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TRYPSIN ISOINHIBITORS OF LUCERNE: ASSOCIATION WITH LEAF FRACTION 1 PROTEIN

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Abstract—Trypsin inhibitory activity occurring in the vegetative portion of lucerne (*Medicago sativa* L.) comprised two heat-labile isoinhibitors with isoelectric points at pH 9 and 9.5. During the fractionation of soluble leaf proteins, both inhibitors associated specifically with Fraction 1 protein, and were freed from combination with this protein by acid agglutination. Inhibitor preparations showed significant activity against the acid proteinase pepsin.

INTRODUCTION

Proteinase inhibitors are widely distributed in plants but their property of competitive inhibition of proteolytic enzymes through the formation of reversible stoichiometric protein–protein complexes has not been positively related to a physiological function. They are in general relatively small monomeric or polymeric proteins (MW 4000 to 80 000) and typically have one or more disulphide linkages and a high degree of stability to heat and proteolytic attack Inhibitors with specificity for the serine proteinases trypsin and chymotrypsin are most common but others have been described with specificities encompassing other classes of endopeptidases as well as other types of serine proteinase [1–3]

Proteinase inhibitors have been found in the seed extracts of several members of the family Leguminosae [4, 5], but only lucerne contained significant inhibitory activity in the leaves [6]. This latter activity has been extracted from the vegetative portion of lucerne, and some of its properties have been elucidated [7–9].

We describe here trypsin inhibitory activity which originates in the vegetative parts of lucerne, associates specifically with Fraction 1 protein (ribulose-1,5-bisphosphate carboxylase, EC 4.1.1.39) during the extraction of soluble leaf proteins and has different properties to the inhibitors previously described The possible effects of interactions between Fraction 1 protein and trypsin inhibitors on the use of lucerne as a fodder crop for ruminants are discussed.

RESULTS

Ten samples of leaf Fraction 1 protein which had all been prepared by a similar method [10] were not equally susceptible to digestion by trypsin, and TCA-soluble products accumulating during 4 hr incubation at 39° varied from 0 3 to 2.9 mg/ml (mean 1.0 mg/ml). After heat denaturation at 100° for 20 min, samples of each of these preparations were equally susceptible to tryptic digestion, liberating 2.0–2 3 mg/ml (mean 2.1 mg/ml) TCA-soluble products, under the same conditions Two samples representing Fraction 1 protein most susceptible (F1-A) and that least susceptible (F1-B) to trypsin digestion were selected for further study F1-A appeared to be devoid of inhibitory activity since heat denaturation did not significantly alter its susceptibility to trypsin, as it did with F1-B.

Table 1 shows typical results obtained when the two protein samples were incubated with trypsin after various treatments Susceptibility of F1-A to proteolysis was unaffected by heat- or pH-induced denaturation, whereas F1-B became more readily degradable after these treatments. The acid-soluble fraction (ASF) which was separated from acid-agglutinated F1-B contained a component which, when added back to incubations already containing either heat-denatured F1-B, F1-A or acid-agglutinated F1-A, resulted in almost total inhibition of tryptic activity. This inhibitory component was completely inactivated by heating at 100° for 20 min, but could not be separated from Fraction 1 protein in F1-B and other preparations of the leaf protein by gel filtration on Sephadex G-50 and G-200 or on Sephacryl S-300

Proteolysis of casein by trypsin was also significantly inhibited by the presence of the soluble component released from acid-agglutinated F1-B (Table 2). Inhibitory activity was partially inactivated by heating at 100° for 5 min, but 20 min at this temperature was necessary to completely eliminate anti-trypsin activity.

Enzyme specificity of the inhibitory activity associated with leaf Fraction 1 protein was examined by incubating a range of commercial proteinases with casein in the presence and absence of the acid-soluble inhibitor(s) from F1-B Typical results are shown in Table 3 The activities of α-chymotrypsin, papain and proteinase K were unaffected, trypsin activity was strongly inhibited (> 90%), and pepsin and pronase E were inhibited to a lesser extent (30 and 16%, respectively)

To investigate the possibility that trypsin inhibitory activity could occur in association with soluble leaf proteins other than Fraction 1 protein, each fraction produced during the separation of lucerne soluble proteins, by ammonium sulphate precipitation and gel filtration [11], was screened for the presence of acid-soluble (pH 4 75) trypsin inhibitory activity using casein as substrate. Inhibition of proteinase activity occurred in the presence of acid-soluble material derived from the

Table 1 Factors affecting the susceptibility of 2 samples of leaf Fraction 1 protein to trypsin

Substrate for trypsin digestion	TCA-soluble products after 30 min incubation (mg/ml)
F1-A	2 0
F1-A Heat denatured	1 8
F1-A Acid-agglutinated, redissolved	2 3
F1-B	0 1
F1-B Heat denatured	1 8
F1-B Acid-agglutinated, redissolved	2 0
F1-B Heat denatured + F1-B acid-soluble fraction (ASF)	0 3
F1-A + F1-B ASF	0 3
F1-A Acid-agglutinated, redissolved + F1-B ASF	0 3
F1-A + F1-B ASF heated at 100° for 20 min	2 1

Substrate (5 mg/ml) in 20 mM KPi buffer, pH 8 0, was incubated for 30 min at 39 with trypsin (100 μg/ml) TCA-soluble products were measured as described in the Experimental Heat-denatured F1, acid-agglutinated F1 and the acid-soluble fraction from F1 were prepared from F1 protein (5 mg/ml) as described in the Experimental

Table 2 Effect of heat-labile inhibitors from a sample of leaf Fraction 1 protein on the proteolysis of casein by trypsin

Addition to substrate	TCA-soluble products after 30 min incubation (mg ml)
None	3 1
F1-B ASF	0 7
F1-B ASF preheated at 100° for 2 min	0 8
F1-B ASF preheated at 100° for 5 min	1 7
F1-B ASF preheated at 100° for 10 min	2 6
F1-B ASF preheated at 100° for 20 min	3 1

Casein (5 mg/ml) in 20 mM KPi buffer, pH 8 0 was incubated with trypsin (10 μg/ml) for 30 min at 39 TCA-soluble products were measured as described in the Experimental The acid-soluble fraction (ASF) was prepared from F1-B (5 mg/ml) as described in the Experimental

Table 3 Effect of inhibitory activity from a sample of leaf Fraction 1 protein on the proteolysis of casein by a range of proteinases

Enzyme	Incubation time/temperature	TCA-soluble products (mg/ml) Without inhibitor	With inhibitor*
α-Chymotrypsin	30 min/37	4 4	4 5
Papain	15 min/37°	3 5	3 7
Pepsin	45 min/37	2 9	2 0
Pronase E	30 min/40	5 9	4 9
Proteinase K	30 min/37°	5 1	4 9
Trypsin	30 min/37°	4 5	0 3

*Each incubation contained 0 1 ml/ml of the acid-soluble fraction (ASF) prepared from Fraction 1 sample B (F1-B) (20 mg/ml) as described in the Experimental

Fraction 1 protein, but no inhibitory activity was released by acid-agglutination of any of the other soluble proteins

The electrophoretic behaviour of the trypsin inhibitory activity associated with leaf Fraction 1 protein (F1-B) and present in lucerne juice, from which most of the protein had been removed by agglutination at pH 4 75, was examined in aqueous gels containing 7 5% (w/v) polyacrylamide The activity was resolved into two components, both having a net positive charge at pH 8 4 and migrating towards the cathode Chromatofocusing, whereby proteins are separated on a column according to their isoelectric points, confirmed the results obtained by PAGE The elution profile for the trypsin inhibitory activity derived from Fraction 1 protein (F1-B) is shown in Fig 1 The two inhibitory components separated had isoelectric points in the region of 9 5 and 9 0, respectively

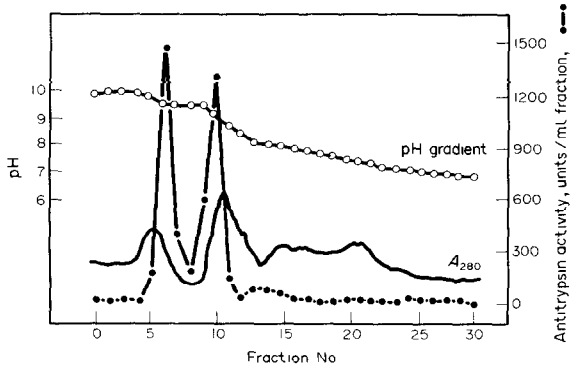

Fig 1 Chromatofocusing of lucerne trypsin isoinhibitors Inhibitors contained in the acid-soluble fraction prepared from F1-B (3 g) were subjected to chromatofocusing as described in the Experimental

DISCUSSION

Our results show that the vegetative portion of lucerne contains trypsin inhibitory activity which can be recovered from the acid-soluble fraction of lucerne juice and associates with leaf Fraction 1 protein during the separation of soluble leaf proteins. The nature and physiological purpose of the association between inhibitors and protein is not yet known but it is tempting to speculate that it illustrates a previously unconsidered role for inhibitors in selectively protecting a single protein from degradation by endogenous plant proteinases

Inhibitory activity was composed of at least two isoinhibitors whose isoelectric points (pH 9 and 9.5) indicate a large content of basic amino acids

The difference in isoelectric points for the isoinhibitors, compared with Fraction 1 protein (isoelectric point pH 5 5), is sufficient to suggest that the inhibitors have very different physical properties to Fraction 1 Co-fractionation of Fraction 1 protein and inhibitor in the absence of strong chemical or physical interaction, therefore, seems unlikely Evidence that such interaction occurs stems from our failure to separate leaf Fraction 1 protein from inhibitors by gel filtration, both during preparation of the protein and subsequently Electrostatic interaction between the isoinhibitors and Fraction 1 protein at pH near neutrality is easy to envisage, but other soluble proteins of the stroma no doubt have isoelectric points in the region pH 4–7 and yet we have obtained no evidence that they can interact with the trypsin inhibitors

The lucerne isoinhibitors strongly inhibited the activity of trypsin towards casein, azocasein and leaf Fraction 1 protein and, to a lesser extent, also protected casein from attack by pepsin and pronase, no inhibitory activity was recorded against the serine proteinases α-chymotrypsin and proteinase K (alkaline) or against the thiol proteinase, papain The lack of activity towards α-chymotrypsin is not particularly surprising as other potent trypsin inhibitors have been described which do not inhibit chymotrypsin [1]. Similarly, partial inhibition of pronase is perhaps to be expected since this is a non-specific preparation from *Streptomyces griseus* containing trypsin-like and alkaline serine proteinases as well as neutral metalloproteinase activity [16]. Our observation that lucerne trypsin inhibitors can significantly inhibit the carboxyl (acid) proteinase pepsin is interesting since, with only one exception, proteinase inhibitors from higher plants are not active against this mechanistic class of endopeptidase [1] According to Laskowski and Kato [3], a particular inhibitory reactive site can inhibit only proteinases belonging to a single one of the four mechanistic classes of Hartley [17], so it is possible that inhibitor preparations derived from leaf Fraction 1 protein or whole lucerne juice contain not only isoinhibitors of trypsin but also a carboxyl proteinase inhibitor. Alternatively, our pepsin samples could contain some serine proteinase, but contamination to the extent of 30% seems unlikely

Available information led Chien and Mitchell [8] to conclude that lucerne forage may contain two distinct trypsin inhibitors Inhibitory activity examined electrophoretically was derived from more than one component, was maximal in the leaves of mature plants, was extractable in aqueous sodium chloride or dilute acid from either dehydrated fresh tissue or commercial dehydrated lucerne meal in which it is likely that soluble proteins would have been rendered largely insoluble, and was completely inactivated only after prolonged heating [7–9] By contrast, the trypsin isoinhibitors described in this paper were readily inactivated by heat Their extraction from fresh tissue appears to depend on initial extraction of leaf Fraction 1 protein with which the inhibitors may associate *in vivo*; Fraction 1 protein and inhibitors may then be separated by agglutinating the leaf protein at pH 4.75 when the inhibitors remain acid-soluble. Leaf protein concentrate, prepared from lucerne by heat- or acid-precipitation of soluble proteins, would probably be free of trypsin inhibitory activity such as we have described here and yet, nevertheless, was found to contain anti-trypsin activity [18]. Such results can only mean that two distinct types of inhibitor exist.

Because of the widespread use of lucerne as a forage crop for ruminants, it would be of considerable interest to know whether the trypsin isoinhibitors present in the vegetative portion of the plant can occur in sufficiently high concentration to affect the proteolytic functions of rumen micro-organisms and, thus, the normal protein metabolism of the animal Rumen bacteria are known to produce trypsin-like serine proteinases [19, 20] and the results of unpublished work carried out in this laboratory indicated that the proteolytic activity of the rumen bacterium, *Bacteroides ruminicola* R8/4, is strongly inhibited by the presence of the trypsin isoinhibitors associated with leaf Fraction 1 protein, with the result that the organism's requirement for peptide nitrogen may not be met and no growth occurs. Similar inhibition of bacterial proteinases within the rumen of a lucerne-grazed ruminant could result in a substantially increased half-life for soluble proteins and since soluble proteins and, in particular, Fraction 1 protein are widely believed to be the foaming agents in legume bloat (see ref [21] for review), persistence of such proteins in the rumen may increase the risk of bloating. Further work is in progress to establish whether a connection exists between the presence of trypsin inhibitors in lucerne and its ability to cause bloat and also to determine whether naturally-occurring proteinase inhibitors, such as those of lucerne, could be used to mediate protein-sparing effects in the rumen and improve nitrogen retention by ruminants

EXPERIMENTAL

Proteins. Fraction 1 protein was prepared from lucerne (up to 15 kg fr wt) by the method of ref [10] Heat denaturation of leaf

Fraction 2 proteins was achieved by passing the lucerne juice through a stainless steel coil held at 62.5°. Gel filtration of the heat-stable protein ppted at 24% (w/v) $(NH_4)_2SO_4$ (mainly Fraction 1 protein) was performed on columns of Sephadex G-50 measuring 5 × 85 cm or 15 × 60 cm. Purified Fraction 1 protein was extensively dialysed against 10 mM KPi buffer, pH 6.8, and stored at −20° as a freeze-dried powder.

When required, simultaneous preparation of both leaf Fraction 1 and Fraction 2 proteins from lucerne juice was achieved by a combination of $(NH_4)_2SO_4$ pptn and gel filtration as described in ref. [11].

Casein (light, white, soluble, 89.5% protein by wt) was obtained from BDH Biochemicals and azocasein from Sigma.

Proteinases. Pronase E, pepsin (EC 3.4.23.1), α-chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) were obtained from Sigma. Proteinase K (EC 3.4.21.14) was purchased from Boehringer and papain (32 mg/ml, 29 units/mg, EC 3.4.22.2) from Millipore.

Lucerne trypsin inhibitors. (1) *Fraction 1 protein* (up to 3 g in 40 ml glass distilled H_2O) was dialysed against three changes of 10 mM KPi buffer pH 6.8. Trypsin inhibitors were released into the acid-soluble fraction (ASF) by adjusting the pH to 4.75 with 0.5 M HCl. The supernatant (2500 *g*, 4°, 20 min) was adjusted to pH 7 with 0.5 M NaOH and made 30–40% (w/v) with $(NH_4)_2SO_4$. The ppted crude inhibitors were centrifuged (30 000 *g*, 4°, 20 min) and, after dissolving in H_2O were extensively dialysed against 10 mM KPi, pH 6.8, separated from insoluble material (3000 *g*, 4°, 20 min) and freeze-dried. Freeze-dried material prepared thus from the batch of Fraction 1 protein, which was the richest source of inhibitors, contained 723 mg total protein and 2.9×10^5 inhibitor units/g. For some expts acid-agglutinated Fraction 1 protein was redissolved at pH 8–9 in NaOH and finally adjusted to pH 7 with 0.1 M HCl. (2) *Fresh leaf extract.* Inhibitors were prepared by homogenizing (Ultraturrax, maximum power for 30 sec) lucerne (up to 1 kg fr. wt) in protein extraction buffer [10] (2 ml/g tissue) and adjusting the pH of the resultant juice to within the range 3.6–4.75 with 0.5 M HCl, acid-soluble material was separated from agglutinated protein by centrifugation (17 000 *g*, 4°, 20 min), dialysed against 10 mM KPi buffer, pH 6.8 at 4°, freeze-dried and stored at −20°.

Inhibitor assay. Trypsin inhibitory activity was quantified by determining its effect on the proteolysis of azocasein by trypsin at pH 8 and 39°. The assay contained inhibitor (up to 0.3 ml), 50 mM KPi, pH 8, azocasein (5 mg) and trypsin (10 μg) made up to 1 ml with glass distilled H_2O. Reaction was stopped after 30 min by the addition of 1 ml 20% (w/v) trichloroacetic acid (TCA) and the mixture was centrifuged (10 000 *g*, 4°, 20 min). To ensure exclusion of fragments of the TCA pellet, the supernatant was passed through a glass fibre filter (type A–E, Gelman). TCA-soluble products were measured by adding NaOH (0.5 M) to the supernatant and comparing A_{440} of the soln with a standard curve for azocasein in 0.5 M NaOH. 1 unit of trypsin activity was defined as the quantity of trypsin catalysing the formation of 1 μg acid-soluble products per min under the above conditions. Correspondingly, 1 unit of inhibitor was defined as the amount of inhibitor which completely inhibited 1 unit of trypsin. Since inhibition of proteinase activity may not always be a linear function of inhibitor concn up to the 100% value [12], inhibition curves were constructed where possible to determine the inhibitor content of unknown samples.

Proteinase assays. In determining the effect of lucerne trypsin inhibitory activity on the proteolysis of casein (10 mg/ml) by different proteinases, pronase E (20 μg/ml), proteinase K (10 μg/ml), pepsin (20 μg/ml), α-chymotrypsin (40 μg/ml), trypsin (20 μg/ml) and papain (10 μg/ml) were assayed under the conditions prescribed for each of these enzymes in the Merck booklet, *Preparations for Biochemistry*. Assays (total vol. 1 ml) were terminated by rapid cooling in an ice bath. TCA (10% w/v final concn) was added to deproteinize samples and TCA-soluble products were determined by the procedure of ref. [13]. Trypsin digestion of lucerne Fraction 1 protein (5 mg/ml) was carried out for up to 4 hr at 39° in 20 mM KPi buffer, pH 8, with trypsin at a concn of 100 μg/ml. Reaction was terminated and TCA-soluble products determined as above.

Protein assay. Protein nitrogen was determined by a micro-Kjeldahl method [14]. Protein concn was routinely estimated by reference to standard curves relating A_{280} to the concn of dissolved protein.

Chromatofocusing. Lucerne trypsin inhibitors were fractionated by the method described by Pharmacia in the booklet, *Chromatofocusing with Polybuffer and PBE*, but with some of the modifications employed in ref. [15].

A column (1 × 33 cm) containing Polybuffer exchanger, PBE 94 (Pharmacia) was packed at a flow rate of 100 cm/hr and equilibrated with degassed 50 mM ethanolamine–HOAc buffer, pH 9.9, containing dithiothreitol (DTT, 1 mM) and polyethylene glycol 4000 (PEG, 10 mg/ml). The sample containing trypsin inhibitory activity, dissolved in the same buffer, was applied to the column and eluted with a pH gradient soln (180 ml) containing Polybuffer 96 (36 ml), pharmalyte 10.5–8.0 (2.6 ml), DTT (1 mM) and PEG 4000 (10 mg/ml) made to 200 ml with H_2O and pH 7 with HOAc and degassed. Any proteins with isoelectric points below 7 were recovered from the column by subsequent elution with 1 M NaCl. The flow rate throughout was 35 cm/hr and 6 ml fractions were collected.

Gel electrophoresis. PAGE was carried out at pH 8.4 on an LKB multiphor flat bed apparatus. Gels contained acrylamide (7.5% w/v), *N,N'*-methylene bisacrylamide (0.15% w/v), Tris (0.0249 M), glycine (0.189 M), ammonium persulphate (0.1% w/v) and *N,N,N',N'*-tetramethylethylene diamine (0.1% v/v). Pre-electrophoresis (1–2 V/cm) was performed for 30–60 min before sample application. Electrophoresis was carried out at 10 V/cm for 2 hr or until bromophenol blue marker had reached the end of the gel.

Polyacrylamide gels were negatively stained for the presence of trypsin inhibitors using the chromogenic substrate α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl (BANA, Sigma). Following electrophoresis, the gels were incubated at 39° for 30 min in pre-warmed 100 mM KPi buffer, pH 7, containing trypsin (0.5 mg/ml). Excess trypsin was then removed with three washes of H_2O, and each gel was incubated at 39° for up to 3 hr in a substrate soln containing 3 ml BANA (10 mg/ml in dimethyl sulphoxide), 3 ml Echtblausaltz B (Fast Blue salts B, Fluka, 20 mg/ml in 0.001 M HCl) and 54 ml 100 mM KPi, pH 7. Trypsin inhibitor bands appeared as colourless zones against a purple background. Stained gels were stored in 10% (w/v) HOAc.

REFERENCES

1 Richardson, M. (1977) *Phytochemistry* **16**, 159
2 Ryan, C. A. (1979) in *Herbivores Their Interaction with Secondary Plant Metabolites* (Rosenthal, G. A. and Janzen, D. H., eds.) p. 599. Academic Press, New York.
3 Laskowski, M. and Kato, I. (1980) *Annu. Rev. Biochem.* **49**, 593
4 Gonzalez, E., Callejas, A., Seidl, D. and Jaffe, W. G. (1979) *J. Agric. Food Chem.* **27**, 912
5 Chavan, J. K. and Hejgaard, J. (1981) *J. Sci. Food Agric.* **32**, 857
6 Walker-Simmons, M. and Ryan, C. A. (1977) *Plant Physiol.* **59**, 437
7 Ramirez, J. S. and Mitchell, H. L. (1960) *J. Agric. Food Chem.* **8**, 393
8 Chien, T. F. and Mitchell, H. L. (1970) *Phytochemistry* **9**, 717

9 Chang, H-Y, Reeck, G. R and Mitchell, H. L (1978) *J Agric Food Chem.* **26**, 1463
10 Jones, W T and Mangan, J L (1976) *J Agric Sci* **86**, 495
11 Horsnell, J M., Harrison, F. A and Mangan, J L (1982) *Biochem Soc Trans* **10**, 33
12 Fritz, H, Trautschold, I and Werle, E (1974) *Methods of Enzyme Analysis* **2**, 1064
13 Miller, G L (1959) *Analyt Chem.* **31**, 964
14 Mangan, J L and West, J (1977) *J Agric. Sci.* **89**, 3
15 Hirasawa, K, Irvine, R F and Dawson, R M C (1982) *Biochem J.* **205**, 437
16 IUB (1979) in *Enzyme Nomenclature Recommendations* (1978) *of the Nomenclature Committee of the International Union of Biochemistry* p 296 Academic Press, New York
17 Hartley, B S (1960) *Annu Rev Biochem* **29**, 45
18. Humphries, C (1980) *J Sci Food Agric* **31**, 1225
19 Hazlewood, G P and Edwards, R (1981) *J Gen Microbiol* **125**, 11
20 Brock, F M and Forsberg, C W (1980) *Abstracts of the 30th Annual Meeting of the Canadian Society of Microbiologists* F8
21 Clark, R T J and Reid, C S W (1974) *J Dairy Sci* **57**, 753