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Inhibition of Human Trypsin, Plasmin, and Thrombin by Naturally Occurring Inhibitors of Proteolytic Enzymes*

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SUMMARY

Human trypsin was strongly inhibited by three apparently unrelated inhibitors: lima bean trypsin inhibitor, bovine colostrum inhibitor, and bovine Kunitz pancreatic inhibitor. It was inhibited to varying degrees by Kunitz soybean inhibitor, soybean inhibitor AA, kidney bean inhibitor, black-eyed pea inhibitor, navy bean inhibitor, and quail ovomucoid. It was essentially uninhibited by bovine Kazal pancreatic inhibitor, porcine Kazal pancreatic inhibitor, potato inhibitor, chicken ovoinhibitor, and 10 avian ovomucoids including that of chicken. Many of these proteins strongly inhibit bovine trypsin. Thus, inhibition of bovine trypsin by naturally occurring inhibitors is no index of their activities against human trypsin. The activities of these inhibitors against human plasmin were generally similar to those against human trypsin, except that the activities of lima bean inhibitor and quail ovomucoid were comparatively weaker and the activity of the Kunitz soybean inhibitor was much stronger. The esterolytic activity of human thrombin was not affected by any of the inhibitors.

Naturally occurring protein inhibitors of proteolytic enzymes differ widely in their inhibitory activity against different enzymes as well as in their physical and chemical structures (1–3). Of major interest in this field has been the inhibition of two bovine pancreatic enzymes, trypsin and α-chymotrypsin. In fact, inhibitors are usually differentiated according to their activity against these two enzymes.

That wide variations in inhibitory capabilities exist, even among homologous inhibitors, is exemplified by the differences in activity of the various avian egg white ovomucoids against these two pancreatic enzymes. For example, chicken[1] ovomucoid inhibits bovine trypsin but has no demonstrable effect on bovine α-chymotrypsin (4). In contrast, golden pheasant ovomucoid has strong inhibitory activity against α-chymotrypsin but only very weak activity against bovine trypsin. Turkey ovomucoid is "double headed," inhibiting both enzymes simultaneously, apparently at noninteracting sites. Homologous multiple forms of inhibitors with similar varying specificities toward trypsin and α-chymotrypsin have also been found in such sources as lima beans (5) and soybeans (6).

Reports on the effects of protein inhibitors on other proteolytic enzymes have appeared, but the amounts of quantitative information on these other enzymes is small compared to that on the bovine pancreatic enzymes (7–13). Limited observations have been made on the inhibition of pancreatic extracts of salmon (10) and on turkey trypsin (13), chicken chymotrypsin (13), and human and ovine trypsins (7). Chicken chymotrypsin was inhibited by turkey ovomucoid, and turkey trypsin was inhibited by both chicken and turkey ovomucoids. These interactions appeared to occur in a manner similar to the inhibition of the respective bovine enzymes. The salmon enzymes were inhibited by the Kunitz soybean trypsin inhibitor (10). Human trypsin was inhibited by a serum trypsin inhibitor and by STI[2] (14), and ovine as well as bovine trypsin was inhibited by chicken ovomucoid (7). However, the fact that human trypsin was not inhibited by chicken ovomucoid was reported in a single preliminary communication (7). More information is available on the inhibition of human plasmin (fibrinolysin), doubtlessly due to the ready availability of purified preparations of this particular enzyme. Plasmin was reported to be inhibited by bovine pancreatic inhibitor (11), the lima bean trypsin inhibitor (8, 11), a kidney bean inhibitor (12), and STI (8, 9, 11), but not by chicken ovomucoid (11). In contrast, the fibrin-clotting activity of thrombin has been reported to be inhibited by the Kazal bovine

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[1] The scientific names of species used in this article are: cassowary, *Casuarius aruensis;* chicken, *Gallus gallus;* duck, *Anas platyrhynchos;* emu, *Dromiceius* n. *hollandiae;* golden pheasant, *Chrysolophus pictus;* ostrich, *Struthio camelus;* penguin, *Pygoscelis adeliae;* Japanese quail, *Coturnix coturnix japonica;* rhea *Rhea americana;* tinamou, *Eudromia elegans;* turkey, *Meleagris gallopavo;* black-eyed pea, *Vigna sinensis;* kidney bean, *Phaseolus vulgaris;* lima bean, *Phaseolus lunatus;* navy bean, *Phaseolus vulgaris;* soybean, *Glycine max;* potato, *Solanum tuberosum;* domestic cow, *Bos taurus;* pig, *Sus scrofa domesticus;* and human, *Homo sapiens.*

[2] The abbreviations used are: STI, Kunitz soybean trypsin inhibitor; PTI, bovine Kunitz pancreatic trypsin inhibitor.

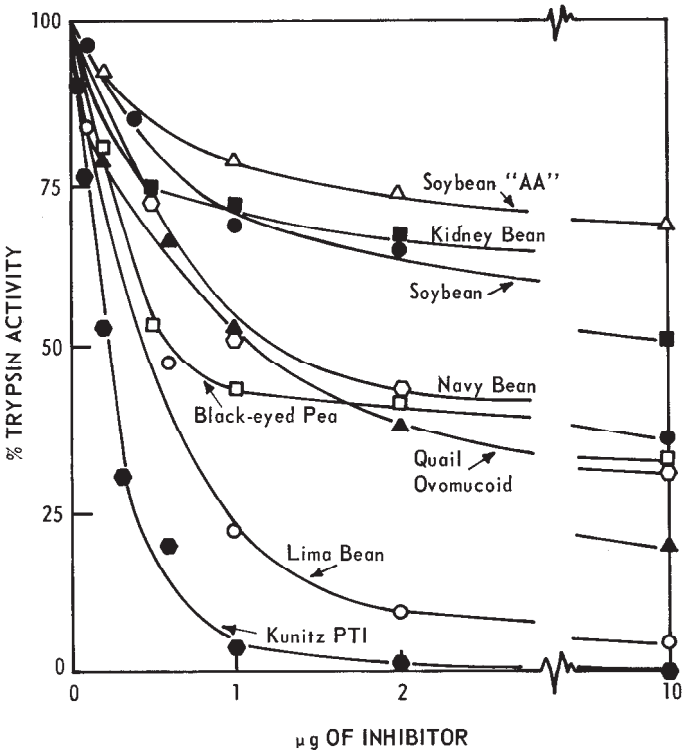

FIG. 1. The inhibition of human trypsin by naturally occurring inhibitors. The substrate was the synthetic substrate toluenesulfonyl-L-arginine methyl ester, and the assay was a modified Hummel assay (26). The amount of enzyme preparation was 4 μg (1.33 μg per ml in a final volume of 3 ml).

pancreatic inhibitor, although the esterase activity was not inhibited (15). Other reports have indicated that thrombin is not inhibited by pancreatic inhibitors (16).

The importance of the inhibition of human enzymes, both as a fundamental problem in comparative biochemistry and as concerns the obvious implications involving human nutrition and medicine, prompted a more quantitative study of the inhibition of human pancreatic trypsin and human blood plasmin and thrombin. The present report describes the inhibitory effects of 23 different naturally occurring protein inhibitors against these enzymes.

METHODS

Ovomucoids from chicken, golden pheasant, turkey, duck, Japanese quail, penguin, cassowary, emu, ostrich, rhea, and tinamou were isolated from egg whites of the appropriate species by trichloracetic acid-acetone precipitation and purified by sequential chromatography on DEAE-cellulose (4, 17). Chicken ovoinhibitor was prepared as previously described (4). Potato inhibitor I was a gift of Dr. C. A. Ryan, Washington State University (18). The porcine and bovine Kazal pancreatic inhibitors (15, 19, 20) were gifts of Dr. P. J. Burck of Eli Lilly and Company, and PTI (21) was a gift from Dr. B. Kassell of Marquette University. Kidney bean component C,[3] black-eyed pea component D (22), and the inhibitor from navy bean (23) were gifts from Dr. J. P. Riehm of the University of California at Santa Barbara. Soybean AA (6) was purchased from Miles Laboratories, Inc., and STI was purchased from Worthington (this is a mixture of several inhibitors). Lima bean trypsin inhibitor was purchased from Nutritional Biochemicals (this is also a mixture, consisting of four to six fractions, all of which inhibit bovine trypsin (5)). Bovine colostrum inhibitor was that prepared by Haynes, Osuga, and Feeney (24).

Human trypsin[4] was prepared by activation of a sample of human trypsinogen by bovine trypsin. The human trypsinogen (25) was a gift from Dr. P. J. Keller of the School of Dentistry, University of Washington. The two samples of urokinase-activated plasmin (11) were gifts from Dr. G. H. Barlow of Abbott Laboratories and Dr. K. C. Robbins of the Michael Reese Research Foundation. Thrombin was a purified fraction and the gift of Dr. A. Pappenhagen of Cutter Laboratories, Berkeley, California.

Trypsin inhibitor activities were determined with the Hummel assay with toluenesulfonyl-L-arginine methyl ester (26) as substrate, modified for inhibitory assays (5), or by the Kunitz assay (27) with casein as substrate.

RESULTS

Effects of Inhibitors on Human Trypsin—Of the 23 inhibitors tested against human trypsin, only lima bean trypsin inhibitor, bovine colostrum inhibitor, and PTI had strong inhibitory activity with toluenesulfonyl-L-arginine methyl ester as substrate (Fig. 1; Table I). The stoichiometries of these three inhibitors indicated 1:1 molar combining ratios with the enzyme. The degree of inhibition indicated dissociation constants of approximately 10^{-9} M. In contrast, both bovine and porcine Kazal pancreatic trypsin inhibitors,[5] the potato inhibitor, chicken ovoinhibitor, and all of the avian ovomucoids (with the exception of one) had essentially no inhibitory activity. The soybean inhibitor (AA) of Birk (6), STI, and the kidney bean trypsin inhibitor had weak but definite inhibitory activity. Inhibitory activity of intermediate strength was found with the navy bean and black-eyed pea inhibitors and quail ovomucoid.[6]

Nearly identical results were obtained in an assay with human trypsin, with casein as the substrate. When tested at a level of 2 μg of inhibitor against 1 μg of human trypsin, the inhibitors effected the indicated per cent inhibition of the enzyme: bovine Kazal pancreatic trypsin inhibitor (<10%), chicken ovomucoid (<10%), kidney bean trypsin inhibitor (35%), STI (68%), black-eyed pea inhibitor (70%), navy bean inhibitor (72%), quail ovomucoid (76%), lima bean trypsin inhibitor (95%), bovine colostrum inhibitor (95%), and bovine Kunitz PTI

[3] The samples of inhibitors from navy bean, kidney bean, and black-eyed pea were gifts of Dr. J. P. Riehm. The kidney bean inhibitor fraction was a strong inhibitor of bovine trypsin.

[4] As received from Dr. P. J. Keller, the sample of human trypsinogen was reported to give, after activation with bovine trypsin, 10 to 15 μM benzoyl arginine ethyl ester split per min per mg. After activation by trypsin in our laboratory, the preparation had approximately 45% the activity of a commercial preparation of bovine trypsin with either toluenesulfonyl-L-arginine methyl ester or casein as a substrate. Values for amounts of trypsin used in different experiments were estimated on the basis of the activity of the activated mixture against toluenesulfonyl-L-arginine methyl ester. The preparation had no detectable (<2%) activity against benzoyltyrosine ethyl ester, a substrate for bovine α-chymotrypsin.

[5] Inhibitory properties of the porcine inhibitor were presented at the 154th Meeting of the American Chemical Society in Chicago, 1967, by P. J. Burck, E. W. Cerwinsky, and E. L. Grinnan.

[6] In the estimation of the inhibitory activities, both the molecular weight and the inhibitions at low and high inhibitor-enzyme ratios must be considered. On this basis, quail ovomucoid should be considered as a very good inhibitor of human trypsin.

TABLE I

Properties of naturally occurring inhibitors of proteolytic enzymes

Source	Type	Mol Wt	Enzymes inhibited[a]					General references
			Pancreatic			Plasma		
			Bovine		Human	Human		
			Tryp	Chym	Tryp	Plasmin	Thrombin	
Colostrum, Bovine		10,500	+++	−	+++	+++	−	1, 24
Egg White								
Chicken	Ovomucoid	28,000	++++	−	−	−	−	1
Chicken	Ovoinhibitor	46,500	++++	+++	−	−	−	1
Quail	Ovomucoid	28,000	++++	−	++	−	−	1
Tinamou	Ovomucoid	28,000	+	+++	−	−	−	1
Turkey	Ovomucoid	28,000	++++	+++	−	−	−	1
Pancreas								
Bovine	Kunitz	6,513	++++	++	++++	++++	−	21
Bovine	Kazal	6,155	++	−	−	−	−	15, 16
Porcine	Kazal	6,024	++	−	−	−	−	15, 16
Soybean	Kunitz	21,000	++++	+	+	+++	−	1
	(AA)	24,000	+	++	+	+	−	6
Lima bean		10,000	++++	+++	++++	+	−	5
Navy bean		23,000	+++	+++	++	+	−	23
Kidney bean		10,000	+++	+++	+	+	−	12[c]
Pea (black-eyed)		17,000	++	+++	++	+	−	22
Potato		38,000	+	+++	−	−	−	13

[a] Tryp and Chym refer to trypsin and α-chymotrypsin, respectively. The degree of inhibition is indicated as −, for extremely weak or inactive, and +, ++, +++, and ++++, for varying degrees, progressing from weak to strong inhibition.

[b] These references are general references for preparation and physical and chemical properties of inhibitors. The data for the enzymes inhibited were obtained in the present investigation.

[c] See text Footnote 3.

(99%). At higher ratios of inhibitor to enzyme, STI is apparently a better inhibitor.

Effects of Inhibitors on Human Plasmin—The bovine Kunitz PTI had strong activity against human plasmin, while all of the inhibitors from legumes had definite but weaker activities. Thus the inhibitions were, in general, similar to those of human trypsin. However, at least three definite differences were noted, the most striking of which was the much weaker activity of lima bean trypsin inhibitor against human plasmin. The stronger activity of STI as well as the apparent lack of activity of quail ovomucoid against plasmin was also significant. As indicated in Fig. 2, PTI inhibited plasmin most extensively, in agreement with the results of Robbins and Summaria (11). STI had a lesser amount of inhibitory activity than PTI, but was much stronger than the other bean inhibitors, all of which were relatively weak. This is in contrast to the results with human trypsin, for which lima bean trypsin inhibitor was strongly inhibitory and STI only moderately inhibitory. Bovine colostrum inhibitor was a good inhibitor of plasmin like STI.

Effects of Inhibitors on Human Thrombin—No inhibition of human thrombin was noted with any of these 23 inhibitors, with toluenesulfonyl-L-arginine methyl ester as substrate.

DISCUSSION

It is becoming increasingly apparent that homologous inhibitors, such as the avian ovomucoids, vary in their inhibitory capacities as well as in their specificities for any particular proteolytic enzyme (1). In addition, the activity of a particular inhibitor may actually be so specific that it fails to inhibit homologous enzymes from different species. One presumed example is reported in this paper. The human trypsin used was not inhibited by chicken ovomucoid, which generally is a good inhibitor of the trypsins of several other species. When the different specificities of ovomucoids toward human trypsin, bovine trypsin, and bovine α-chymotrypsin are considered, the possible different combinations of inhibitory relationships become numerous. An obvious extension of the current study would be the examination of the inhibition of human chymotrypsin by those proteins which inhibit bovine α-chymotrypsin. Other interesting studies would include comparisons of the inhibitory activities of homologous inhibitors toward bacterial proteinase (subtilisin), bovine α-chymotrypsin, and other chymotrypsins. Recent studies have indicated that avian ovomucoids vary in their relative degrees of inhibition of bovine α-chymotrypsin and subtilisin. For example, subtilisin will displace bovine α-chymotrypsin from its complex with penguin ovomucoid, while other avian ovomucoids have a stronger affinity for α-chymotrypsin.[7] Similar relationships most probably exist with the plasmins from different species, as well as between plasmin and trypsin.

The practical significance of these findings is directly related to human nutrition and medicine. Whether or not human trypsin and human chymotrypsin are inhibited by protein inhibitors in

[7] BIGLER, J. C., AND FEENEY, R. E., *Abstracts Pacific Slope Biochemical Conference at the University of California, Santa Barbara, California, September 4 to 6, 1968.*

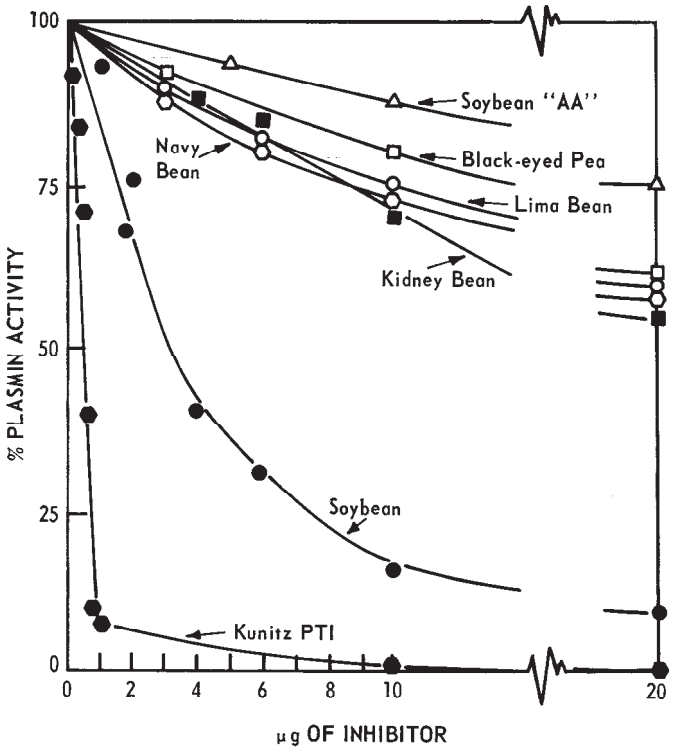

FIG. 2. The inhibition of human plasmin by naturally occurring inhibitors. The substrate was casein, and the assay was according to Kunitz (27). The amount of enzyme preparation was 10 μg (5 μg per ml in a final volume of 2 ml).

the diet is obviously important. Studies have been made to determine whether various protein inhibitors are inactivated by digestive enzymes from various animal sources, but similar studies do not appear to have been made with human enzymes. There are thus at least two questions of interest from a nutritional standpoint. (*a*) Does the inhibitor arrive in an active form in the intestine? (*b*) Is the inhibitor capable of inhibiting one of the human enzymes in the intestine? From the present results it seems desirable to undertake just such a detailed study of the various lima bean inhibitors recently described (5). Also, of current practical importance is the bearing which these results have on the general approach to the testing of biological activities. Many of these tests are based on the belief that the fundamental metabolism of most living cells is similar and that, with the exception of minor metabolic differences, one animal may be used to test the biological effects of various biochemicals on other animals. This is the basis for the extensive testing of chemicals on experimental animals, such as rats and mice, and extrapolation of these results to man. The results of the present study indicate that such extrapolation may be a poor method for macromolecules. Macromolecular interactions require fittings which are more complicated as compared to those between smaller molecules. The dogma concerning similarity of metabolic pathways does not extend to include interactions of macromolecules with one another.

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