

Enzyme inhibitors from plants: Enterokinase inhibitors in tubers and seeds

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Abstract. Of the 22 tubers and 9 pulses screened for inhibitors of enterokinase activity, the following 12 tubers, *Curcuma amada*, *Kyllinga monocephala*, *Solanum tuberosum*, *Canna indica*, *Helianthus tuberosus*, *Coleus parviformis*, *Mirabilis jalapa*, *Colocasia antiquorum* (red variety), *Alium cepa*, *Arnorphophalus companulatus*, *Maranta arundinacea*, *Daucus carota*, and 9 pulses namely, *Vigna sinensis*, *Arachis hypogea*, *Pisum sativum*, *Phaseolus vulgaris* (white bean), *Phaseolus vulgaris* (kidney bean), *Phaseolus mungo*, *Cicer arietinum*, *Dolichos lablab* and *Cajonus cajan* contained inhibitory activity. Three tubers, *Arnorphophalus companulatus*, *Maranta arundinacea* and *Daucus carota* and all the nine pulses exhibited endogenous esterase activity towards benzoyl arginine ethyl ester. Among the 8 pulses and 3 tubers processed by affinity chromatography on trypsin-sepharose, to separate trypsin inhibitor from enterokinase inhibitor, *Phaseolus vulgaris* (kidney bean), *Phaseolus vulgaris* (white bean) and *Dolichos lablab* contained distinct enterokinase inhibitors. These fractions were devoid of trypsin inhibitor activity. The trypsin inhibitor from *Coleus parviformis* tubers alone did not bind to trypsinsepharose and was recovered in the unbound fraction along with the enterokinase inhibitor.

Keywords. Enterokinase inhibitors; trypsin inhibitors; proteinase inhibitors; tubers; pulses.

Introduction

Enterokinase (EC 3.4.21.9) is a serine proteinase required for the activation of pancreatic proenzymes in the duodenum. Porcine enterokinase specifically cleaves a peptide bond lys 6-ile 7 of bovine trypsinogen (Blackburn, 1976) to convert the proenzyme to active trypsin and initiates the cascade of reactions leading to the activation of many digestive proteinases (Blackburn, 1976). The occurrence of enterokinase deficiency in human infants and the consequent protein malabsorption in such individuals have provided *in vivo* evidence for the pivotal role of the enzyme in the digestion process (Hadorn *et al.*, 1969; Howarth *et al.*, 1975). Any factor in food which is capable of suppressing the enterokinase activity would lead to disturbances in digestion comparable to enterokinase deficiency. In view of this, a systematic survey of edible plant tissues for

Abbreviations used: N-B_z-L-arg, α-N-benzoyl-L-arginine; N-B_z-DL-arg, α-N-benzoyl-DL-arginine.

enterokinase inhibitor is of interest. Although inhibitors of pancreatic proteinases like trypsin and chymotrypsin have been extensively investigated, the presence of enterokinase inhibitors in food material was reported only recently (Lau *et al.*, 1980).

In this paper, we report the occurrence of enterokinase inhibitors in a wide variety of plants, and also demonstrate for the first time the presence of a specific enterokinase inhibitor, free of trypsin inhibitory activity in some plant seeds.

Materials and methods

Materials

The tubers and seeds were procured locally from commercial sources. A few wild varieties of non-edible tubers were collected during the rainy season.

α -N-Benzoyl L-arginine (N-B_z-L-arg)ethyl ester, α -N-benzoyl DL-arginine(N-B_z-DL-arg) *p*-nitroanilide and Trizma base (tris hydroxymethyl amino methane) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Cyanogen bromide activated-Sepharose 6MB and Sephadex G-15 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine trypsin (EC 3.4.21.4, twice crystallized) was the product of Worthington Biochemical Corporation, Freehold, New Jersey, USA.

Bovine trypsinogen was prepared as described by Wilimowska-Pelc and Mejbaum-Katzenellenbogen (1978) taking care to obtain the trypsinogen free of trypsin contamination. Bovine enterokinase was partially purified from duodenum according to Liepnicks and Light (1979). The procedure was followed upto the ammonium sulphate fraction stage and this fraction was used as the source of bovine enterokinase in all the studies. Trypsin-Sepharose was prepared as described earlier (Shivaraj and Pattabiraman, 1981).

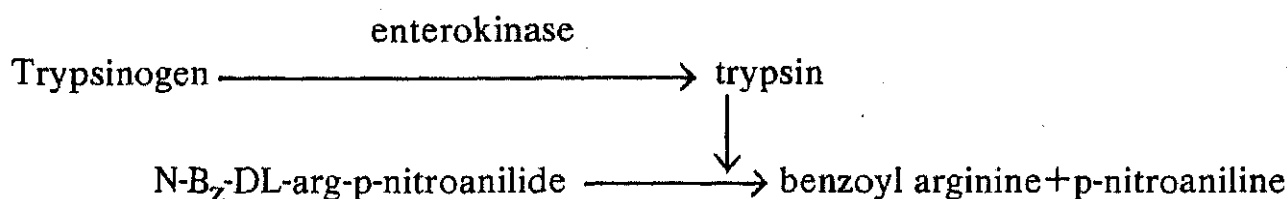
Methods

Preparation of cell-free extracts: Tubers were homogenized in a glass mortar with 3 volumes of 0.1 M Tris-HCl buffer, pH 8.0 at 28-30°C. The homogenate was centrifuged at 10,000 *g* for 20 min, the supernatant was separated and used to estimate enterokinase inhibitory activity and endogenous esterase activity. Seeds were extracted with 10 volumes of the same buffer as described above.

Assay of enterokinase: Esterase activity of bovine enterokinase was determined using N-B_z-L-arg ethyl ester as substrate by the method described by Bhat *et al.* (1978) for trypsin. The assay system contained 60 μ mol of Tris-HCl buffer pH 8.0; 1 μ mol of CaCl₂; 2 μ mol of N-B_z-L-arg ethyl ester and suitable aliquots of enterokinase preparation in a final volume of 1.0 ml. The reaction was initiated by the addition of the substrate. The other details of assay were similar to those described elsewhere (Bhat *et al.*, 1978). The crude extracts of different plant tissues (0.1-0.6 ml) were included in the assay system to determine the inhibitory activity. The difference between the esterase activity of enterokinase control and test containing the enterokinase plus the crude extracts was the measure of inhibitory activity. At the concentrations used, the substrate was not a limiting

factor. To estimate esterase activity of the plant extract, the assay system was modified by including the crude extract in a reaction mixture containing N-B_z-L-arg ethyl ester, CaCl₂ and buffer as described above but omitting enterokinase. One unit of esterolytic activity is defined as the amount of enzyme which liberates 1 μmol of benzoyl arginine from N-B_z-L-arg ethyl ester in 1 min under the assay conditions. One unit of esterolytic inhibitor is the amount which suppresses the esterolytic activity by one unit.

Assay of enterokinase activity using trypsinogen as substrate was performed based on the following reactions.



Trypsinogen was activated to trypsin by preincubation with enterokinase at pH 6.0 and trypsin formed was assayed by the method of Erlanger *et al.* (1961).

The preincubation system contained 50 μmol Tris acetate buffer pH 6.0; 1 μmol, CaCl₂; 100 μg trypsinogen; and suitable amounts of enterokinase preparation in a final volume of 1.0 ml. The reaction was initiated by adding trypsinogen and incubated at 37°C for 10 min. To this assay mixture, 2.0 ml of 2 mM N-B_z-DL-arg p-nitroanilide in 50 mM Tris-HCl, buffer, pH 8.0 was added and incubated for further period of 15 min. The reaction was stopped by adding 1.0 ml of 30% acetic acid. The colour was measured at 410 nm.

One unit of enterokinase estimated is defined as that amount which produced one amidolytic unit of trypsin under the specified assay conditions. One amidolytic unit of trypsin is that amount which liberated 1 μmol of p-nitroaniline in 1 min. Enterokinase inhibitor activity was estimated by this method by including suitable aliquots of fractions in the preincubation systems. One unit of enterokinase inhibitor is the amount which suppressed the enterokinase activity by one unit.

Amidolytic activity of bovine crystalline trypsin was estimated as follows. One μmol of CaCl₂, 100 μmol Tris-HCl buffer pH 8.0; 4 μmol N-B_z-DL-arg p-nitroanilide and 20 μg of trypsin in a final volume of 3.0 ml were incubated at 37°C for 15 min. The reaction was stopped by the addition of 1.0 ml of 30% acetic acid and the absorption measured at 410 nm. To estimate trypsin inhibitor activity, the aliquots of crude extracts and chromatographic fractions were included in the assay system and the amidolytic activity was determined. The difference between the amidolytic activity of control and test determined in the absence and presence of plant extracts was a measure of the inhibitor activity. One amidolytic unit of trypsin is that amount which hydrolyzed 1 μmol of N-B_z-DL-arg p-nitroanilide in 1 min. One unit of trypsin inhibitor is that amount which suppressed the activity of trypsin by one unit. Protein was estimated by the method of Lowry *et al.* (1951).

Affinity chromatography on trypsin-sepharose: To explore the possibility of the

presence of a specific enterokinase inhibitor devoid of trypsin inhibitor activity, crude extracts were subjected to affinity chromatography on trypsin-Sepharose at 4°C. The crude extract (2.7 ml) was mixed with 0.3 ml of 5 M NaCl and applied to a 5.5 ml (0.9×8.6 cm) column of trypsin-sepharose previously equilibrated with 10 mM Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. The flow rate was 10 ml/h. The column was washed with 50 ml of the equilibration buffer and then eluted with 50 ml of 50 mM HCL. Ten ml fraction were collected. Each fraction was dialyzed overnight against 1 litre of 10 mM Tris-a-acetate buffer, pH 6.0 at 4°C. Suitable aliquots (5 µl-300 µl) were used for measuring enterokinase and trypsin inhibitor activities.

Results

Twentytwo edible as well as non-edible tubers and 9 pulses were screened for enterokinase inhibitory activity with N-B_z-L-arg ethyl ester as substrate. The results are presented in table 1. The plants were classified into two groups based on the presence of enterokinase inhibitor and endogenous esterase activity.

Table 1. Enterokinase inhibitor and endogenous esterase activities of tubers and seeds.

	Endogenous esterase		Enterokinase inhibitor	
	Specific activity (units/g protein)	Units ^a /100 g tissue	Specific activity	Units ^a /100 g tissue
A				
Mango ginger (<i>Curcuma amada</i>)	Nil	Nil	47.1	8.9
Kylinga (<i>Kyllinga monocephala</i>)	„	„	28.3	18.9
Potato (<i>Solanum tuberosum</i>)	„	„	21.5	18.9
Canna (<i>Canna indica</i>)	„	„	21.3	12.3
Dahlia (<i>Helianthus tuberosus</i>)	„	„	20.8	12.3
Coleus (<i>Coleus parviformis</i>)	„	„	13.2	17.9
Mirabilis (<i>Mirabilis jalapa</i>)	„	„	10.7	15.4
Colocasia (<i>Colocasia antiquorum</i> , red variety)	„	„	8.2	7.9
Onion (<i>Allium cepa</i>)	„	„	5.2	3.5
B				
Amorphophalus (<i>Amorphophalus companulatus</i>)	98.2	229.8	21.5	50.4
Arrowroot (<i>Maranta arundinacea</i>)	2.9	4.9	15.6	26.6
Carrot (<i>Daucus carota</i>)	24.6	14.0	12.3	7.0
Cow pea (<i>Vigna sinensis</i>)	6.6	112.5	5.2	87.8
Pea nut (<i>Arachis hypogea</i>)	0.9	20.7	3.8	83.7
Green pea (<i>Pisum sativum</i>)	4.4	72.0	3.4	56.0
White bean (<i>Phaseolus vulgaris</i>)	5.2	77.0	3.3	49.0
Kidney bean (<i>Phaseolus vulgaris</i>)	9.7	105.0	3.1	34.0
Black gram (<i>Phaseolus mungo</i>)	6.0	91.0	2.8	42.0
Bengal gram (<i>Cicer arietinum</i>)	9.9	77.0	2.7	21.0
Field bean (<i>Dolichos lablab</i>)	3.1	37.5	2.5	30.0
Red gram (<i>Cajanus cajan</i>)	2.5	38.3	1.3	20.3

^aEnterokinase esterase activity is assessed using N-B_z-L-arg ethyl ester as substrate.

The values are the mean of three different estimations.

Group A includes nine tubers which exhibit only enterokinase inhibitor activity. Three tubers and 9 pulses showed both enterokinase inhibitor activity and endogenous esterase activity and are included in group B. Other plant materials namely tubers of *Dioscorea alata*, *Curcuma longa*, *Musa paradisiaca*, *Colocasia antiquorum* (garden variety), *Elettaria cardamomum* were devoid of inhibitor activity as well as endogenous esterase activity while *Dioscorea esculenta*, *Amorphophalus companulatus*, *Arginia indica*, *Alocasia macrohiza* and *Ipomea batata* exhibited only endogenous esterase activity.

Among the tubers, *Curcuma amada* exhibited the highest specific activity of enterokinase inhibitor (47.1×10^{-3} units/mg protein). The specific activity of the inhibitor was found to be highest in *Vigna sinensis* among pulses. The specific activity of the inhibitor in pulses varied from 1.3 to 5.2×10^{-3} units/mg protein and was lower than that in tubers ($5.2-47.1 \times 10^{-3}$ units/mg protein). Maximum specific activity of endogenous esterase was observed in *Amorphophalus companulatus*. Endogenous esterase specific activity varied from 0.9 to 9.9×10^{-3} in different pulses. The activity of enterokinase inhibitor in pulses in terms of units/100 g tissue was higher than in tubers.

An attempt at separation of enterokinase inhibitor from, trypsin inhibitor was made by chromatography of the crude extracts on trypsin-sepharose. The data are presented in table 2.

Table 2. Quantitation of enterokinase inhibitor and trypsin inhibitor activities after trypsin-sepharose affinity chromatography.

	Total protein applied (mg)	Enterokinase inhibitor in washings (Units ^a /g)	Trypsin inhibitory activity in bound fraction (Units ^b /g)
Kidney bean (<i>Phaseolus vulgaris</i>)	39.0	6.42	11.51
Field bean (<i>Dolichos lablab</i>)	43.5	1.04	12.46
White bean (<i>Phaseolus vulgaris</i>)	57.0	0.77	5.42
Coleus (<i>Coleus parviformis</i>)	20.2	0.25 ^b	Nil
Black gram (<i>Phaseolus mungo</i>)	58.5	Nil	2.03'
Green pea (<i>Pisum sativum</i>)	74.0	„	2.07
Bengal gram (<i>Cicer arietinum</i>)	40.0	„	5.82
Cow pea (<i>Vigna sinensis</i>)	39.0	„	8.33
Pea nut (<i>Arachis hypogea</i>)	27.0	„	1.29
Arrowroot (<i>Maranta arundinacea</i>)	23.2	„	1.52
Potato (<i>Solanum tuberosum</i>)	4.5	„	1.27

^a Enterokinase inhibitor activity was estimated by trypsinogen activation followed by amidolytic determination of trypsin.

^b The enterokinase inhibitor activity was the difference between the total inhibitory activity and the activity against trypsin. The trypsin inhibitory activity was 0.20 units/g.

The unbound fractions were found to contain enterokinase inhibitors in the cases of kidney bean, field bean, white bean and coleus tuber. The trypsin inhibitor was found in the bound fraction and was eluted in 50 mM HCl except in the case of coleus where trypsin inhibitor was not bound to trypsin-sepharose at all. In all other cases investigated, no separate enterokinase inhibitor could be detected in the unbound fraction. Due to dilution of the fractions during trypsin-sepharose affinity chromatography, assessment of inhibitor activity against the esterolytic action of enterokinase could not be carried out. Elution pattern of enterokinase inhibitor and trypsin inhibitor on trypsin-sepharose affinity chromatography of extracts of kidney bean, white bean and field bean are shown in figure 1. The early fractions which contained enterokinase inhibitor activity were devoid of trypsin inhibitor activity.

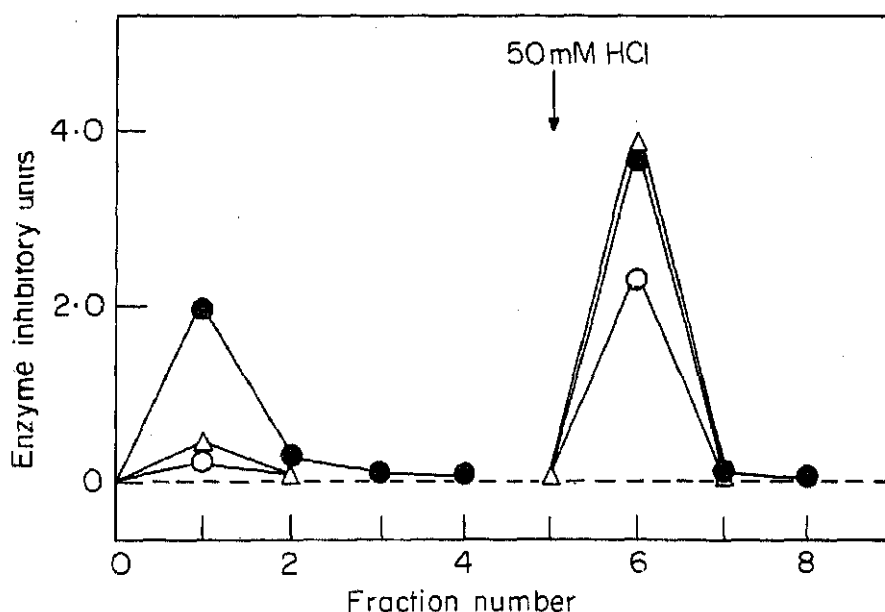


Figure 1. The crude extracts from kidney bean, field bean and white bean were subjected to affinity chromatography on trypsin sepharose as described under methods. O, kidney bean; Δ, field bean; ●, white bean.

The proteinase inhibitors in coleus provided a distinct picture. The trypsin inhibitor and enterokinase inhibitor of coleus could not be separated by this method, as the trypsin inhibitor did not bind to the column.

Discussion

The presence of proteinaceous inhibitors against the digestive proteinases in nature is well documented (Ryan, 1973). Although the similarity of enterokinase to other serine proteases like trypsin and chymotrypsin has been studied, this enzyme was not investigated in detail with respect to its susceptibility to inhibition by various natural proteinase inhibitors. Recently Liepnicks and Light (1979) reported that pancreatic trypsin inhibitor is a potent inhibitor of enterokinase.

Lau *et al.* (1980) demonstrated the presence of enterokinase inhibitor in potato tubers, tomato leaves and peanut seeds. The peanut seed enterokinase inhibitor was purified and has been fractionated into two inhibitors which showed trypsin inhibitory and chymotrypsin inhibitory activities, indicating the non-specific nature of the inhibitors (Lau *et al.*, 1980).

The results presented revealed the presence of enterokinase inhibitor in storage tissues of 21 plants. Among these plant materials, 8 pulses and 3 tubers were further investigated by affinity chromatography. Specific enterokinase inhibitors in 3 of them namely, kidney bean, white bean and field bean need to be highlighted as the tissues which possess the enterokinase inhibitor and were devoid of trypsin inhibitor activity. The presence of inhibitor with such specificity towards enterokinase has not been reported so far. The presence of trypsin inhibitors in the plant storage tissues investigated limits the assessment of enterokinase inhibitory activity with trypsinogen as substrate. In view of this, esterase activity of enterokinase with N-B_z-L-arg ethyl ester as substrate was used in the screening studies. The extracts of *Alocasia macrorhiza* and ragi (*Eleusine coracana*) were found to exhibit no inhibitor activity against the esterase activity of our enterokinase preparation. This observation confirmed the assumption that the enterokinase preparation is free of trypsin contamination. If the enterokinase preparation were to be contaminated with trypsin, significant esterase inhibitor activity would have been observed in alocasia (Sumathi and Pattabiraman, 1977) and ragi (Shivaraj and Pattabiraman, 1981) extracts as these two are known to contain potent trypsin inhibitors.

The inhibition of esterase activity of enterokinase by crude extracts may be due to the nonspecific action of trypsin inhibitors in the case where separation of enterokinase inhibitor was not possible by affinity chromatography. Coleus tuber presented a different type of trypsin inhibitor which did not bind to trypsin-sepharose. This may be due to very mild interaction of the inhibitor with trypsin or due to the cleavage of the inhibitor during interaction and its release. The present report adds many pulses and tubers to the list of sources of enterokinase inhibitors. Further studies to isolate and characterize enterokinase inhibitor from pulses are in progress.

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