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Comparative study of the action of cereal protease inhibitors on human, bovine and porcine pancreatic enzymes

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Abstract

Extract of corn, barley, ragi, sorghum, pearl millet, setaria, echinocloa and kodo seeds were tested for inhibitory activity against human and bovine pancreatic proteases. Setaria was equally active against the two systems. Kodo was more effective in inhibiting the caseinolytic, tryptic (hydrolysis of benzoyl arginine p-nitroanilide-BAPNA) and chymotryptic (hydrolysis of acetyl tyrosine ethyl ester-ATEE) activities of the human pancreatic preparation whereas corn, barley and echinocloa were potent against the bovine system. Other seeds showed mixed patterns. Inhibitory activity against elastase (hydrolysis of succinyl trialanyl p-nitroanilide-STANA) activity in bovine pancreatic extract was relatively poor in all the seeds. Comparative studies on the inhibition of crystallized porcine elastase with casein and STANA as substrates showed that magnitude of inhibition was more (1.70 fold in barley to 13.2 fold in pearl millet) with all the seeds when casein was used. Similar studies with crystalline bovine trypsin and chymotrypsin revealed significant difference only with kodo, which was found to be eight times more powerful in blocking tryptic activity when BAPNA was used as substrate.

Key words; Millet proteinase inhibitors, action on human, bovine, porcine pancreatins, natural and synthetic substrates.

1. Introduction

Protease inhibitors from millets and minor grains like sorghum[1], ragi[2], pearl millet[3], corn[4] and barley[5] have been isolated and characterized. However, detailed investigations on the action of these inhibitors on human pancreatic proteases are lacking. Since some of these cereals are the staple diet for man in semi-arid regions in many parts of the world, such a study will be nutritionally important. In this communication, we report the studies on the action of eight cereal extracts on the caseinolytic, tryptic and chymotryptic activities of bovine and human pancreatic preparations. The data are correlated to the inhibitory patterns obtained with pure bovine trypsin, bovine chymotrypsin and porcine elastase using protein and synthetic substrates.

2. Material and methods

2.1 Seeds

The following seeds were procured from Tamil Nadu Agricultural University, Coimbatore - Setaria (*Setaria intalica*, CO-5), echinocloa (*Echinocloa fructeneucea*, CO-1), ragi (*Eleusine*

coracana, CO-10), pearl millet (*Pennisetum typhoideum*, USH-9), corn (*Zea mays*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*, CO-23) and kodo (*Paspalum scorbiculatum*, CO-3).

2.2 Chemicals and enzymes

Bovine trypsin (twice crystallized), bovine α-chymotrypsin (thrice crystallized) and porcine elastase (twice crystallized) were obtained from Millipore Corporation, Freehold, N.J., U.S.A. Enterokinase was partially purified up to the ammonium sulphate stage from bovine duodenum according to the method of Liepnicks and Light[6]. Benzoyl DL-arginine p-nitroanilide (BAPNA), N-acetyl L-tyrosine ethyl ester (ATEE), succinyl tri-L-alanyl p-nitroanilide (STANA) were procured from Sigma Chemical Company, St. Louis, MO, U.S.A. Other reagents were analytical grade chemicals.

Bovine pancreas was procured from a local slaughter house. Human pancreas was obtained at autopsy. Acetone powder preparations of the frozen pancreatic tissues were the sources of the proenzymes. The preparations were activated by incubating the suspension of 1 g of the powder in 25 ml of 0.01 M phosphate buffer, pH 7.6 with 0.1 ml of the enterokinase solution for 2 hr at 37°C in presence of 15 mg of NaCl. The mixtures were centrifuged at 10000 × g for 20 min at 4°C and the supernatants were used as the sources of the enzymes.

2.3 Assay of protease activities

Caseinolytic activities of crystalline bovine trypsin, bovine chymotrypsin, porcine elastase, activated preparations of human and bovine pancreatic extracts were determined as described earlier[7]. In routine experiments 10 μg of bovine trypsin, 12.5 μg of bovine α-chymotrypsin, 75 μg protein of the bovine pancreatic preparation and 150 μg protein of the human pancreatic preparation were used to give an absorbance of 0.6 (Spectronic 20, cuvette diameter, 1.3 cm λ 540) under the assay conditions (pH 7.6, 37°C, 10 min incubation). 12.5 μg protein porcine elastase was used under similar conditions to give an absorbance of 0.45. This was necessitated because porcine elastase action was not linear beyond this value. One unit of caseinolytic activity was defined as the amount that produced trichloroacetic acid soluble fragments equivalent to an absorbance of 1.0.

Trypsin amidolytic activity was determined using BAPNA as substrate[8]. Under the assay conditions (pH 7.6, 37°C, 30 min incubation) 10 μg of bovine trypsin, 160 μg protein of the bovine pancreatic preparation and 225 μg protein of the human pancreatic preparation gave an O.D. value of 0.60 (λ 410 nm). One unit of enzyme activity is equivalent to the amount of enzyme that released *p*-nitroaniline amounting to an absorbance of 1.0. Elastase amidolytic activity was measured with STANA as substrate[9]. Under the assay conditions (pH 7.6, 37°C, 15 min incubation) 7 μg of porcine elastase and 70 μg protein of bovine pancreatic preparation gave an O.D. value of 0.6 (λ 410 nm). Human pancreatic extract had very poor STANA hydrolysis activity. Six mg protein of the extract gave an O.D. value of 0.6 under similar assay conditions. One unit of enzyme activity is the amount of enzyme that liberated *p*-nitroaniline equivalent to an absorbance of 1.0. Esterase activity of chymotrypsin was measured with ATEE as substrate[10]. Under the assay conditions (pH 7.6, 37°C, 10 min incubation) 1.25 μg of bovine crystalline α-chymotrypsin, 6 μg protein of the bovine

pancreatic extract and 12 μg protein of human pancreatic extract yielded an absorbance value of 0.6 (λ 540 nm). One unit of enzyme activity is defined as the amount that released acetyl tyrosine equivalent to an absorbance value of 10.

2.4 Preparation of seed extracts

Seed extracts were prepared as follows. The grains were finely powdered and homogenized with 4 volumes (w/v) of 0.02 M sodium phosphate buffer, pH 7.6 containing 0.15 M NaCl. The suspensions were stirred continuously for 30 min and centrifuged at 10000 × g for 20 min at 4°C and dialyzed against the same buffer for 16 h at 4°C. The supernatants were tested for inhibitory activity by including a wide range of aliquots in duplicate in the assay systems for proteolytic activities as described above. The seed extracts by themselves did not have any measurable hydrolytic activity on casein, BAPNA, ATEE or STANA under the assay conditions.

2.5 Inhibitory units

One unit of inhibitory activity is the amount of the seed extract that suppressed one unit of proteolytic activity (caseinolytic, BAPNA hydrolysis, ATEE hydrolysis or STANA hydrolysis as the case may be) under the assay conditions. The inhibitory units for routine purposes were calculated based on inhibition in the linear range.

2.6 Protein determination

Protein was determined by the method Lowry *et al*[11], using bovine serum albumin as standard.

3. Results and discussion

The relative inhibitory capacities of the extracts of millets and cereals (in the linear range of inhibition) on the caseinolytic, amidolytic (BAPNA) and esterolytic (ATEE) activities of the human and bovine pancreatic preparations are shown in Table I. Setaria had comparable action on both the pancreatic preparations with respect to the inhibition of all the three activities. Echinocloa was nearly 50% more active in inhibiting the caseinolytic, amidolytic and esterolytic reactions of the bovine pancreatic preparation. Similarly, sorghum was also slightly more active on the bovine system. Corn and barley also exhibited higher inhibition of the bovine preparation but some differences in the individual reactions are discernable. Corn inhibited the amidolytic activity of the bovine system more than two fold effectively whereas with reference to caseinolytic and esterolytic inhibitions the preference is not so highly magnified. Barley was characteristic in that it inhibited the esterolytic activity of the bovine preparation about 5.6 fold more effectively. Kodo was the only seed that had preferential action on the human system, the ratios of the relative activities being 2.3 (caseinolytic), 2.9 (amidolytic) and 1.7 (esterolytic) in favour of the inhibition of the human pancreatic preparation. Ragi and pearl millet exhibited mixed patterns. Even though ragi was 1.7 and 4.0 times, respectively, more active in blocking the caseinolytic and esterolytic activities of the bovine extract, it was 1.4 times more effective in inhibiting the amidolytic activity of the human extract. Pearl millet which was equally effective in inhibiting the caseinolytic activities of the two pancreatic preparations, was nearly two times more active on the bovine system in regard to inhibition of the amidolytic and esterolytic activities.

Table I

Relative inhibitory activities in seed extract against human and bovine pancreatic preparations

Seed extract	Caseinolytic inhibition (units/mg protein)		Amidolytic (BAPNA) inhibition (units/mg protein)		Esterolytic (ATEE) inhibition (units/mg protein)	
	Human	Bovine	Human	Bovine	Human	Bovine
Setaria	0.47	0.52	1.05	0.98	0.04	0.04
Echinocloa	0.40	0.60	0.93	1.33	0.21	0.32
Sorghum	0.27	0.31	0.20	0.27	0.058	0.062
Corn	1.37	1.83	2.29	5.00	1.03	1.37
Barley	0.64	1.44	1.12	1.92	0.17	0.96
Kodo	0.25	0.11	1.00	0.35	0.120	0.07
Ragi	1.50	2.60	5.20	3.60	0.4	1.60
Pearl millet	0.40	0.40	0.36	0.64	0.014	0.03

The diminution of amidolytic and esterolytic activities of the pancreatic preparations was proportional to inhibitor concentration up to 50-70% inhibition with different seed extracts. Complete inhibition (95-100%) of the amidolytic activity could be demonstrated with the bovine system with extracts of corn (0.18 mg protein), ragi (0.4 mg), barley (0.5 mg), echinocloa (1.2 mg), setaria (1.3 mg) and pearl millet (2.0 mg) and in human system with ragi (0.4 mg), corn (0.7 mg), echinocloa (1.2 mg), setaria (1.3 mg) and pearl millet (4.0 mg). Similarly, complete inhibition of ATEE hydrolysis in bovine pancreatic preparation was observed with ragi (0.1 mg protein), barley (0.2 mg), corn (0.18 mg), echinocloa (0.6 mg) and sorghum (1.8 mg) and in the human system with ragi (0.1 mg), corn (0.18 mg), barley (0.8 mg), and echinocloa (1.2 mg). Complete inhibition could not be shown in other cases either due to relatively lower inhibitory capacities of the seeds or poor linearity of inhibition at higher ranges.

The caseinolytic inhibition unlike the amidolytic and esterolytic inhibition was linear only over a narrow range (up to 20-40%) with respect to the volume of the seed extract in all cases. In the case of setaria alone a limiting maximal inhibition of one third of the caseinolytic activity was observed with both the pancreatic preparations. The inhibition profiles of setaria extract are shown in fig. 1 (A,B,C). For comparison, the action of ragi, one of the millets with relatively high inhibitory activity, is also represented (fig. 1 D,E,F). Since setaria extract had no action on the caseinolytic activity of the bovine chymotrypsin and very poor activity on elastase (see below), it can be suggested that the limiting inhibition observed represents the caseinolytic activity contributed by trypsin in both the human and bovine pancreatic preparations.

The inhibitory capacities of the eight seed extracts on crystalline trypsin, chymotrypsin and elastase, the three important endopeptidases of pancreatic origin, by the caseinolytic method are shown in Table II. Unlike with crude pancreatic preparations, the caseinolytic inhibition of pure trypsin or chymotrypsin was linear with respect to concentration of the seed extracts up to 50-70% inhibition (except with kodo which had very poor activity).

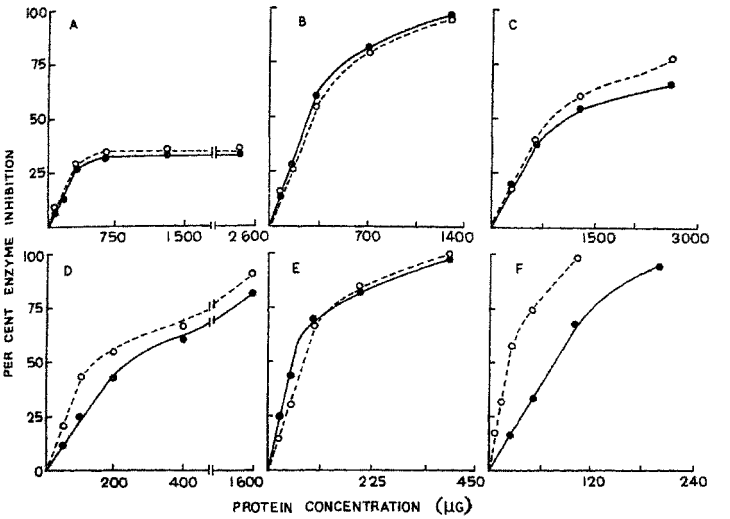

FIG. 1. Inhibition of proteolytic activity in human (•——•) and bovine (0——0) pancreatic extracts by setaria seed extract (A,B,C) and by ragi seed extract (D,E,F).

A,D — Caseinolytic inhibition; B,E — Amidolytic (BAPNA) inhibition; C,F — Esterolytic (ATEE) inhibition.

Table II

Relative inhibitory activities in seed extract against pure enzymes

Seed extract	Bovine trypsin	Bovine chymotrypsin units/mg protein	Porcine elastase
Barley	1.68	0.40	0.16
Corn	1.37	0.46	0.34
Ragi	2.00	0.60	0.30
Echinocloa	0.60	0.23	0.20
Setaria	0.46	Nil	0.05
Pearl millet	0.40	Nil	0.12
Sorghum	0.16	0.31	0.27
Kodo	0.05	0.06	0.13

However, with elastase the linearity of inhibition was generally poor (15-30%). Barley, corn, ragi and echinocloa were respectively 4.2, 3.0, 3.3, and 2.6 times more active on trypsin than on chymotrypsin. Sorghum and kodo, on the other hand, were more active on chymotrypsin. Setaria and pearl millet did not exhibit any action on chymotrypsin. These data are in agreement with the patterns obtained with purified enzyme inhibitors of setaria[12], pearl millet[3], ragi[2], corn[4], sorghum[1], and barley[5]. The elastase inhibitory capacity relative to antitryptic activity was poor in all the seeds except with sorghum and kodo. These data indicate the wide differences in the spectrum of action of the inhibitors from grains.

Analysis of the data presented above reveals some apparent anomalies. While the caseinolytic inhibition of pure trypsin and chymotrypsin by the seed extracts was proportional to inhibitor concentration over a wide range, the patterns with pancreatic preparations were not. Yet, the esterolytic or amidolytic inhibition of the crude pancreatic extracts by the seed extracts was linear over a broader range. Comparison of amidolytic and esterolytic inhibition of bovine pancreatic preparation (Table I) indicate that bovine tryptic activity (BAPNA hydrolysis) is more powerfully inhibited than the chymotryptic activity (ATEE hydrolysis) by sorghum and kodo. Yet, the studies with pure enzymes (Table II) show that these seeds block bovine chymotrypsin rather than trypsin more effectively based on the caseinolytic inhibition. One of the reasons for these differences could be that the inhibitors when mixed with a crude pancreatic preparation could be bound to different target enzymes reducing the observed potency or they could be subjected to limited proteolysis by non-target enzymes altering their activity. Alternatively, the discrepancies could be due to the differences in magnitudes of inhibition depending on the nature of the substrate. It is pertinent to point out that cationic human trypsin was weakly inhibited when assayed with BAPNA as the substrate compared to the magnitude of inhibition with casein as substrate by Kunitz soybean inhibitor[13]. On the other hand, human chymotrypsin was more effectively inhibited by the same inhibitor when a synthetic substrate rather than casein was used[13]. Differential patterns of inhibition depending on the nature of the substrate were also reported for acacia seed inhibitor[14]. To clarify some of the unexpected patterns, studies comparing the inhibitory activities with pure enzymes in presence of casein and synthetic substrates were undertaken.

In Table III, the magnitude of inhibition of trypsin, chymotrypsin and elastase with casein as well with a corresponding synthetic substrate by the seed extracts is compared. In regard to antitryptic activity, wide difference in the degree of inhibition depending on the nature of the subtrate was observed only with kodo and pearl millet. Kodo extract was eight times more powerful in its antitryptic activity when BAPNA was used as the substrate. Conversely, pearl millet was two times more effective in blocking tryptic activity when casein was the substrate. Even though data in Table I suggest that sorghum and kodo extracts will be poor inhibitors of chymotrypsin with ATEE as substrate, the studies with pure enzymes belied this expectation. In regard to chymotrypsin inhibition, a significant difference depending on the substrate was observed only with kodo. Profound effect of substrate on the magnitude of inhibition was found in studies with elastase. In all the cases, inhibition was more with casein than with STANA. Differences even of an order of magnitude was seen with pearl millet and sorghum, emphasizing the importance of substrate in determining inhibition patterns by protease inhibitors.

Table III
Relative inhibition of pure enzymes with casein and synthetic substrates by seed extracts

Seed extract	Inhibition of bovine trypsin in presence of		Inhibition of bovine chymotrypsin in presence of		Inhibition of porcine elastase in presence of	
	Casein	BAPNA	Casein	ATEE	Casein	STANA
	(μg of enzyme inhibited by one mg protein of the seed extract)					
Kodo	0.83	6.92	1.30	2.70	3.47	1.56
Sorghum	2.59	2.59	6.48	6.22	7.41	1.11
Ragi	33.30	30.00	12.50	9.40	8.33	1.56
Corn	22.90	25.70	7.62	7.14	9.52	4.29
Barley	28.00	26.60	8.33	9.17	5.00	3.00
Echinocloa	10.00	8.80	4.86	4.86	6.94	1.24
Setaria	7.18	6.60	Nil	—	1.28	0.38
Pearl millet	6.60	3.35	Nil	—	3.30	0.25

The human pancreatic preparation showed very poor STANA hydrolytic activity and hence the effect of seed extracts on this activity could not be investigated. The effect of the seed extracts on STANA hydrolysis by bovine pancreatic preparation is shown in Table IV. The inhibitory activities were generally low and unlike with inhibition of BAPNA and STANA hydrolyses, the linearity was restricted to a narrow range of 20-30% except in the cases of corn, barley and ragi. Near complete inhibition of STANA hydrolysis was found with ragi (2.0 mg protein), corn (2.8 mg) and barley (5.0 mg). It is reasonable to assume that as was observed with porcine elastase, inhibition of bovine and other animal elastases could be more in presence of a protein substrate. It has been reported that elastase accounts for

Table IV
Inhibition of STANA hydrolytic activity of the bovine pancreatic preparation by seed extracts

Seed extracts	Inhibition of STANA hydrolysis (units/mg protein)
Ragi	0.40
Corn	0.40
Kodo	0.20
Echinocloa	0.20
Barley	0.20
Setaria	0.038
Sorghum	0.025
Pearl millet	0.02

20-25% of total proteolytic activity of the human pancreatic system [15,16] and for 15% in bovine system[13]. In view of these points, detailed studies on the effect of plant protease inhibitors on elastases will be nutritionally relevant.

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References

1. HARISH KUMAR, P.M., VIRUPAKSHA, T.K. AND VETHAYATHIL, P.J. Sorghum proteinase inhibitors. Purification and some biochemical properties. *Int. J. Peptide Res.*, 1978, **12**, 185-196.

2. SHIVARAJ, B. AND PATTABIRAMAN, T.N. Natural plant enzyme inhibitors. Characterization of an unusual α-amylase trypsin inhibitor from ragi (*Eleusine coracana Gaertn*), *Biochem. J.*, 1981, **193**, 29-36.

3. CHANDRASHEKAR, G. AND PATTABIRAMAN, T.N. Natural plant enzyme inhibitors. Isolation and characterization of two trypsin inhibitors from bajra. *Indian J. Biochem. Biophys.*, 1982, **19**, 1-7.

4. JOHNSON, R., MITCHELL, A.L., WASSOM, C.E. AND REECK, G.R. Comparison on trypsin inhibitors from several types of corn. *Phytochemistry*, 1980, **19**, 2749-2750.

5. MIKOLA, J. AND SUOLINNA, E.M. Purification and properties of a trypsin inhibitor from barley. *Eur. J. Biochem.*, 1969, **9**, 555-560.

6. LIEPNICKS, J.J. AND LIGHT, A. The preparation and properties of bovine enterokinase, *J. Biol. Chem.*, 1979, **254**, 1677-1683.

7. SUMATHI, S. AND PATTABIRAMAN, T.N. Natural plant enzyme inhibitors VI. Studies on trypsin inhibitors of *Colocasia antiquorum* tubers. *Biochim. Biophys. Acta*, 1979, **556**, 115-127.

8. ERLANGER, B.F., KOKOWSKY, N. AND COHEN, W. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.*, 1961, **95**, 271-278.

9. BIETH, J., SPIESS, B. AND WERMUTH, C.G. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem. Med.*, 1974, **11**, 350-357.

10. PRABHU, K.S. AND PATTABIRAMAN, T.N. A colorimetric method for the estimation of the esterolytic activity of chymotrypsin. *Indian J. Biochem. Biophys.*, 1977, **14**, 96-98.

11. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 1951, **193**, 265-275.

12. UDUPA, S. AND PATTABIRAMAN, T.N. Natural plant enzyme inhibitors. Studies on a proteinase inhibitor from Italian millet (*Setaria italica*). *J. Sci. Fd. Agric.*, 1984, **6**, 679-688.

13. MALLORY, P.A. AND TRAVIS, J. Human pancreatic enzymes. Characterization of anionic human trypsin. *Biochemistry*, 1973, **12**, 2847-2851.

14. PRABHU, K.S., SAIDHNAA, K. AND PATTABIRAMAN, T.N. Natural plant enzyme inhibitors: A comparative study of the action of legume inhibitors on human and bovine pancreatic proteases. *J. Sci. Fd Agric.*, 1984, **35**, 314-321.

15. MALLORY, P.A. AND TRAVIS, J. Inhibition spectra of the human pancreatic endopeptidases. *Am. J. Clin. Nutr.*, 1975, **28**, 823-830.

16. KROGDAHL, A. AND HOLM, H. Inhibition of human and rat pancreatic proteinases by crude and purified soybean proteinase inhibitor. *J. Nutr.*, 1979, **109**, 551-558.