DISTRIBUTION OF PROTEASE INHIBITORY ACTIVITY IN SOME RAPESEED GENOTYPES AND OTHER CULTIVATED CRUCIFERS

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INTRODUCTION

In recent years plant families have been characterized not only by phenotypic traits but also by the presence and different contents of specific compounds. Among these are proteinase inhibitors (PI), the most studied being inhibitors of serine proteases, viz. trypsin and chymotrypsin. These two enzymes are present in animals and microorganisms, but not in plants. Of the roles hypothesized for PI, the most important seems to be protection against herbivorous insects and plant pathogens via inhibition of their proteolytic enzymes (Green and Ryan, 1972; Peng and Black, 1976; Broadway and Duffey, 1986; Gatehouse et al. 1985).

The molecular properties of most plant PI have been well described by Ryan (1981) and Richardson (1981). Generally these compounds appears to be rather similar, with a single polypeptide chain of low molecular weight containing a high percentage of S-S cross-links. This property affords stability against thermal and acid-base denaturation.

PI in the Cruciferae have never been studied in as much detail as in other families, particularly Leguminosae. Nevertheless, several authors have described the presence of some PI activities in this plant family and discussed it from different points of view, emphasizing the importance of these compounds in insect-attack protection, human and animal nutrition, and plant metabolism (Ogawa et al. 1971; Chen and Mitchell 1973; Broadway, 1989; Broadway et al. 1990).

This study is a continuation of our previous work in this field aimed at the isolation and characterization of a trypsin inhibitor from sound seeds of Sinapis alba (Menegatti et al. 1985). We report the distribution of trypsin inhibitory activity (TIA) in some cruciferous seeds and discuss its relationship with some genotypic characters.

MATERIALS AND METHODS

Chemicals. Bovine ß-trypsin (TRL 3X crystallized, salt free) and bovine α-chymotrypsin were supplied by Worthington Chem. Co. (NJ, USA); Bovine serum albumin, chymotrypsinogen, ribonuclease A and polybuffer 96 were obtained from Pharmacia (Sweden). The Kunitz and Bowman-Birk trypsin inhibitor from soybean, BApNA and BTpNA were supplied by Sigma Chem. Co. (MO, USA). The other reagents were of analytical grade.

Preparation of the crude extract. The seeds used are described in Table 1. The seeds (30 g) of each cultivar or species were defatted by grinding in n-hexane (1:10 w/v) twice. The defatted meals were dried under vacuum and stored. Each defatted meal (5 g) was homogenized in 250 ml 50 mM phosphate buffer (pH 7.0) in an ice bath in an Ultraturrax mod. TP18/2N homogenizer at medium speed for ca. 20 min. After centrifugation the limpid extract was lyophilized.

Isolation of protease inhibitors. A suitable amount of lyophilized powder of each sample, corresponding about to 0.3 units of inhibitor (UI), was dissolved in 1 ml distilled water and filtered through a 0.22 µm Millex-GV (Millipore) filter. A 200 µl sample was applied on a Superose 12 HR 10/30 gel filtration column connected to a FPLC system (Pharmacia) and eluted with a 0.2 ml/min flow of 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The chromatofocusing trials were also carried out with the FPLC system using a Mono P HR 5/20 column. The samples were dissolved in the starting buffer, 25 mM ethanolamine-acetic acid (pH 9.4) and eluted at 0.7 ml/min with polybuffer 96 titerd to pH 6 with acetic acid. The pH was monitored continuously by a special glass electrode in the FPLC elution line. In both cases 1 ml fractions were collected and the TIA of each fraction was assayed.

Thermal denaturation. An aliquot of the sample (2 ml for crude extracts or 500 µl for purified fractions) was poured into a screw cap test tube and heated in a boiling water bath for 3 min. After cooling and centrifugation (for the crude extracts only) the samples were assayed for TIA.

Inhibition assay. To determine the TIA we measured the inhibition of tryptic hydrolysis in 0.2 M triethanolamine buffer (pH 7.8) at 25 °C using BApNA as substrate by following the change of the absorbance at 405 nm (Kassel, 1970). The activity of bovine α-chymotrypsin was measured essentially according the method of Di Pietro and Liener (1989). One unit of TIA (UI) was defined as the amount inhibiting 1 mg trypsin.

RESULTS AND DISCUSSION

TIA in cruciferous seed extracts. For this study we chose six commercial rapeseed varieties, which included "double high", "0" and "00" genotypes, plus two other cultivated cruciferous

Table 1. Description of the crucifer samples.

Specie	Cultivar	Genotype	Source	Country
Brassica napus	Matador	"double high"	SIS Foraggera	Italy
	Bienvenue	"0"	SIS Foraggera	Italy
	Jet Neuf	"0"	SIS Foraggera	Italy
	Anima	"00"	Semundo	Germany
	Tapidor	"00"	Ringot	France
	Drakkar	"00"	Ringot	France
S. alba	Emergo	====	SIS Foraggera	Italy
B.carinata	Dodolla	====	University of Pisa	Italy

seeds, Sinapis alba and Brassica carinata (Table 1). Table 2 shows the TIA of crude seed extracts before and after boiling for 3 min. The table also reports the soluble protein content and the percent decrease of TIA after heat treatment. The TIA found for the chosen samples fall into roughly four different behavior groups, both for inhibitory activity and, to a lesser extent, soluble protein content. In fact, the Matador ("double high"), Bienvenue ("0"), Jet Neuf("0") rapeseed cultivars can be put into one group and all the "00" genotypes into another. Sinapis alba and Brassica carinata show two other specific behaviors. On the average, the TIA for the four

groups are 3.9, 6.9, 0.9 and 2.4 UI/g, respectively, while the soluble protein contents are 2.2, 2.7, 2.0 and 3.1 mg/ml. The data for the samples within each group are quite similar, although the cv. Anima stands out as the genotype with the highest TIA. This grouping remains about the same even after heat treatment of the extracts. In fact, the residual inhibitory activity showed a percent decrease of ca. 80% for the first group, 45% for the second, 56% for Sinapis alba, and 42% for Brassica carinata.

Table 2. TIA (UI/g defatted meal) of crude seed extract before and after heat treatment (h.t.) (100°C for 3 min).

Rapeseed Cultivar or crucifer species	Before h.t.		After h.t.		TIA decrease
	TIA (UI/g)	Sol. Prot. (mg/ml)	TIA (UI/g)	Sol. Prot. (mg/ml)	(%)
Matador	4.0	2.2	0.9	0.5	78
Bienvenue	3.7	2.2	1.0	0.4	73
Jet Neuf	4.1	2.2	0.7	0.5	83
Anima	9.6	2.8	5.7	0.5	41
Tapidor	5.4	2.7	3.0	0.5	44
Drakkar	5.7	2.7	3.0	0.6	47
Sinapis alba	0.9	2.0	0.4	0.5	56
B. carinata	2.4	3.1	1.4	0.7	42

Distribution of the inhibitors by chromatofocusing technique. The chromatofocusing profiles of the crude rapeseed extracts are very similar, showing a main TIA peak generally collected in the sixth or seventh fraction. This inhibitor was sometimes preceded by another small peak (patterns not shown). By chromatofocusing we observed that the pIs fall in the alkaline region, from 9 to 9.5. Fig. 1 shows the chromatofocusing profile of a crude extract of Sinapis alba, in which the first two peaks presumably correspond to those observed in the rapeseed extracts. In fact, both inhibitors found in these two species have similar pIs and resistance against temperature, although with an inverted percentage of TIA distribution: the first peak isolated in these two species is thermolabile, unlike the second, which maintains a large part of its inhibitory activity even after a treatment of 100 °C for 3 min. The third small peak, with a pI nearly to 8.0, was sometimes present with even smaller intensity in the rapeseed extracts.

Identification of the inhibitor family by gel filtration. In addition to chromatofocusing, we used gel filtration chromatography to determine the distribution of TIA in crude seed extracts and, where possible, to identify the inhibitor types, e.g. Kunitz and Bowman-Birk families, on the basis of molecular weight (MW). All crude rapeseed extracts showed very similar gel filtration patterns for TIA. The typical profile showed three well-separated TIA peaks (Fig. 2), which were collected, with good reproducibility, in fractions 16, 18 and 21. The main peak can be ascribed to an inhibitor (III) of low molecular weight and generally characterized by a considerable resistance towards heat denaturation, mainly for the "00" genotypes. In addition, this inhibitor ranged from 55 to 80% of the total TIA of the crude rapeseed extracts. The other two inhibitors, which have apparent MWs of ca. 11000 (I) and

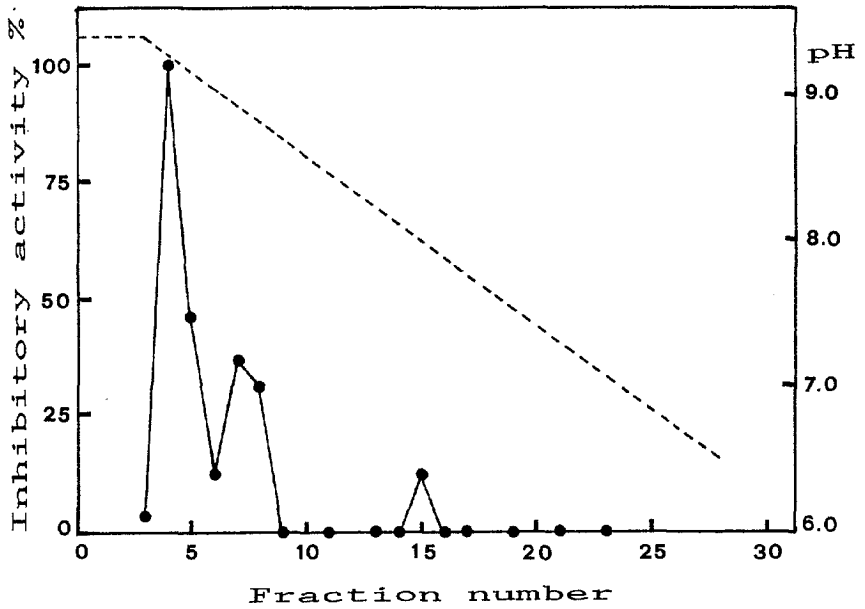

Fig. 1. Chromatofocusing separation of Sinapis alba trypsin inhibitory activity in the pH 9-6 range.

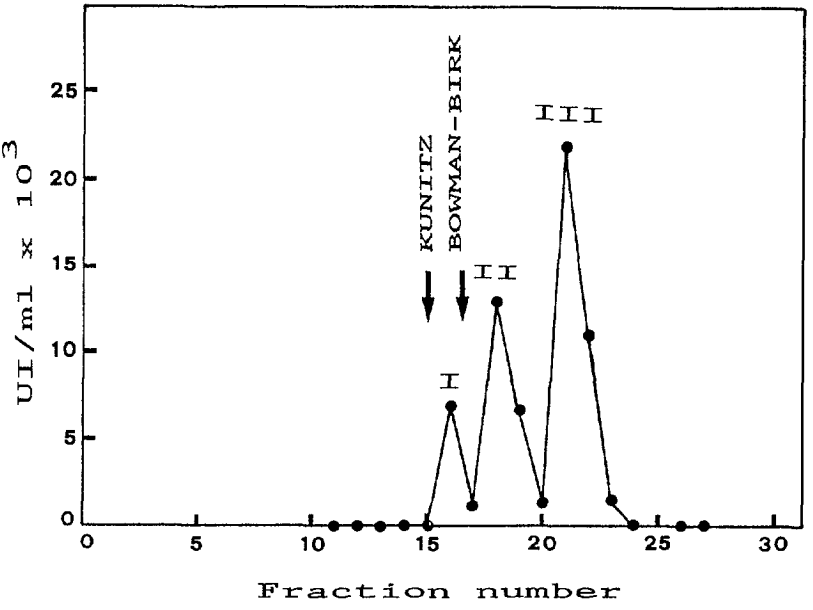

Fig. 2. Gel filtration chromatography of rapeseed (cv. Bienvenue) trypsin inhibitory activity on a Superose 12 HR 10/30 column-FPLC system. The Kunitz and Bowman-Birk inhibitors from soybean were chromatographed in a previous experiment.

5000 (II) dalton, were easily denatured by increasing the temperature. Strong denaturation towards the first inhibitor was observed even at low temperatures. The chromatographic patterns for crude seed extracts of Sinapis alba and Brassica carinata were quite different. The former (not shown) revealed the presence of at least three inhibitors, as in the rapeseed extracts, although with some MW differences. In addition, the pattern showed a different TIA percentage distribution for the three peaks. In Sinapis, they are completely inverted compared to rapeseed. Some molecular properties of the two major inhibitors in Sinapis have been reported by Menegatti et al. (1985; 1990). Although a complete study of rapeseed inhibitors is still in progress in our laboratory, preliminary findings indicate that these proteins have no measurable activity of chymotrypsin inhibition.

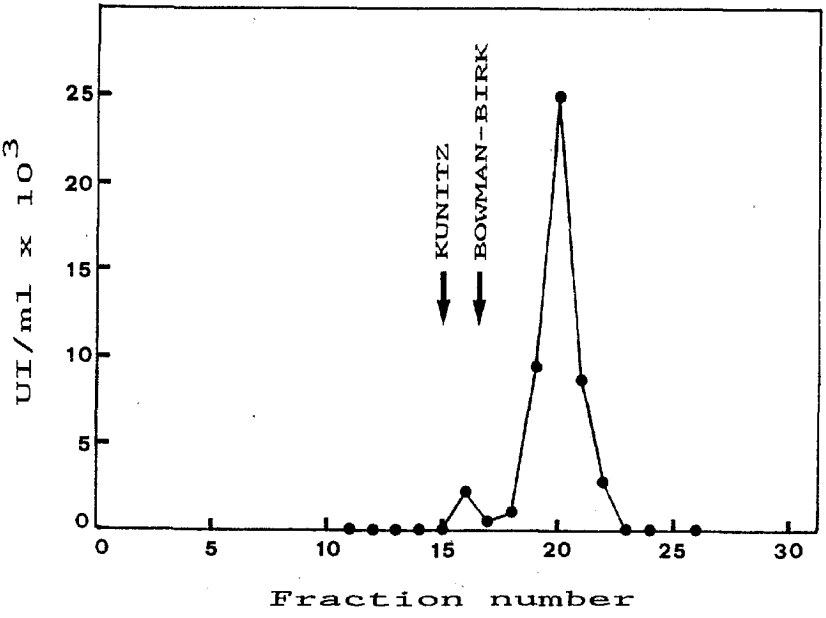

Fig. 3. Gel filtration chromatography of Brassica carinata trypsin inhibitory activity on a Superose 12 HR 10/30 column-FPLC system. The Kunitz and Bowman-Birk inhibitors from soybean were chromatographed in a previous experiment.

Fig. 3 shows the chromatographic profile of Brassica carinata, in which it is apparent that this species contains almost exclusively one specific inhibitor with a particular low molecular weight (fraction n. 20). This specific inhibitor, in addition to having a pI similar to the most important rapeseed and Sinapis PI, also appears to be rather thermoresistant. As to the possibility of classifying all these inhibitors in the Kunitz and Bowman-Birk families, it seems important to emphasize that their molecular weights are generally underestimated (see Fig. 2 and 3). In fact, Norioka et al. (1988) reported that high basic inhibitors elute in gel filtration chromatography much later than soybean inhibitors (pI ≈ 4) because of an interaction between the basic protein and the gel.

In addition, if one considers the similar chromatographic results for rapeseed and Sinapis extracts and the fact that the main inhibitors isolated from white mustard seeds have MWs, as determined by analytical centrifuging and aminoacid composition, of ca. 20000 (Menegatti et al. 1985) and ca. 7000 dalton (Menegatti, 1990), and are thermolabile and thermoresistant respectively, it is reasonable to suppose that the inhibitors found in rapeseed and Brassica carinata are also basic inhibitors of the Kunitz and Bowman-Birk types. The main rapeseed peak (III), while of lower MW than Bowman-Birk (Fig. 2), can be ascribed to this family on the basis of its resistance to thermal denaturation.

CONCLUSIONS

TIA of two types of inhibitors, the Kunitz and Bowman-Birk families, were found and isolated from all crude seed extracts of the crucifers considered. There seemed to be characteristic patterns of highly basic inhibitors, which are able to differentiate the species and genotypes. If one considers the hypothesis of Norioka et al.(1988) regarding the relationship between the different types of TIA and evolution, Sinapis alba would be the most primitive crucifer because it contains mainly the Kunitz type inhibitor. In rapeseed and Brassica carinata the Bowman-Birk type prevails. Among the rapeseed genotypes the "00" are characterized by a preponderance of the thermoresistant inhibitor, which presumably belongs to the Bowman-Birk family. In this case, too, it seems that in more advanced genotypes the Bowman-Birk type inhibitors gradually tend to replace the Kunitz type.

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