RAPESEED PROTEINASE INHIBITORS

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INTRODUCTION

Proteinase inhibitors are widely distributed in the plant kingdom but only those of the Leguminosae, Graminae and Solanaceae have been investigated intensively because of their nutritional significance and economic importance (Liener and Kakade, 1981). Many proteinase inhibitors are concentrated in the seed but these antinutritional factors can be found in leaves, tubers, corms, bulbs and fruits.

Information on the proteinase inhibitors of the Cruciferae is limited. Trypsin inhibitor activity has been found in the edible parts of broccoli, (*Brassica oleracea var. italica*), Brussels sprouts, (*B. oleracea var. gemmifera*), white and red radish (*Raphinus sativus*) and cauliflower (*B. oleracea var. botrytis*) (Chen and Mitchell, 1973). Two low molecular weight inhibitors (8 and 12 kd) have been isolated from the seed of Japanese radish and characterised (Ogawa *et al*, 1971).

Since rapeseed meal is widely incorporated into a range of animal feedstuffs, investigations of rapeseed and other species of crucifers were initiated to quantitate and characterise the PI therein. Full details of this work will be presented elsewhere.

MATERIALS AND METHODS

Various cultivars of winter rapeseed (*B.napus ssp. oleifera*) were obtained from commercial crops grown on the University of Nottingham Farms. Other crucifer seeds were purchased from local seedsmen. Seeds were ground and defatted by extracting three times with petroleum ether at room temperature. PI were extracted from the defatted meal with 0.05 M phosphate buffer pH 7.0 and purified by affinity chromatography on Trypsin-Sepharose-4B. Individual iso-inhibitors were isolated by chromatography on CM-52 Cellulose. Molecular weights (mol.wt) were determined by gel filtration (Sephadex G-50) and dissociating polyacrylamide gel electrophoresis (SDS-PAGE). pI were determined by isoelectric focussing on polyacrylamide gels. Proteinase and proteinase inhibitor activities were assayed as follows: Trypsin, chymotrypsin and pronase (Kunitz, 1947); Carboxypeptidase A (Folk and Schirme, 1963); Leucine aminopeptidase (Appel, 1974); Pepsin (Anson and Mirsky, 1932). Amino acid composition was determined on acid hydrolysates of the PI. Cysteine was determined as cysteic acid following performic acid oxidation. Stability studies were performed with the pure inhibitor only according to the conditions listed in the results.

RESULTS

Rapeseed contains very low PI levels. Average values of the specific antitryptic activity obtained from the affinity columns amounted to 10 Trypsin Inhibitor units (TIU) per gram defatted meal. [Kunitz (1947) defined the TIU as the amount of inhibitor that reduces the activity of trypsin by one unit. A tryptic unit (TU) is defined as the activity using casein as substrate, that gives rise to an increase

of one unit of optical density at 280 nm (1 cm light path at 37°C) in 10 minutes].

Affinity purified PI from rapeseed contained two isoinhibitors as revealed by inhibitor staining on non-dissociating PAGE. The major component designated Rapeseed Proteinase Inhibitor-1 (RPI-1), was obtained in a pure state by chromatography on CM-52 cellulose. The minor rapeseed inhibitor was not investigated further.

RPI-1 has a mol. wt. of around 7 kd as determined by SDS-PAGE and filtration on Sephadex-G 50 (Table 1). The RPI-1 molecule contains 64 amino acids with glycine (11), proline (8), glutamate (7), half-cystine (6) and aspartate (5) predominating.

Stability studies with respect to trypsin inhibitor activity revealed that RPI-1 was completely stable over the pH range 2-10 at 37°C. At 70°C the inhibitor was stable within the pH range 2-8 but rapidly lost activity at pH 10 (75% in 60 min). RPI-1 was stable (45 min) at 100°C at pH 2-8. Rapid inactivation occurred at this temperature above pH 8.0. At higher temperatures (123°C), RPI-1 was completely inactivated above pH 9.0 within 45 min. The inhibitor was more stable at acid pH at this temperature with around 30% activity remaining after heating 45 min at pH 2.0. At pH 8.0 only 15% inhibitory activity remained with the same heating regime.

A range of different classes of proteinases were tested for sensitivity towards RPI-1. The inhibitor was inactive towards pepsin (Table 2) but inhibited trypsin, chymotrypsin and pronase. Both leucine aminopeptidase and carboxypeptidase A were inhibited strongly. Inhibitor-proteinase complexes were examined for residual inhibitory activity. All the RPI-1-enzyme complexes retained almost all the activity against the proteinases except those involved in the complex.

As with other PI, RPI-1 was a competitive inhibitor of trypsin using the synthetic peptide TAME as substrate. Other cruciferous seeds including cabbage (*B. oleracea var. capitata*) and kale (*B. oleracea var. acephala*) were found to contain similar isoinhibitors (purified by affinity chromatography) at equivalent concentrations on non-dissociating PAGE (enzyme stained).

DISCUSSION

Rapeseed contains PI that can be isolated in a highly purified form by affinity chromatography on Trypsin-Sepharose 4B. The inhibitors are present at extremely low concentrations (10 $TIU.g^{-1}$ defatted meal) compared with certain legume seeds such as soyabean (*Glycine max*) with 2×10^3 $TIU.g^{-1}$ defatted meal and kidney bean (*Phaseolus vulgaris*) 6.5×10^2 $TIU.g^{-1}$ meal. PI at such low concentrations clearly do not pose any nutrition problems.

Two isoinhibitors have been identified by PAGE of the affinity chromatography purified inhibitors and the major (RPI-1) component was obtained pure following chromatography on CM-52 cellulose.

RPI-1 exhibited unusual specifity characteristics. It was found to inhibit the serine proteinases trypsin, chymotrypsin and pronase albeit the last two enzymes less effectively than trypsin. The metalloproteinases leucine aminopeptidase and carboxypeptidase were also inhibited strongly by RPI-1. Pepsin was insensitive to the inhibitor. Such a broad specificity against different classes of proteinases has not been observed previously. Thus it would appear that RPI-1 is unique amongst plant PI.

Proteinase-RPI-1 (E-I complexes) generally retained inhibitory activity against proteinases apart from the enzyme involved in the complex. An exception to this was

the chymotrypsin-inhibitor complex which inhibited trypsin weakly. The reactive site for chymotrypsin on the inhibitor is probably close to that for trypsin with which it interferes in the chymotrypsin-inhibitor complex. Notwithstanding this observation it is concluded that RPI-1 probably possesses different reactive sites for each of the serine proteinases inhibited.

The two metalloproteinase-inhibitor complexes retained inhibitory activity against the metalloproteinase not involved in the complex, chymotrypsin and pronase but not trypsin. At this stage no explanation can be offered for this loss of activity.

RPI-1 was found to be a small molecule (mol.wt. approximately 7 kd) containing 64 amino acids. The amino acid composition of the inhibitor was different from those of other classes and families of PI.

By virtue of the fact that RPI-1 inhibits two classes of proteinase, has a mol. wt. of 7 kd and possesses an unusual amino acid composition precludes its inclusion in any of the existing four classes of inhibitor or any of the six families of serine proteinase inhibitors.

Similar PI have been isolated from other crucifers by means of affinity chromatography but these have not been characterised fully at this time. Japanese radish seeds were found to contain three trypsin inhibitors, two of which had mol. wt. of 8 and 12 kd (Ogawa *et al*, 1971). At this stage it is not known whether the radish and rapeseed PI are similar.

CONCLUSIONS

Rapeseed has been shown to contain an unusual PI with activity against both serine- and metallo-proteinases. RPI-1 cannot be classified into any of the six families of plant serine proteinase inhibitor because it does not belong solely to this inhibitor class and its mol. wt. and amino acid composition are incompatible with other members of this class.

REFERENCES

ANSON, M.L. and MIRSKY, A.E. 1932. The estimation of pepsin with haemoglobin. J. Gen Phys., 16: 59-63.

APPEL, W. 1974. In Methods of Enzymatic Analysis. H.U. Bergmeyer, (ed), pp 958-963, Verlag Chemie. Wienheim.

CHEN, I. and MITCHELL, H.L. 1973. Trypsin inhibitors in plants. Phytochemistry, 12: 327-330.

FOLK, J.E. and SCHIRME, E.W. 1963. The porcine pancreatic carboxypeptidase A system. 1. Three forms of the active enzyme. J. Biol. Chem., 238: 3884-3894.

KUNITZ, M. 1947. Crystalline soybean inhibitor. II. General Properties. J. Gen. Physiol., 30: 291-310.

LIENER, I.E. and KAKADE, M.L. 1980. Proteinase inhibitors. In Toxic constituents of Plant Foodstuffs. I.E. Liener (ed), pp 7-71, Academic Press, New York.

ODANI, S. and IKENAKA, T. 1978. Studies on soybean trypsin inhibitors. XIII. Preparation and characterisation of active fragments from Bowman-Birk proteinase inhibitors. J. Biochem., 83: 747-753.

OGAWA, T., HIGASA, T. and HATA, T. 1971. Proteinase inhibitors in plant seeds. V. Inhibitors in rape seeds. Mem. Res. Inst. Food Sci. Kyoto Univ., 32: 1-6.

PEARCE, G., SY. L., RUSSELL, C., RYAN, C.A. and HASS, G.M. 1982. Isolation and characterisation from potato tubers of two polypeptide inhibitors of serine proteinases. Arch. Biochem. Biophys., 213: 456-462.

RYAN, C.A., HASS, G.M., KUHN, R.W. 1974. Purification and properties of a carboxypeptidase inhibitor from potatoes. J. Biol. Chem., 249: 5495-5499.

Table 1. Amino acid composition[1] and physical of RPI-1 and other PI

	Soyabean[2] BBI	Potato[3] CPI	Potato[4] PTI	RPI-1
Residues				
Aspartic acid	11	5	7	5
Threonine	2	2	2	0
Serine	3	2	3	3
Glutamic acid	7	2-3	2	7
Proline	6	3	3	8
Glycine	-	3	4	11
Alanine	4	4	4	3
Half-Cystine	14	6	8	6
Valine	1	1	1	3
Isoleucine	2	1	2-3	2
Leucine	2	-	1	3
Tyrosine	2	1	3-4	1
Phenylalanine	2	1	1	4
Lysine	5	2	0	4
Histidine	1	2	0	1
Arginine	2	1	3	4
Tryptophan	-	1-2	0	-
Total residues	71	37-39	42-44	64
Mol. Wt. (kd)	8	3.1	5.3	7.1
pI	-	-	-	6.8

(1) Residues per molecule

(2) Odani, S. and Ikenaka, T. (1978)

(3) Ryan, C.A., Hass, G.M. and Kuhn, R.W. (1974)

(4) Pearce, G., Sy, L., Russell, G., Ryan, C.A. and Hass, G.M. (1982)

Table 2. Specificity of RPI-1

Enzyme	% Inhibition relative to trypsin
Trypsin	100%
Chymotrypsin	58%
Pronase	36%
Leucine aminopeptidase	150%
Carboxypeptidase A	66%
Pepsin	0