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INHIBITION OF TRYPSINS AND CHYMOTRYPSINS FROM DIFFERENT ANIMAL SPECIES: A COMPARATIVE STUDY

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Abstract—1. The effect of 3 purified trypsin inhibitors and 4 legume seed extracts on the trypsins and chymotrypsins of the activated pancreata of 11 animal species, including man, was measured.

2. The activation was performed by either homologous enterokinase or by bovine trypsin. Several trypsinogens were not activated by the latter.

3. Rabbit trypsin was the most sensitive to all inhibitor preparations, while the human trypsin was the most resistant, except to the black bean extract.

4. The response of the chymotrypsins was more variable and those of capybara and rabbit showed extreme sensitivity.

5. Considerable differences between the extracts of black and white garden beans, both *Phaseolus vulgaris*, with respect to their reactivity toward different animal enzymes were detected.

6. No relation between relative pancreas weight and susceptibility toward soybean trypsin inhibitor could be observed.

INTRODUCTION

The inhibition of pancreatic proteolytic enzymes by plant extracts, especially those from legume seeds may have nutritional consequences (Liener and Kakade, 1980). Although the inhibitors are thermolabile, some residual activity remains in standard heat-treated plant foods (Churella *et al.*, 1976). Research regarding the effect of trypsin inhibitors in foods has been performed mostly on soybeans. From the point of view of human nutrition other legume seeds are even more important than soybeans, depending on the population groups involved. In animal feeding the addition of uncooked legumes to feed with their inhibitors intact, may reduce its nutritional value.

Contradictory results have been reported with respect to the relative inhibitory activity of soybean trypsin inhibitors toward trypsins of different origins, and considerable differences in the susceptibility of the pancreatic proteases of different animal species have been observed (Krogdahl and Holm, 1983). The use of the commercially available bovine trypsin as a reference enzyme has been advocated by Belitz *et al.* (1982) and criticized by Krogdahl and Holm (1979) and Holm and Krogdahl (1982).

The relative sensitivity of the pancreatic proteases of animals toward different inhibitors may bear upon the overall physiological process of digestion. In the present work we have compared the activity of seven inhibitor preparations, four seed extracts and three commercial inhibitors on the enzymes of 12 animal species including bovine and human, as a contribution to the study of their possible undesirable nutritional effects.

MATERIALS AND METHODS

Zymogen activation

Pancreata from recently killed animals were weighed, cut into small cubes of about 0.6 g and either processed immediately or frozen for later use.

Each cube was suspended in 10 vol. (w/v) of a solution of 5 mM Tris–HCl, 40 mM $CaCl_2$, pH 7.8, and finely chopped with scissors. The human pancreas, that of a young man killed in an accident, was cut into cubes and frozen immediately after autopsy. For activation of the zymogens a piece of finely minced duodenum from the same animal was added to the suspension or else a solution of crystalline bovine trypsin (EC 3.4.21.4) (Sigma, type III) was added up to a final concentration of 1 μg/ml. The human pancreas was activated by bovine trypsin only, because either human duodenum or pancreatic juice were available. The suspensions were incubated in a shaker-waterbath at 37°C and aliquots were removed at regular intervals for the determination of trypsin activity. When activity reached maximum the suspension was centrifuged and the supernatant adjusted to pH 4.0 with 1 M HCl and again clarified by centrifugation. This activated enzyme extract could be stored at 4°C for several days without loss of activity. Aliquots were also frozen in individual vials for later use. The pancreas of two animals or two groups of animals were activated and assayed three times each with the only exception of human pancreas, where all experiments were performed with the one organ available.

The pancreata of the following animals were processed: rabbit (*Oryctolagus cuniculus*), mouse (*Mus musculus*), rat (*Rattus rattus*), hamster (*Cricetus cricetus*), guinea pig (*Cavia* sp.), sheep (*Ovis musimon*), capybara (*Hydrochoerus hydrochoeris*), dog (*Canis familiaris*), pig (*Sus* sp.), horse (*Equus equus*), and human. The pancreas-body weight relation was calculated in each case.

Protease activities

Tryptic activity was measured as described by Erlanger

et al. (1966) using benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) (Sigma) as substrate. The volume of the extract used in each test was adjusted to give an activity equal to 20 μg of crystalline bovine trypsin, that is an absorbance of 0.400 at 410 nm, in a 10 min experiment.

Chymotryptic activity was determined following the procedure of Schwert and Takenaka (1955) with *N*-benzoyl-L-tyrosine-ethylester (BTEE) (Sigma) as substrate. The volume of pancreas extract per assay was equivalent to the activity of 2.4 μg of bovine chymotrypsin (EC 3.4.21.1) (Sigma) giving a slope of 0.500 at 257 nm.

Plant extract preparations and purified inhibitors

Finely ground black beans (*Phaseolus vulgaris*, Cubagua variety), white beans (*Phaseolus vulgaris*), black lima beans (*Phaseolus lunatus*, Panguita variety), and defatted seeds of soya (*Glicine max*) were suspended in 10 vol. of distilled water (w/v) and stirred for 24 hr at 4°C. After clarifying by centrifugation the protein concentration of the supernatant was determined by the Lowry *et al.* (1951) procedure and adjusted to 300 mg/ml by dilution with water.

The Kunitz soybean trypsin inhibitor (SBTI) (Sigma) and the lima bean inhibitor (LBI) (Sigma) were dissolved in distilled water at a concentration of 20 μg/ml and the egg white inhibitor (EWI) (Calbiochem) was prepared at 100 μg/ml also in water.

Each inhibitor preparation was tested at different concentration levels selected as to decrease the initial activity by 50%. Assays were run in triplicate and the results were expressed as the amount of inhibitor required to reduce the activity of 1 μg of trypsin or chymotrypsin by 50%.

RESULTS

Zymogen activation caused by bovine trypsin and by homologous enterokinase preparations showed remarkable differences between species. In rat, mouse, capybara, sheep and pig twice as much trypsin activity was obtained when homologous small intestine extracts, instead of bovine trypsin were used for activation. The time curves were similar to those represented in Fig. 1. Dog trypsinogen was activated by both enterokinase and bovine trypsin to the same extent. Bovine trypsin caused no detectable activation of rabbit, hamster, horse or guinea pig zymogens under the experimental conditions used.

Rabbit trypsin was the most sensitive to all the plant inhibitor preparations, while human trypsin was the most resistant, except to the black bean extract. A remarkable similarity between horse and human trypsins was evident with most plant inhibitors (Table 1). Between the two varieties of *P. vulgaris*, the black bean extract was more active than the white one in all cases except with guinea pig trypsin, which was inhibited to the same extent by both. LBI was less active than SBTI, even though the lima bean extract was always more active than the soybean extract. The EWI reacted with all the pancreatic preparations to nearly the same extent, with the notable exception of the human enzyme, which was only very slightly affected. In general, the plant inhibitors showed a great variability toward the trypsins of the different species. The differences between the most and the less active inhibitor preparations were more than six-fold as illustrated by their reaction with rabbit and human trypsin, respectively (Table 1). The response of the chymotrypsins to the plant inhibitors tested was even more variable, capybara and rabbit being extremely sensitive, while hamster generally the less reactive (Table 2). EWI-

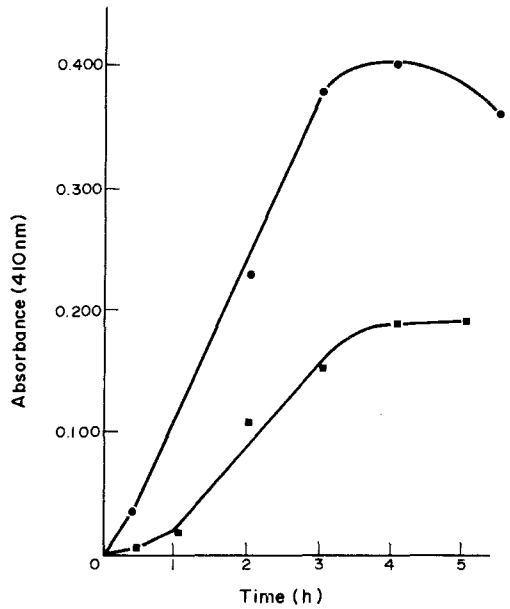

Fig. 1. Trypsinogen activation in sheep pancreatic extract. ●——●: Enterokinase activation. ■——■: Trypsin activation.

Table 1. Inhibition of trypsin of different origins*

Animal	Inhibitors (nmoles × 10^{-2}) SBTI†	LBI‡	EWI§	Extracts (μg) Black bean	White bean	Lima bean	Soybean
Mouse	0.55	1.20	0.90	1.05	1.72	1.16	1.53
Hamster	0.35	0.80	0.60	0.57	1.31	0.62	0.84
Rat	0.40	1.20	0.95	0.56	1.41	0.61	1.15
Guinea pig	0.25	1.10	0.80	1.56	1.34	0.84	1.60
Rabbit	0.10	0.40	0.75	0.35	0.51	0.35	0.53
Capybara	0.60	1.30	0.95	0.68	1.88	0.94	1.63
Dog	0.35	1.30	0.90	0.75	1.50	0.75	1.50
Sheep	0.50	1.30	0.95	0.60	1.48	0.85	1.28
Pig	0.60	1.40	1.10	0.85	1.98	1.03	1.78
Horse	0.45	2.20	1.10	1.36	2.50	0.92	1.79
Human	0.85	2.20	∞	0.99	3.38	1.22	3.57
Bovine¶	0.50	2.30	1.50	2.25	3.10	2.44	3.52

*nmoles of inhibitor and μg of bean extracts causing 50% inhibition of 1 μg of bovine trypsin or its equivalent activity of pancreas preparation as measured by BAPNA hydrolysis.
†SBTI: Soybean trypsin inhibitor.
‡LBI: Lima bean inhibitor.
§EWI: Egg white inhibitor.
¶Commercial bovine trypsin (Sigma).

Table 2. Inhibition of chymotrypsin of different origins*

Animal	Inhibitors (nmoles × 10^{-2}) SBTI†	LBI‡	EWI§	Extracts (μg) Black bean	White bean	Lima bean	Soybean
Mouse	7.4	2.2	23.8	3.13	5.24	6.58	15.72
Hamster	8.9	5.2	19.5	28.82	26.63	17.75	33.83
Rat	7.5	4.4	27.7	8.75	10.08	16.05	18.30
Guinea pig	2.0	1.8	15.2	1.56	2.57	6.80	10.59
Rabbit	1.9	0.7	4.2	4.38	2.82	2.71	4.14
Capybara	0.2	1.9	2.1	0.52	0.96	1.17	13.67
Dog	11.5	1.7	25.7	0.52	1.47	1.19	4.38
Sheep	∞	3.3	22.3	0.54	3.75	2.67	∞
Pig	20.9	2.3	22.3	0.88	2.02	1.50	25.00
Horse	36.1	3.6	28.2	1.25	4.59	2.63	12.29
Human	4.8	4.2	∞	0.94	3.96	5.50	6.44
Bovine¶	2.7	1.7	∞	0.25	0.72	0.36	0.78

*nmoles of inhibitor and μg of bean extracts causing 50% inhibition of 1 μg of bovine chymotrypsin or its equivalent activity of pancreas preparation as measured by BTEE hydrolysis.
†SBTI: Soybean trypsin inhibitor.
‡LBI: Lima bean inhibitor.
§EWI: Egg white inhibitor.
¶Commercial bovine chymotrypsin (Sigma).

inhibition of chymotrypsin also varied widely, capybara being the most, bovine and human chymotrypsins the less affected. SBTI showed to be a potent inhibitor of capybara and rabbit chymotrypsins, while not recognizing the sheep enzyme at all. This was also true for the soybean extract used in a parallel experiment. The difference between the responses of the capybara and horse chymotrypsins was more than 100-fold. Rabbit chymotrypsin was about 8 times more sensitive towards LBI than the hamster enzyme. A parallelism between the activities of the seed extracts and their corresponding purified commercial plant inhibitors was observed. No relation between relative pancreas weight and the susceptibility toward SBTI (Liener, 1979) could be detected.

DISCUSSION

The susceptibility of the trypsins and chymotrypsins of the 12 animal species studied toward the different inhibitors shows a wide range of variability. A general conclusion about "the range of sensitivity" can, therefore, not be drawn from these experiments. SBTI, although more active on trypsins, also inactivated chymotrysins of all species except sheep. The relation of SBTI and soybean extract activities on trypsins was rather constant, but showed some unusual behaviour toward chymotrypsins; the capybara enzyme, for example, was relatively more sensitive to SBTI than to the soybean extract, while dog chymotrypsin responded more to the crude preparation than to the commercial product. EWI, although generally considered to be a trypsin inhibitor, reacted with most chymotrypsins, with the notable resistance of both the human and bovine enzymes, results described previously by Feeney *et al.* (1969), Mallory and Travis (1975) and by Krogdahl and Holm (1979).

It is interesting to note that the two bean samples, the black and the white varieties, both *P. vulgaris* reacted differently with the various trypsins and chymotrypsins. These facts are in accord with the observations of Holm and Krogdahl (1982) who showed that genetically different soybeans had different inhibitory activities on the pancreatic proteases of three animal species. The differences observed are not surprising since a large number of trypsin inhibitors exist in seeds and their concentrations and solubilities, as well as those of other extractable proteins, vary greatly from one variety to another (Seidl *et al.*, 1978; Liener, 1979). These findings indicate that the experimental results obtained with one cultivar of seeds should only very cautiously be extrapolated to another even of the same species. Belitz *et al.* (1982) found important differences between 20 plant inhibitors in their action on human and bovine proteinases. In many cases human chymotrypsin was more sensitive than the bovine enzyme, and in other cases this was true for both human trypsin and chymotrypsin. We found that the lima bean extract inhibited bovine chymotrypsin much more than the human enzyme, while the forementioned authors obtained opposite results. On the other hand, in Krogdahl and Holm's (1979) experiments, as in ours, rat trypsin was more sensitive to inactivation by SBTI than human trypsin. Similarities as well as differences between results reported in literature may be due to the level of inhibitor used in testing, as pointed out by Holm and Krogdahl (1982); however, the affinities of inhibitors to proteases of different animals, when tested on one and the same level as reported in Tables 1 and 2, are significant and real.

It is widely but maybe erroneously accepted, that the small amounts of enzyme inhibition of soybean products, which may have withstood thermal inactivation in human food preparations, are without nutritional and physiological significance (Rackis and McGee, 1975; Churella *et al.*, 1976). This inhibitory activity, however, has not been measured on the human enzymes. Also, no long term feeding experiments have been reported about other vegetable products with inhibitors that were not completely destroyed by the cooking procedures. The *in vitro* inhibition of pancreatic proteinases, as studied in the present paper, does not indicate the *in vivo* effect of the inhibitor in the animal. Struthers *et al.* (1983) compared rats, pigs and monkeys fed raw soybean flour. The response indicated that the control mechanisms for synthesis of pancreatic nucleic acids and enzymes are dissimilar in these animals; therefore extrapolation of results from one animal species to

another is rather risky and subject to the same reasoning as extrapolation from one seed species to another.

It is evidently not enough to know the affinity of trypsin inhibitors to the trypsin of a certain animal for the assessment of its respective physiological significance. The susceptibility of the inhibitors to digestion by gastric juice and their effect on the pancreata may also be different.

The estimations of the pancreatic function *in vivo* in animals fed inhibitor-containing diets by the procedure of Lee and Liener (1981) is a useful method, but its ability to detect slight effects, such as residual inhibitor activity in heat-treated human food products has to be proven in long term feeding studies. These products as well as others of vegetable origin consumed regularly, and whose inhibitors are not completely destroyed, should be tested on the human proteases before accepting the proposal that they do not possess nutritional and physiological significance (Rackis and McGee, 1975; Churella *et al.*, 1976); and finally, the relative ability of inhibitors to reduce proteolytic activity is only one of several factors to be taken into account for the prediction of the overall effect of the food stuff in a given animal.

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