Food Chemistry **32** (1989) 59–67

Thermal Stability of Protease Inhibitors in Some Cereals and Legumes

H. S. Saini

New South Wales Department of Agriculture and Fisheries,
North Coast Agricultural Institute,
Wollongbar, NSW 2480, Australia

(Received 15 February 1988; revised version received and accepted 29 June 1988)

ABSTRACT

The thermal stability of trypsin and chymotrypsin inhibitor activities in soybeans, mungbeans, rye and triticale was investigated. Both trypsin and chymotrypsin inhibitors were resistant to dry heat and retained more than 50% activity after heating at 105°C for 15 min. Autoclaving for 5 min at 121°C virtually destroyed trypsin and chymotrypsin inhibitor activities both in legumes and cereals. Trypsin and chymotrypsin inhibitors were readily extractable in tris-HCl buffer (pH 8·0, 0·1M, containing 10 mM $CaCl_2$) and were assayed using chromogenic substrates. Heating the extracts at 100°C for 15 min did not remove trypsin and chymotrypsin inhibitors completely. If the dry heat treatment is to be applied to inactivate protease inhibitors, high temperatures above 120°C should be selected.

INTRODUCTION

Protease inhibitors are naturally occurring proteins with an ability to inhibit the proteolytic activity of certain enzymes (Ryan, 1973; Laskowski & Kato, 1980). They have been studied extensively in a variety of legumes (Wagner & Riehm, 1967; de Lumen & Salamat, 1980; Al-bakir *et al.*, 1982; Tan *et al.*, 1984); cereals (Erickson *et al.*, 1979) and tubers (Ryan, 1973) because these species are considered important food sources. These substances have

Food Chemistry 0308-8146/89/$03·50

attracted the attention of scientists in many disciplines, especially food chemists and nutritionists, because of their possible effect on the nutritive value of food proteins (Erickson *et al.*, 1979; Tan & Wong, 1982; Rackis *et al.*, 1975). Several inhibitors are double headed, i.e. capable of inhibiting both trypsin and chymotrypsin (Haynes & Feeney, 1967; Richardson, 1977) and occur in multiple molecular forms (Haynes & Feeney, 1967; Kunitz, 1947*a*; Wilson & Laskowski, 1973; Tsukamoto *et al.*, 1983).

The nutritional significance of plant protease inhibitors has been extensively investigated (Liener & Kakade, 1980). Increased intake of raw beans and cereals, high in trypsin and chymotrypsin inhibitor activity, stimulates pancreatic juice secretion and causes pancreatic hypertrophy and growth inhibition. To counteract these effects, heating and autoclaving the grains prior to ingestion is often recommended and practised. Although the effect of heat on protease inhibitors has been evaluated in some legumes, information on the comparison of various heat treatments, particularly in cereal grains, is still fragmentary. In this paper, the trypsin and chymotrypsin inhibiting abilities of some food grains, under different modes of heat inactivation are evaluated.

EXPERIMENTAL

Preparation of seed meals

The seeds used were soybeans (*Glycine max* cv. Forrest), mungbeans (*Vigna radiata* cv. Berken), rye (*Secale cereale* L. cv. Univeta) and triticale (× *Triticoscale* cv. Satu). Whole dry seeds were ground in a Udy cyclone mill to pass through a 100 μm mesh sieve.

Preparation of dry heat-treated seeds

Seeds in duplicate lots (20 g) were placed to a depth of approximately 10 mm in metal Petri-dishes and heated in a forced-draught oven to temperatures of 90, 105, 120, 135 and 150°C for 15 min. The oven was allowed to reach the desired temperature before heating the seeds. After heating, the seeds were cooled to room temperature (20°C) and fine ground.

Preparation of autoclaved seeds

The autoclaved seeds were prepared by placing 20 g seeds in Petri-dishes to a depth of approximately 10 mm and autoclaving at 121°C for 5, 15, 25, 35 and 45 min. The time taken for the autoclave to reach 121°C was approximately

10 min and, after autoclaving, approximately 12 min to return to atmospheric pressure. Seeds were taken out and allowed to cool at room temperature. All seed samples subjected to autoclaving, including an untreated sample, were then dried at 60°C in a forced-draught oven for 24 h. All samples were prepared in duplicate.

Extraction of trypsin and chymotrypsin inhibitors

Finely ground samples (1·0 g) were suspended in 25 ml tris-HCl buffer (pH 8·0, 0·1 M, containing 10 mM $CaCl_2$) in 50 ml quickfit tubes. Tubes were vigorously shaken and occasionally stirred on a vortex-mixer. The extraction was carried out exactly for 2 h and the tubes stored at 4°C between each stirring. The suspensions were centrifuged (14 000 *g*, 15 min, 4°C) and clear supernatant solutions used for the assay of trypsin and chymotrypsin inhibitors. All extractions were carried out in duplicate and the determinations were performed on duplicate aliquots of each extract.

Assay of trypsin and chymotrypsin inhibitor activities

The extracts were diluted to a point where 0·5 ml solution produced 40–50% inhibition. Trypsin inhibitor activity was measured using *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as substrate. An aliquot (0·5–1·0 ml) of trypsin inhibitor solution (final volume made to 1·0 ml with buffer, 0·1 M, pH 8·0 containing 10 mM $CaCl_2$) was mixed with trypsin stock solution (100 µl, 0·2 mg/ml in tris-HCl buffer). The mixture was incubated at 37°C for 5 min followed by the addition of 50 µl BAPNA (10 mg/ml in dimethyl-formamide) and reincubated. Exactly after 10 min the reaction was stopped by adding 2·0 ml of N acetic acid. Absorption was then read at 410 nm against a reagent blank. The values were corrected for a sample blank run simultaneously. The concentration of active trypsin in the commercial preparation was determined according to the method of Chase & Shaw (1967). Trypsin inhibitor activity was then calculated as mg enzyme inhibited/g sample.

Chymotrypsin inhibitor activity was determined by using a method modified from Hirado *et al.* (1981). Portions of appropriately diluted extracts were pipetted and adjusted to 1·0 ml with buffer (Tris-HCl, 0·1 M, pH 8·0). Chymotrypsin (bovine) stock solution (200 µl, 0·2 mg/ml in tris-HCl buffer, 0·1 M, pH 8·0) was added and the mixture incubated for 5 min at 37°C. Substrate (*N*-benzoyl-L-tyrosine-*p*-nitroanilide) was then added, mixed thoroughly, and tubes reincubated. After exactly 10 min the reaction was stopped by adding 2·0 ml of 1 N acetic acid. The mixture was then centrifuged at 10 000 *g* for 10 min and the absorbance of the supernatant was read at

410 nm against a reagent blank. A sample blank was prepared simultaneously and the net change in absorbance was used to calculate chymotrypsin inhibitor activity as mg enzyme inhibited/g sample and calculated by assuming the concentration of active chymotrypsin in the commercial preparation as 100%.

Thermal stability of trypsin and chymotrypsin inhibitors in meal extracts

Thermal stability of trypsin and chymotrypsin inhibitors in rye and soybean meal extracts (pH 8·0) was determined after heating the extracts at 40, 60, 80 and 100°C for 15 min in a water bath. Portions (4·0 ml) of the extracts in duplicate were transferred to 15 ml quickfit, stoppered test tubes and heated at nominated temperatures for specified times. Tubes were then taken out, immediately placed in ice and, after cooling, stored at 4°C for further analysis.

RESULTS AND DISCUSSION

Extractability and assay of trypsin and chymotrypsin inhibitors

Kakade *et al.* (1969) developed a procedure for the measurement of trypsin inhibitor activity in soybeans using an uncentrifuged extract, an extraction pH of 8·4–10·0 and the synthetic substrate BAPNA. Methods of assaying trypsin inhibitor using this substrate and different extraction mediums have been reported for a variety of plant materials (Baintner, 1981; Al-bakir *et al.*, 1982; Tan & Wong, 1982). Similar procedures have also been reported for the measurement of chymotrypsin inhibitor activity using synthetic substrate BTPNA (Hirado *et al.*, 1981; Tan *et al.*, 1984). In the present study the inhibitors were extracted in tris-buffer (pH 8·0, 0·1M containing 10 mM $CaCl_2$) and a complete extraction was achieved at 2 h both for soybean and rye meals (Table 1). When compared with the extraction medium and assay procedure (AOCS official method) developed by Kakade *et al.* (1969), a close agreement between the trypsin inhibitor activities was achieved (Table 2). For the assay of chymotrypsin inhibitor, BTPNA was prepared in dimethylformamide (DMF) and the solution thus prepared was stable for 2 weeks. Tan *et al.* (1984) reported an assay procedure using BTPNA as the substrate where it was necessary to centrifuge the reaction mixture due to flocculation before reading the absorbance. In the assay procedure employed in the current investigations, flocculation was eliminated by adding 0·1 ml DMF, immediately after the addition of substrate solution, followed by vigorous shaking. Absorbance was read immediately after the reaction was stopped to avoid the recurrence of flocculants.

TABLE 1
Effect of Time on the Extractability of Protease Inhibitor in Tris-HCl Buffer (pH 8·0, 0·1M) containing 10mM $CaCl_2$

Time of extraction (h)	*Rye*		*Soybeans*	
	Trypsin inhibitor	*Chymotrypsin inhibitor*	*Trypsin inhibitor*	*Chymotrypsin inhibitor*
	(mg enzyme inhibited/g sample, dry wt.)			
1	0·83	0·80	13·40	10·90
2	0·81	0·80	13·55	10·70
4	0·79	0·85	13·00	10·65
6	0·78	0·80	13·40	10·70
8	0·76	0·85	13·00	10·85

Thermal stability of the inhibitors

No apparent difference was observed in cereals and legumes in their response to wet heat-treatments (Table 3). Autoclaving for 15 min at 121°C virtually destroyed the trypsin inhibitor activity in rye and triticale and chymotrypsin inhibitor was completely eliminated after 5 min autoclaving. In soybeans and mungbeans, more than 80% of trypsin inhibitor was removed after 5 min autoclaving and there was a slow depletion at subsequent times. More than 95% chymotrypsin inhibitor was destroyed after 5 min autoclaving. Mungbeans were devoid of chymotrypsin inhibitor activity.

The patterns of trypsin and chymotrypsin inhibitor depletion were slightly different when the seeds were subjected to dry heat treatment. A substantial increase (~68%) in trypsin inhibitor activity was observed in

TABLE 2
Extractability of Trypsin Inhibitor (mg enzyme inhibited per g sample, dry wt.)

Grain type	*Tris-HCl extraction*[a]	0·01N *NaOH extraction*[b]
Soybeans	10·30	9·97
Mungbeans	2·30	2·06
Rye	0·56	0·63
Triticale	0·30	0·35

[a] Extraction medium employed in current investigations.
[b] Extraction medium employed by Kakade *et al.* (1969).

TABLE 3
Effect of Autoclave Times on Trypsin and Chymotrypsin Inhibitor Activity in Legumes and Cereals

Autoclaving time (min)	*Trypsin inhibitor activity*[a]				*Chymotrypsin inhibitor activity*[a]			
	Soybeans	*Mungbeans*	*Rye*	*Triticale*	*Soybeans*	*Mungbeans*	*Rye*	*Triticale*
0	13·5 ± 0·10	2·2 ± 0·10	0·57 ± 0·02	0·38 ± 0·02	11·67 ± 0·32	0·00	1·20 ± 0·04	1·30 ± 0·06
5	1·7 ± 0·13	0·38 ± 0·03	0·12 ± 0·02	0·08 ± 0·01	0·33 ± 0·03	0·00	0·34 ± 0·04	0·00
15	0·31 ± 0·04	0·14 ± 0·02	0·03 ± 0·01	0·02 ± 0·01	0·00	0·00	0·15 ± 0·03	0·00
25	0·15 ± 0·03	0·04 ± 0·00	0·00	0·00	0·00	0·00	0·00	0·00
35	0·13 ± 0·03	0·02 ± 0·00	0·00	0·00	0·00	0·00	0·00	0·00
45	0·12 ± 0·02	0·02 ± 0·00	0·00	0·00	0·00	0·00	0·00	0·00

[a] mean ± standard error of mean; $n = 2$.
See Table 2 for units.

triticale heated at 90°C for 15 min compared to an 18% increase in rye (Fig. 1a). There was a gradual decrease in the activity at subsequent treatments and almost a complete elimination occurred at 135°C. The standard error of means was less than 0·10 for mungbeans and it varied from 0·06 to 0·45 for soybean samples. In the case of soy and mungbeans (Fig. 1b) no increase was observed at 90°C and the activity was 80 and 100%, respectively. As in cereal grains, the activity decreased gradually with only 6% remaining after 135°C treatment. A similar pattern was observed in chymotrypsin inhibitor activity (Fig. 1c and d). At 90°C, it increased slightly (7–8%) in cereal grains, against a 13% decrease observed in soybeans. More than 70% chymotrypsin inhibitor was eliminated after heating at 120°C. The standard error of mean for all samples was less than 0·05 except a control of soybeans (mean 11·6, se, 0·32).

It appears that, in both cereals and legumes, these inhibitors are extremely resistant to dry heat. Autoclaving, on the other hand, is quite effective in eliminating both trypsin and chymotrypsin inhibitors from these grains. Similar differences in the thermal stability of trypsin and chymotrypsin

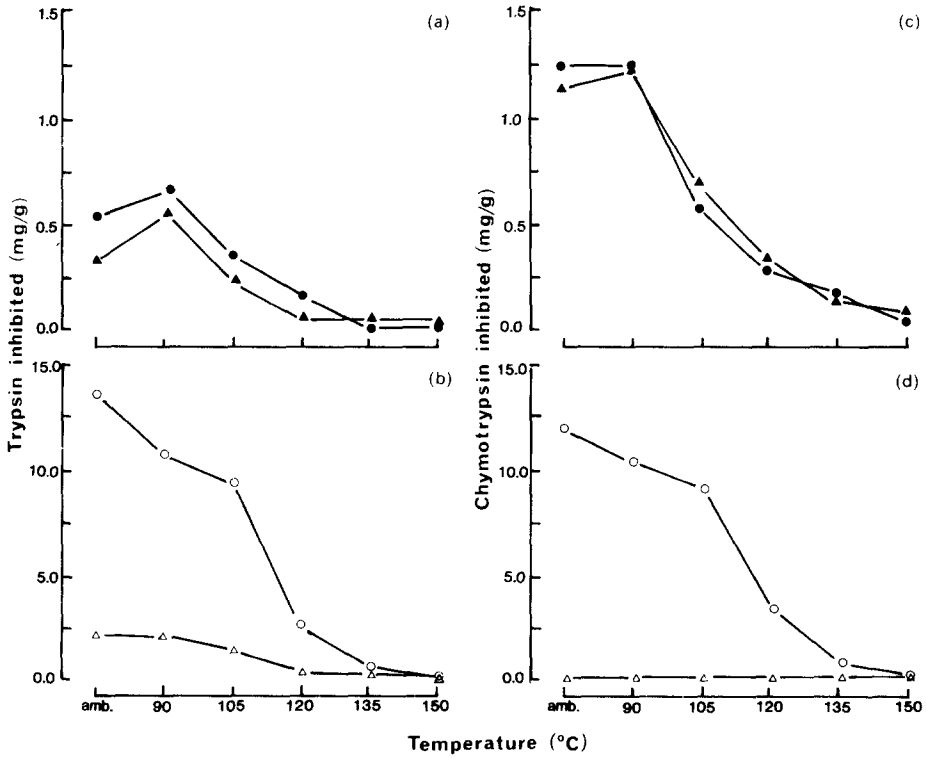

Fig. 1. Trypsin and chymotrypsin inhibiting abilities of legume and cereal grains heated at different temperatures for 15 min. ●, rye; ▲, triticale; ○, soybeans; △, mungbeans.

TABLE 4
Thermal Inactivation of Protease Inhibitors in Rye and Soybean Extracts

Heating temperature (°C)	*Rye*		*Soybeans*	
	Trypsin inhibitor	*Chymotrypsin inhibitor*	*Trypsin inhibitor*	*Chymotrypsin inhibitor*
	% inactivation[a]		*% inactivation*[a]	
40	40 ± 1·5	15 ± 2·0	6 ± 0·40	5 ± 0·20
60	45 ± 2·0	35 ± 1·5	16 ± 1·00	31 ± 1·00
80	55 ± 1·0	62 ± 2·0	38 ± 2·12	43 ± 3·00
100	65 ± 3·0	69 ± 1·5	42 ± 2·12	47 ± 2·00

[a] mean ± standard error of mean; $n = 2$.

inhibitors in winged beans have been reported (Tan & Wong, 1982; Tan *et al.*, 1984). The increase in trypsin and chymotrypsin inhibitor activity in cereals after heating at 90°C perhaps occurred due to an increased solubility of the protein. Therefore, it is important that if dry heat-treatment is applied, high temperatures above 120°C should be selected. This was further substantiated by heating the meal extracts (pH 8·0) at various temperatures (Table 4). In soybeans more than 50% of both trypsin and chymotrypsin inhibitor activity persisted after heating at 100°C for 15 min whereas, in rye grains, this activity remained above 30%.

Thermal inactivation of protease inhibitors and nutritional significance

A number of deleterious effects may occur in monogastric animals fed raw legumes and cereal meals or meal fractions with high protease inhibitor activity. In general, hypersecretion of pancreatic enzymes is initiated by protease inhibitors in the protein of raw food legumes and cereals. Rackis *et al.* (1975) demonstrated that 70–80% of trypsin inhibitor in soybeans needed to be destroyed in order to achieve maximal gain in weight and PER's with rats. No pancreatic hypertrophy occurred in rats fed soy flour in which the trypsin inhibitor activity had been destroyed. These effects could explain the interrelationship between the depressed utilization of nutrients, particularly protein and amino acids, growth inhibition and protease inhibitors as well as the thermal inactivation of such inhibitors.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swine Compensation Fund of N.S.W.

REFERENCES

Al-bakir, A. Y., Sachde, A. G. & Naoum, I. E. (1982). Occurrence and stability of trypsin inhibitors in Iraqi local legumes. *J. Agric. Food Chem.*, **30**, 1184–5.

Baintner, K. (1981). Trypsin-inhibitor and chymotrypsin inhibitor studies with soybean extracts. *J. Agric. Food Chem.*, **29**, 508–13.

Chase, T., Jr & Shaw, E. (1967). *p*-Nitrophenyl-*p*-guanidinobenzoate HCl: A new active site titrant for trypsin. *Biochem. Biophys. Res. Commun.*, **29**, 508.

Erickson, J. P., Miller, E. R., Elliot, F. C., Ku, P. K. & Ullsey, D. E. (1979). Nutritional evaluation of triticale in swine starter and grower diets. *J. Ani. Sci.*, **48**, 547–59.

Haynes, R. & Feeney, R. E. (1967). Fractionation and properties of trypsin and chymotrypsin from lima beans. *J. Biol. Chem.*, **242**, 537–43.

Hirado, M., Iwata, D., Niinobe, M. & Fuji, S. (1981). Purification and properties of thiol protease inhibitors from rat liver cytosol. *Biochem. Biophys. Acta*, **669**, 21–7.

Kakade, M. L., Simons, N. & Liener, I. E. (1969). An evaluation of natural vs synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem.*, **46**, 518–26.

Kunitz, M. (1947*a*). Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen.* Physiol., **30**, 291–6.

Laskowski, M., Jr & Kato, I. (1980). Protein inhibitors of proteinases. *Ann. Rev. Biochem.*, **49**, 593–626.

Liener, I. E. & Kakade, M. L. (1980). In *Toxic Constituents of Plant Foodstuffs* (2nd edn), ed. I. E. Liener, Academic Press, New York, p. 7.

de Lumen, B. O. & Salamat, L. A. (1980). Trypsin inhibitor activity in winged beans (*Psophocarpus tetragonolobus*) and the possible role of tannins. *J. Agric. Food Chem.*, **28**, 533–6.

Rackis, J. J., McGhee, J. E. & Booth, A. N. (1975). Biological threshold levels of soybean trypsin inhibitor by rat bioassay. *Cereal Chem.*, **52**, 85–9.

Richardson, M. (1977). The protease inhibitors of plants and microorganisms. *Phytochemistry*, **16**, 159–69.

Ryan, C. A. (1973). Proteolytic enzymes and their inhibitors in plants. *Ann. Rev. Plant Physiol.*, **24**, 173–96.

Tan, H-H & Wong, K-C (1982). Thermal stability of trypsin inhibitor activity in winged beans (*Psophocarpus tetragonolobus*). *J. Agric. Food Chem.*, **30**, 1140–3.

Tan, N-H, Rahim, Z. H. A., Khor, H-T & Wong, K-C (1984). Chymotrypsin inhibitor activity in winged beans (*Psophocarpus tetragonolobus*). *J. Agric. Food Chem.*, **32**, 163–6.

Tsukamoto, I., Miyoshi, M. & Hamaguchi, Y. (1983). Purification and characterization of three trypsin inhibitors from beans, *Phaseolus vulgaris* 'Kintoki'. *Cereal Chem.*, **60**, 281–5.

Wagner, L. P. & Riehm, J. P. (1967). Purification and partial characterization of a trypsin inhibitor isolated from navy beans. *Arch. Biochem. Biophys.*, **121**, 672–9.

Wilson, K. A. & Laskowski, M., Sr (1973). Isolation of three inhibitors of trypsin from garden bean (*Phaseolus vulgaris*). *J. Biol. Chem.*, **248**, 756–63.