T., Shaw E., 1970. Titration of trypsin, plasmin and thrombin with p-nitrophenyl p-guanidinobenzoate HCl. In: Methods in enzymology. Perlman G. E., Lorand L. (eds.). Acad. Press, New York-London. Vol. 19, pp. 20-27.
- Davis B. J., 1964. Disc electrophoresis II. Method and application to human serum proteins. Ann N. Y. Acad. Sci. USA 121: 404-427.
- Erlanger B. F., Kokowsky N., Cohen W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271-278.
- Frankel-Conrat H., 1957. Methods for investigating the essential groups for enzyme activity. In: Methods in enzymology. Colowick S. P., Kaplan N. O. (eds.). Acad. Press, New York. Vol. 4, pp. 247-269.

- Goa J., 1953. A microbiuret method for protein determination. Determination of total protein in cerebrospinal fluid. *Scand. J. Clin. Lab. Investig.* 5: 218-222.
- Karlsson Ch., Davis H., Ohman J., Anderson U., 1973. LKB 2117 Multiphor I. Analytical thin layer gel electrophoresing in polyacrylamide gel. Application Note LKB 75.
- Kassel B., Marcinišzyn B. M., 1971. A simple method of purification of basic trypsin inhibitor of bovine organs. In: *Proceedings of the International Research Conference on Proteinase Inhibitors*. Munich, Nov. 1970. Fritz H., Tschesche H. (eds.). Berlin-New York. pp. 43-46.
- Kunitz M., 1947. Crystalline soybean trypsin inhibitor II. General properties. *J. Gen. Physiol.* 30: 291-310.
- Menegatti E., Palmieri S., Walde P., Luisi P. L., 1985. Isolation and characterization of a trypsin inhibitor from white mustard (*Sinapis alba* L.). *J. Agr. Food Chem.* 33: 784-789.
- Ogawa T., Higasa T., Hata T., 1968. Proteinase inhibitors in plant seeds. Part II. Purification and some properties of three different types of inhibitors from Japanese radish seed (*Raphanus sativus*). *Agr. Biol. Chem.* 32: 484-491.
- Ogawa T., Higasa T., Hata T., 1971a. Proteinase inhibitors in plant seeds. Part III. Characterization of two different types of inhibitors obtained from Japanese radish seed (*Raphanus sativus*). *Agr. Biol. Chem.* 35: 712-716.
- Ogawa T., Higasa T., Hata T., 1971b. Proteinase inhibitors in plant seeds. Part V. Inhibitors in rape seeds. *Mem. Research Inst. Food Sci. Kyoto University* 32: 1-6.
- Panyim S., Chalkley R., 1969. High resolution acrylamide gel electrophoresis of histons. *Arch. Biochem. Biophys.* 130: 337-346.
- Pathy L., Smith E. L., 1975. Identification of functional arginine residues in ribonuclease A and lysosome. *J. Biol. Chem.* 250: 565-569.
- Polanowski A., Cieślak E., Otlewski J., Nienartowicz B., Wilimowska-Pelc A., Wilusz T., 1987. Protein inhibitors from the seeds of *Cucurbitaceae* plants. *Acta Biochem. Polon.* 34: 395-406.
- Richardson M., 1977. The proteinase inhibitors of plant and microorganism. *Phytochemistry* 16: 159-169.
- Weber K., Osborn M., 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244: 4406-4412.
- Wilimowska-Pelc A., 1985. Isolation and partial characterization of the trypsin inhibitor from the seeds of *Brassica oleracea* var. *sabellica*. *Acta Biochim. Polon.* 32: 351-361.
- Wilimowska-Pelc A., Malicka-Błaszczewicz M., Mejbaum-Katzenellenbogen W., 1973. Ribonuclease and polypeptide trypsin inhibitors from bovine and porcine pancreas. *Acta Biochim. Polon.* 20: 355-363.
- Wilimowska-Pelc A., Mejbaum-Katzenellenbogen W., 1978. A simple method for isolating trypsin from trichloroacetic acid extracts of bovine pancreas. *Anal. Biochem.* 90: 816-820.

Inhibitory trypsyny z nasion rzepy (Brassica rapa L.)

Streszczenie

W pracy opisano metodę izolacji inhibitorów trypsyny z nasion rzepy. Inhibitory ekstrahowano 0,01 N HCl, zagęszczano przez wysalanie siarczanem amonu, a następnie oczyszczano przez chromatografię jonowymienną na Sp-Sephadex C-25, QAE-Sephadex A-25 i chromatografię powinowactwa na immobilizowanej trypsynie. Spośród trzech wyizolowanych inhibitorów jedynie ITR I o masie 15,9 kDa, pI 6,4 hamuje tylko aktywność trypsyny. Inhibitory ITR II i ITR III

hamują ponadto aktywność chymotrypsyny, mają zbliżoną masę cząsteczkową (około 10 kDa), a pI wynoszą odpowiednio 7,5 i ponad 10.

W pozycji P_1 centrum aktywnego inhibitorów ITR I i ITR III znajduje się reszta argininy, a w przypadku inhibitora ITR II reszta lizyny. W elektroforezie w żelu poliakryloamidowym preparat każdego z inhibitorów wykazuje obecność dwóch frakcji białkowych stanowiących najprawdopodobniej formy natywną (virgin form) i zmodyfikowaną (modified form) z rozerwanym przez trypsynę wiązaniem peptydowym centrum reaktywnego.