Acidic Bovine Pancreatic Trypsin Inhibitor. II. Specificity of Inhibition*

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ABSTRACT: An acidic polypeptide trypsin inhibitor (APTI), identical with pancreatic trypsin inhibitors previously isolated by Kazal *et al.* (Kazal, L., Spicer, D., and Brahinsky, R. (1948), *J. Am. Chem. Soc. 70*, 3034) and Greene *et al.* (Green, L. J., Fackre, D. S., and Rigbi, M. (1966), *J. Biol. Chem. 241*, 5610), has been purified from bovine pancreas. This inhibitor has a specificity markedly different from other trypsin inhibitors, in particular the basic Kunitz pancreatic trypsin inhibitor (KPTI). APTI reacts with trypsin to form a stoichiometric complex similar to that observed with KPTI. The stability of the APTI–trypsin complex is much less than that of the complex with KPTI. This is identical with the phenomenon of "temporary inhibition" observed with the Kazal inhibitor by Laskowski and Wu (Laskowski, M., and Wu, F. C. (1953), *J. Biol. Chem. 204*, 797). APTI does not inhibit chymotrypsin, kallikrein, plasmin, or urokinase. It is a potent inhibitor of thrombin clotting activity, but it does not inhibit thrombin esterase activity.

Kunitz and Northrop (1936) crystallized a polypeptide trypsin inhibitor from bovine pancreas which also inhibited chymotrypsin (Wu and Laskowski, 1955), kallikrein (Werle and Appel, 1959), and thrombin (Ferguson, 1942). Kazal *et al.* (1948) reported the isolation of a crystalline trypsin inhibitor from bovine pancreas which was a potent thrombin inhibitor, but did not inhibit chymotrypsin (Wu and Laskowski, 1955). More recently Greene *et al.* (1966) purified an anionic trypsin inhibitor from bovine pancreatic juice which appears to be identical with the Kazal inhibitor. Fritz *et al.* (1966a,b) have isolated a very specific trypsin inhibitor from dog pancreas and pancreatic juice. This polypeptide does not inhibit chymotrypsin, kallikrein, thrombin, plasmin, or pepsin.

The present study was carried out to compare the inhibitory properties of a pancreatic trypsin inhibitor isolated in our laboratory (Cerwinsky *et al.*, 1967) from bovine pancreas with other known trypsin inhibitors.

Materials and Methods

Inhibitors and Enzymes. All assays involved a comparison of the inhibitory properties of our acidic pancreatic trypsin inhibitor (APTI)[1] and KPTI. In some cases the inhibitory activities of LBTI and SBTI were also investigated. The APTI used was that described in the previous paper (Cerwinsky *et al.*, 1967). This inhibitor was homogeneous upon electrophoresis in acrylamide or starch gel and had a specific activity greater than 2.50. In all cases the KPTI used was a crystalline, salt-free material (Worthington Biochemical Corp.). The SBTI was a three-times-crystallized preparation (Worthington Biochemical Corp.), and the LBTI (Worthington Biochemical Corp.) was fraction III of the preparation described by Fraenkel-Conrat *et al.* (1952). The trypsin used was a two-times-crystallized preparation obtained from Worthington Biochemical Corp. The chymotrypsin was a crystalline product obtained from the Armour Laboratories. The kallikrein used for the inhibition assays was a commercial preparation of *Padutin* (Bayer, Leverkusen, Germany). The thrombin used was a commercial preparation of Thrombin, Topical (Parke Davis and Co.). The urokinase was the Urokinase International Reference Standard supplied by the Leo Pharmaceutical Products Co., Denmark. The human plasmin was kindly supplied by the Michigan Department of Health, Division of Laboratories, through the American Red Cross.

Inhibition of Proteolytic Enzymes. The inhibition of the tryptic digestion of casein was determined by a modification of the tryptic assay method of Kunitz (1947). Trypsin (1 ml, 20 μg/ml) in 0.0025 N HCl was incubated with 1.0 ml of the trypsin inhibitor (0–5 μg/ml) in 0.1 M sodium phosphate (pH 7.6) for 5 min at 25°. This solution (1 ml) was then added to 1.0 ml of 1% casein in 0.1 M sodium phosphate (pH 7.6) at 35°. After a 20-min incubation at 35°, the reaction was stopped by the addition of 3.0 ml of 5% TCA. After standing for 1 hr at 25°, the samples were centrifuged at 1000*g* for 10 min, and the absorbance of

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[1] Abbreviations used: APTI, acidic pancreatic trypsin inhibitor; KPTI, Kunitz pancreatic trypsin inhibitor; LBTI, lima bean trypsin inhibitor; SBTI, soybean trypsin inhibitor; BAEE, benzoylarginine ethyl ester; ATEE, acetyltyrosine ethyl ester; TAME, *p*-toluenesulfonylarginine methyl ester; TCA, trichloroacetic acid; KIU, kallikrein inhibition unit.

the clear supernatant fluids was measured at 280 mμ.

The stability of the complex of trypsin with APTI and KPTI was determined by assaying a solution of the complex for the presence of free trypsin using the method of Schwert and Takenaka (1955) or of free inhibitor using the method of Kassell *et al.* (1963). In both cases the Determatube TRY (Worthington Biochemical Corp.) was substituted for the BAEE solution. The complex was formed by incubating 40 mg of trypsin with 10.5 mg of the inhibitor in 10 ml of 0.07 M Tris-HCl (pH 8.0). A slight excess of the inhibitor was used to impart initial stability to the APTI–trypsin complex.

The inhibition of the chymotryptic digestion of casein was determined using a modification of the method of Wu and Laskowski (1955) for the measurement of chymotrypsin activity. Chymotrypsin (1 ml, 20 μg/ml) in 0.0025 N HCl–0.02 M $CaCl_2$ was incubated with 1.0 ml of the inhibitor (0–100 μg/ml) in 0.1 M sodium borate (pH 8.0) for 5 min at 25°. This solution (1 ml) was then added to 1.0 ml of 1% casein in 0.1 M sodium borate (pH 8.0) at 35°. After a 20-min incubation at 35°, the reaction was stopped by the addition of 3.0 ml of 5% TCA. After standing for 1 hr at 25°, the samples were centrifuged at 1000*g* for 10 min, and the absorbance of the clear supernatant fluids was measured at 280 mμ.

Chymotrypsin esterase inhibition was determined by a modification of the method of Schwert and Takenaka (1955) for the measurement of chymotrypsin activity. The Determatube CHY (Worthington Biochemical Corp.) was substituted for their solution of ATEE. Chymotrypsin (1 ml, 100 μg/ml) in 0.0025 N HCl–0.02 M $CaCl_2$ was incubated with 1.0 ml of the inhibitor (0–500 μg/ml) in 0.07 M Tris-HCl (pH 8.0) for 5 min at 25°. This solution (0.2 ml) was then added to 2.8 ml of the Determatube CHY solution, and the decrease in absorbance was followed at 237 mμ.

The inhibition of kallikrein esterase activity was determined using the method of Trautschold and Werle (1961). The Determatube TRY was substituted for their substrate solution of BAEE. Kallikrein (1 ml, 10 KIU/ml) in 0.0025 N HCl was incubated with 1.0 ml of the inhibitor (0–10 μg/ml) in 0.07 M Tris-HCl (pH 8.0) for 5 min at 25°. This solution (0.2 ml) was then added to 2.8 ml of the Determatube TRY solution, and the increase in absorbance at 253 mμ was determined.

Inhibition of Blood Coagulation Enzymes. Thrombin inhibition was determined by incubating 0.5 ml of bovine thrombin (20 NIH units/ml) in 0.9% NaCl with 0.5 ml of the inhibitor (0–5000 μg/ml) in 0.9% NaCl for 15 min at 37°. This solution (0.2 ml) was then added to 0.2 ml of citrated human plasma (outdated blood bank plasma) and the clotting time was measured (Kazal *et al.*, 1948).

The inhibition of clotting of recalcified, citrated plasma (modification of Ferguson, 1942) was determined by incubating 0.5 ml of citrated human plasma, 0.5 ml of 0.9% NaCl, and 0.3 ml of the inhibitor (0–1000 μg/ml) in 0.9% NaCl for 15 min at 37°. $CaCl_2$ (0.2 ml of 1%) was then added, and the clotting time of the recalcified plasma was determined.

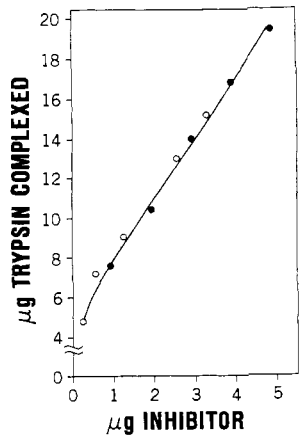

FIGURE 1: Inhibition of the tryptic digestion of casein by KPTI (●) and APTI (○). The amount of free trypsin was determined (as described in Materials and Methods) and subtracted from the total amount of trypsin in the system (20 mg) to determine the amount of trypsin complexed by the inhibitors.

Inhibition of Fibrinolytic Enzymes. Inhibition of urokinase was determined indirectly by incubating 0.5 ml of urokinase (4 CTA units/ml)[2] in 0.9% NaCl with 0.5 ml of the inhibitor (0–2000 μg/ml) in 0.9% NaCl for 15 min at 25°. The incubation solution (30 μl) was then pipetted onto a 0.2% fibrin plate (Alkjaersig *et al.*, 1959). The plate was incubated for 16 hr at 37°, and the zone of lysis obtained was compared with those obtained with various dilutions of urokinase without inhibitor.

The same procedure was followed for the determination of plasmin inhibition using plasminogen-free fibrinogen (Bergstrom and Wallen, 1961) for the preparation of the fibrin plates and using human plasmin (4 casein units/ml) in place of urokinase.

Results

Inhibition of Proteolytic Enzymes. APTI and KPTI complex the same amount of trypsin when inhibition of the tryptic digestion of casein is measured. The results shown in Figure 1 indicate that APTI must react with trypsin on an equimolar basis similar to the formation of a complex of KPTI and trypsin. The APTI–trypsin complex is much less stable than the KPTI–trypsin complex, however. This can be seen in Figure 2. As soon as free trypsin is present in the solution of the APTI–trypsin complex, the complex is no longer stable because the APTI is now a substrate for the unbound trypsin. A rapid increase in the level

[2] Provisional standard adopted by the Committee on Thrombolytic Agents of the National Heart Institute.

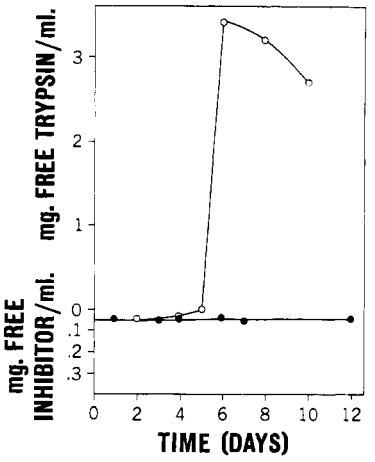

FIGURE 2: Stability of the complex of trypsin with KPTI (●) and APTI (○).

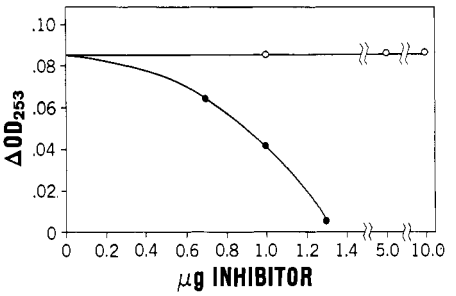

FIGURE 3: Inhibition of kallikrein esterase activity by KPTI (●) and APTI (○). The release of benzoyl arginine from BAEE by kallikrein was followed at 253 mμ.

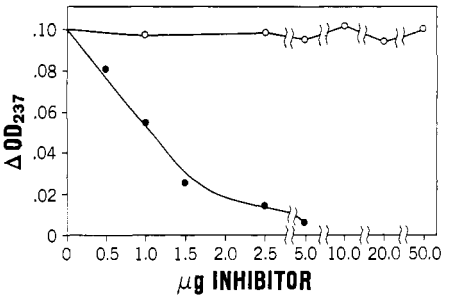

FIGURE 4: Inhibition of chymotrypsin esterase activity by KPTI (●) and APTI (○). The liberation of acetyltyrosine from ATEE by chymotrypsin was followed at 237 mμ.

of free trypsin is observed as the APTI is digested, and trypsin is liberated from the complex.

At the concentrations used APTI did not inhibit kallikrein as did KPTI. The Kunitz inhibitor caused a 50% inhibition of kallikrein esterase activity at a concentration of 1 μg of KPTI/KIU of kallikrein.

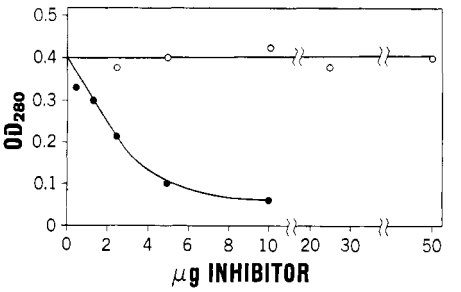

FIGURE 5: Inhibition of the chymotryptic digestion of casein by KPTI (●) and APTI (○). The digestion of casein by chymotrypsin was followed at 280 mμ (as described in Materials and Methods).

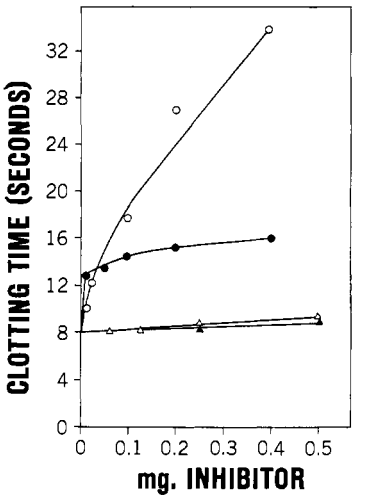

FIGURE 6: Inhibition of thrombin clotting activity by KPTI (●), APTI (○), SBTI (▲), and LBTI (△).

Even at a concentration of 10 μg of APTI/KIU of kallikrein, there is no inhibition of kallikrein esterase activity (Figure 3).

APTI also did not inhibit the proteolytic or esterase activity of chymotrypsin at the concentrations used. At a level of 1 μg of KPTI/10 μg of chymotrypsin, a 50% inhibition of the chymotrypsin esterase activity against ATEE is observed. However, even at a level of 50 μg of APTI/10 μg of chymotrypsin, no inhibition is observed (Figure 4). When 2.5 μg of KPTI is preincubated with 10 μg of chymotrypsin, a 50% inhibition of the chymotrypsin proteolytic activity against casein is observed. At a level of 50 μg of APTI/10 μg of chymotrypsin, no inhibition of chymotrypsin proteolytic activity occurs (Figure 5).

Inhibition of Blood Coagulation and Fibrinolytic Enzymes. KPTI is a weak inhibitor of thrombin clotting activity, and the ability to delay the clotting time of plasma treated with thrombin rapidly reaches a level at which there is only a small delay in the clotting time with increasing levels of KPTI (Figure 6). Much

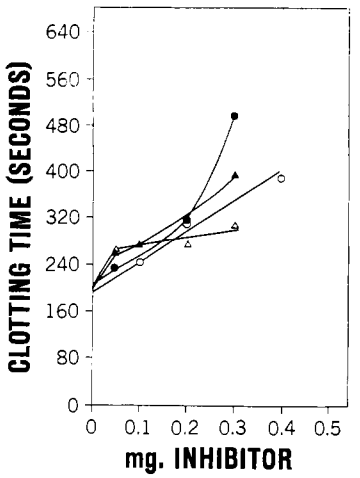

FIGURE 7: Inhibition of the clotting of recalcified plasma by KPTI (●), APTI (○), SBTI (▲), and LBTI (△).

greater delays in clotting time are observed with APTI at levels above 50 μg of inhibitor/2 units of thrombin. LBTI and SBTI were also inactive at the levels used.

APTI, KPTI, LBTI, and SBTI are all inhibitors of the clotting of recalcified plasma, although at high-dose levels KPTI appears to be the best inhibitor (Figure 7).

APTI is not nearly as effective an inhibitor of the fibrinolytic enzymes, plasmin, and urokinase, as KPTI. Only at very high-dose levels does one observe any inhibition of these enzymes by APTI (Table I). LBTI and SBTI also appear to be poor inhibitors of urokinase.

TABLE I: The Inhibition of Plasmin and Urokinase by Trypsin Inhibitors.

Inhibitor	Level in Assay (mg/ml)	% Plasmin Inhibn	% Urokinase Inhibn
APTI	2.00	100	20
	1.00	33	15
	0.50	10	12.5
	0.25	0	0
KPTI	0.1000	100	100
	0.0500	100	100
	0.0250	100	100
	0.0125	8	100
	0.0020		100
	0.0010		45
	0.0005		15
LBTI	1.0000		100
	0.5000		100
	0.2500		85
	0.1250		45
	0.0625		25
SBTI	1.0000		100
	0.5000		100
	0.2500		100
	0.1250		55
	0.0625		40

TABLE II: Comparative Inhibitor Activity of APTI and KPTI.

Type of Inhibition	APTI	KPTI
Trypsin protease	+	+
Trypsin esterase	+	+
Chymotrypsin protease	−	+
Chymotrypsin esterase	−	+
Kallikrein esterase	−	+
Thrombin clotting	++	+
Thrombin esterase	−	−
Recalcified plasma clotting	+	+
Urokinase	−	+
Plasmin	−	+

Discussion

We have characterized a trypsin inhibitor which was isolated and purified from a fraction obtained in a commercial process for the isolation of insulin from bovine pancreas. The trypsin inhibitor we have isolated is different in several respects from KPTI when the inhibitory properties of the two inhibitors are compared (Table II).

APTI inhibits trypsin activity in a manner analogous to that observed with KPTI when inhibition of the tryptic digestion of casein is determined. It is apparent that like KPTI and the trypsin inhibitor of Greene *et al.* (1966), which are about the same molecular weight, APTI reacts with trypsin on an equimolar basis. However, APTI is digested by trypsin, and as a result the APTI–trypsin complex is not as stable as that of KPTI and trypsin. This similarity to the "temporary inhibition" of trypsin observed with the Kazal inhibitor by Laskowski and Wu (1953) and more recently observed by Greene *et al.* (1966) with their inhibitor prepared from bovine pancreatic juice is further proof of the identity of APTI with these inhibitors. As with the Kazal inhibitor, a slight excess of APTI must be present when the complex with trypsin is formed to ensure initial stability of the APTI–trypsin complex. As soon as free trypsin is present, the complex is destroyed by digestion of the APTI.

The identity of APTI with the Kazal inhibitor, an inhibitor from pancreatic juice (Greene *et al.*, 1966), and an inhibitor from bovine pancreas (Fritz *et al.*, 1966b) is further established by the fact that APTI also does not inhibit chymotrypsin esterase or proteolytic activity. KPTI, however, is a very potent chymotrypsin inhibitor.

APTI differs markedly from other trypsin inhibitors in its ability to inhibit enzymes involved in blood coagulation. APTI is a much more potent inhibitor of thrombin clotting activity than are KPTI, LBTI, and SBTI. Fritz *et al.* (1967) have reported that the inhibitor they have isolated from bovine pancreas does not inhibit thrombin. However, they studied only the inhibition of thrombin esterase activity with BAEE. APTI, likewise, does not inhibit thrombin esterase activity when the inhibition of the hydrolysis of TAME is measured. In this respect, APTI is similar in its specificity to the α-2-trypsin-binding macroglobulin described by Lanchantin *et al.* (1966). This inhibitor, isolated from Cohn plasma fraction III-O, forms a complex with thrombin which is inactive against fibrinogen but hydrolyzes TAME. Both APTI and this macroglobulin may react with free amino groups in the thrombin molecule, since acetylation of these groups by acetic anhydride results in a modified thrombin which has no clotting ability but which still possesses esterase activity (Seegers *et al.*, 1960).

KPTI is a more potent inhibitor of clotting of recalcified plasma than APTI, LBTI, and SBTI, although these inhibitors all possess significant inhibitory activity. KPTI also inhibits two enzymes involved in fibrinolysis, plasmin, and urokinase. APTI is a very poor inhibitor of these enzymes. LBTI and SBTI are inhibitors of urokinase only at very high dose levels.

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