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PROTEIN BINDING OF PANCREATIC PROTEOLYTIC ENZYMES *

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In human serum there are two protein fractions that are potent inhibitors to trypsin. These inhibitors travel with the α_1 -globulin and α_2 -globulin fractions when serum proteins are separated by electrophoresis (1-3). One ml of normal serum has sufficient amounts of trypsin inhibitor to neutralize the proteolytic effect of 1.15 (± 0.10) mg of crystalline trypsin. Approximately 90 per cent of the trypsin inhibitory proteins in serum migrate in the electrophoretic cell with the α_1 -globulin fraction, and the remainder with the α_2 -globulin fraction (3).

It is known that in many diseases of unrelated etiology the level of total serum trypsin inhibitor may be increased. This increase, therefore, is not specific for any disease, including acute pancreatitis. Also, it is likely that an increase in the α_1 -globulin trypsin inhibitor is no more specific in the various diseases than is a sedimentation rate. However, the results of a previous study indicate that in acute pancreatitis the α_2 -globulin inhibitor decreases, and in severe pancreatitis it frequently disappears. The divergent changes of the two serum globulin trypsin inhibitors in acute pancreatitis result in a marked increase in the ratio of the α_1 - to α_2 -globulin trypsin inhibitors. The decrease in the α_2 -globulin trypsin inhibitor in acute pancreatitis has been helpful in our laboratory in the diagnosis of acute pancreatitis (3).

The determination of the serum α_1 - and α_2 -globulin trypsin inhibitors involves the lengthy procedure of serum protein separation by electrophoresis, elution of protein fractions which involves many hours, and then the determination of the trypsin-inhibiting capacity of the protein fractions. The present complexities of this procedure make the determination difficult for the routine hospital laboratory. Because of these factors we

sought methods that would permit an easier assay of the α_2 -globulin trypsin inhibitor. Many studies were made which included the determination of the inhibiting activities of the α_1 - and α_2 -globulin fractions to chymotrypsin, elastase, thrombin, plasmin, and collagenase. Although interesting data were obtained, these studies did not permit the differential assay of the serum α_1 - and α_2 -globulin trypsin inhibitors unless electrophoresis was done.

Continuing along this line of investigation, we determined that when trypsin was added to serum and the mixture subjected to electrophoresis, a substance with trypsin-like activity was detected, migrating with the α_2 -globulin fraction. This was an unexpected finding, since the serum to which trypsin was added contained sufficient trypsin inhibitor to completely neutralize many times the amount of trypsin added. Also, other known inhibitors of trypsin and plasmin failed to inhibit this activity.

The aims of this study were: 1) to investigate the properties of the substance with the trypsin-like activity that appears in the α_2 -globulin fraction when trypsin is added to serum (this substance is designated *trypsin-protein esterase*); and 2) to determine whether the addition of chymotrypsin to serum will produce a substance with proteolytic activity which migrates with the α_2 -globulin fraction (*chymotrypsin-protein esterase*).

METHODS AND MATERIALS

The following substances were used in this study: 1) Benzoyl-L-arginine-paranitroanilide (BAPNA), originally developed by Karmen, was synthesized as previously reported (4). 2) A supersaturated aqueous solution of BAPNA in a concentration of 1 mg per ml was prepared by heating to 85° C until dissolution was complete and then cooling in an ice bath. The solution may be stored at room temperature for at least 1 month without appreciable change. 3) 0.1 M Tris buffer, pH 7.67, 0.005 M in CaCl_2 ; 0.005 M Tris buffer, 0.5 M in NaCl .

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4) Trypsin standards; lyophilized crystalline trypsin (Worthington Biochemical Corp.) was used. A stock standard of trypsin in a concentration of 1 mg per ml was prepared in 0.0025 N HCl. Working standard solutions of trypsin were prepared by diluting the stock standards to the desired concentration. Protein content of trypsin standards was determined by measuring at 280 m μ in the Beckman DU spectrophotometer and using the conversion factor of 0.585 as described by Kunitz (5). 5) Chymotrypsin; lyophilized crystalline chymotrypsin (Worthington Biochemical Corp.) was used. 6) Casein substrate; 3 g of vitamin-free casein (Pfanstiehl) was suspended in 100 ml of Tris buffer adjusted to pH 7.6 with HCl. The suspension was heated in a boiling water bath for 15 minutes to dissolve the casein. The solution had a final pH of 7.0. 7) Veronal buffer, pH 8.6, 0.075 ionic strength. 8) Bromphenol blue dye; 0.10 g of dye and 50 g ZnSO₄·H₂O in 50 ml of glacial acetic acid were diluted to 1 L with water. 9) Paper strips; Whatman 3 MM, 3.0 cm wide \times 30.6 cm long (Spinco, 300-028). 10) BAPNA substrate for spraying; a 400 mg per 100 ml solution was prepared by dissolving the substrate in a 1:1 solution of dimethyl-formamide:Tris buffer. 11) Partially purified α_1 -globulin trypsin inhibitor was prepared by a modification of the method described by Bundy and Mehl (6). A diethylaminoethyl cellulose chromatographic column was used in place of Dowex-1. Alpha₁-globulin trypsin inhibitor was prepared by ammonium sulfate precipitation and elution from a diethylaminoethyl cellulose column.¹ 12) β -Toluene sulfonyl-L-arginine methyl ester (TAME) (Mann Research Labs.); a 0.1 M solution was prepared in the following buffer: 0.005 M Tris containing 0.04 M NaCl and 0.02 M CaCl₂, adjusted to pH 8.0 with HCl. 13) N-acetyl-L-tyrosine ethyl ester (ATEe; Mann Research Labs.) was dissolved in a solution of 50 per cent 0.01 M Tris buffer (pH 7.8, 0.005 M in CaCl₂) and 50 per cent methanol, to a concentration of 0.36 M. 14) For starch block electrophoresis, purified potato starch washed four times with barbital buffer (0.075 M, pH 8.67) was used.

The following methods were used:

1. *Measurement of trypsin.* 1) Trypsin digestion of casein was measured according to the method of Kunitz as modified by Bundy and Mehl (2). 2) Esterase activity of trypsin was measured, with the TAME substrate and the automatic titrator with titrographic recording (Copenhagen). To 1.0 ml of the substrate and 8.0 ml of buffer, incubated at 25° C, was added 1 ml of the enzyme dissolved in saline, or 1 ml of starch block eluates. A constant pH of 8 was maintained by the addition of NaOH in a concentration of 0.02 N or 0.05 N. The volume of alkali added is recorded as a function of time and computed as the mean number of micromoles of acid liber-

¹ The authors are grateful to Dr. John Mehl, Professor and Chairman, Department of Biochemistry, University of Southern California School of Medicine, for a supply of the partially purified α_1 - and α_2 -globulin trypsin inhibitors.

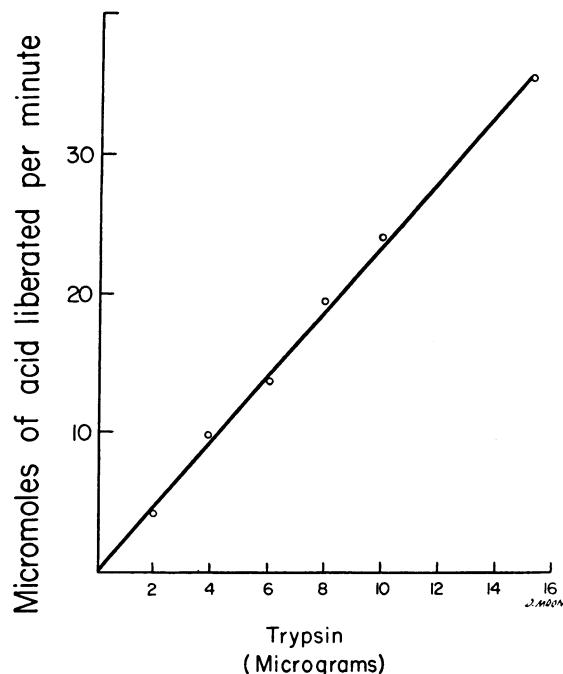


FIG. 1. THE HYDROLYSIS OF β -TOLUENE SULFONYL-L-ARGININE METHYL ESTER (TAME) BY TRYPSIN. Automatic titrator with titrographic recording; 0.05 N NaOH was used as titrant.

ated per unit time (Figure 1). 3) Activity of trypsin also was measured, with the synthetic substrate BAPNA (4). Trypsin splits this substrate to yield benzoyl-L-arginine and the yellow dye, paranitroaniline. Trypsin activity in a 3.0-ml BAPNA system, measured in Beckman absorbance units per minute at 383 m μ , is directly proportional to the amount of trypsin added. When the change in absorbance per minute is recorded, trypsin can be assayed accurately in the range of 0.5 to 1.0 μ g. To increase the sensitivity, trypsin was incubated with BAPNA and buffer for 5 minutes or longer and the reaction stopped by the addition of 1 ml of 0.25 N HCl. The total increase in absorbance compared with blanks can accurately assay trypsin in the range of 0.25 μ g (Figure 2).

2. *Measurement of chymotrypsin.* 1) Chymotrypsin digestion of casein was measured according to the method of Kunitz as modified by Bundy and Mehl (2). 2) Esterase activity of chymotrypsin was measured, with the substrate ATEE, in the automatic titrator with titrographic recording. To 3 ml of the ATEE substrate solution and 2 ml of buffer, incubated at 25° C, was added 1 ml of the enzyme dissolved in saline, or 1 ml of the starch block eluates. A constant pH of 7.8 was maintained by the addition of NaOH in a concentration of 0.02 or 0.05 N.

3. *Paper electrophoretic separation of serum or mixtures of serum and enzyme.* Duplicate or triplicate 50- μ l samples of serum were applied to filter paper strips (Whatman 3MM) and the protein fractions separated

electrophoretically in the Spinco model R electrophoresis cell. Veronal buffer, pH 8.6, 0.075 ionic strength, was used and the system allowed to run 20 to 24 hours at approximately 150 v and 15 ma. Protein localization was obtained by staining one strip with bromphenol blue. Tryptic-like activity was located by spraying with a concentrated BAPNA solution. Protein eluates were prepared by cutting a strip in 1.0-cm sections and eluting each fraction in 1.0 ml of 0.1 M Tris buffer, pH 7.67, overnight at 5° C.

4. *Starch block electrophoretic separation of serum or mixtures of serum and enzyme.* Four ml of serum or 4 ml of serum containing 3.36 mg of crystalline chymotrypsin was applied to starch blocks measuring 38 × 5 × 1 cm. The system was allowed to run for 25 to 30 hours at 5° C with 122 v across the block. Protein fractions were eluted from 1-cm sections with 10 ml of cold isotonic saline for 16 hours. The eluates were filtered and the protein content determined by measuring absorbance at 280 m μ in the Beckman DU spectrophotometer.

5. *Trypsin inhibitor assay of serum protein fractions.* 1) Paper electrophoresis; 0.8 ml of the eluate from each centimeter section (except in the α_1 -globulin region where high inhibitor levels permitted the use of smaller quantities) was incubated for 5 minutes with the trypsin standard; the residual trypsin then was assayed, with the BAPNA substrate. After a 10-minute incubation period the hydrolysis of the substrate was stopped by the addition of 1.0 ml of 0.25 N HCl and the absorbance recorded.

2) Starch block electrophoresis; 0.2 ml of starch block eluates (0.1 ml of fractions containing large amounts of inhibitor) was incubated with 20 μ g of trypsin for 5 minutes at 37° C; residual trypsin then was assayed, with TAME as the substrate in the automatic titrator.

6. *Determination of trypsin-binding protein of serum.* 1) Paper electrophoretic fractions; to 1 ml of serum,

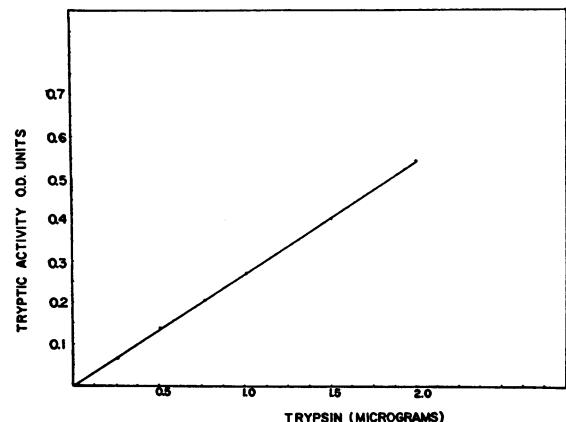


FIG. 2. THE HYDROLYSIS OF THE SUBSTRATE BENZOYL-L-ARGININE-PARANITROANILIDE (BAPNA) BY TRYPSIN. The tryptic activity is expressed as absorbance units per 80-minute incubation period; measurements were made in the Beckman DU spectrophotometer at 383 m μ .

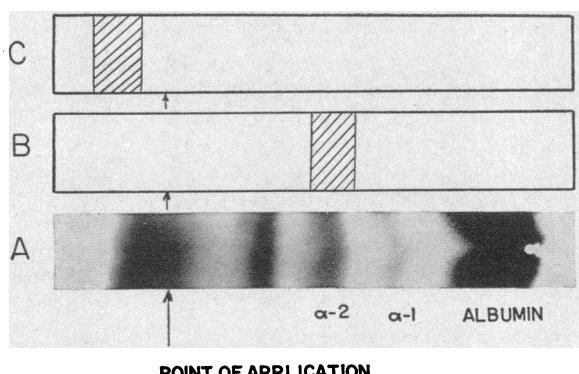


FIG. 3. PAPER STRIPS A AND B REPRESENT ELECTROPHORESIS OF 50 μ l OF A MIXTURE OF 800 μ g CRYSTALLINE TRYPSIN IN 1 ML NORMAL SERUM. A WAS STAINED WITH BROMPHENOL BLUE FOR PROTEIN LOCALIZATION AND B WAS SPRAYED WITH A SOLUTION OF BAPNA IN DIMETHYLFORMAMIDE. C REPRESENTS ELECTROPHORESIS OF 40 μ g CRYSTALLINE TRYPSIN WHICH WAS THEN SPRAYED WITH A SOLUTION OF BAPNA. The development of a yellow band depicted by the hatched area in B indicated the presence of tryptic activity in the α_2 -globulin zone. Crystalline trypsin alone migrated toward the cathode as illustrated by the hatched area in C.

amounts of trypsin varying from 50 to 2,000 μ g were added. The proteins in the mixture of trypsin and serum were separated by electrophoresis according to the procedure described in paragraph 3. Triplicate electrophoretic strips were made; one was stained with bromphenol blue for protein localization; another was sprayed with a solution of BAPNA in dimethylformamide and Tris buffer; the third was cut into 1-cm sections, the protein fractions eluted in Tris buffer for 16 hours, and the eluate assayed for tryptic or tryptic inhibitory activity. In addition, serum alone was electrophoresed, and trypsin followed by α_2 -globulin trypsin inhibitor was added to the eluates from the centimeter sections of electrophoretic paper strips of serum proteins. Alpha₂-globulin trypsin inhibitor was added to aliquots of the same eluates and was followed by the addition of trypsin. This mixture was incubated with BAPNA for 80 minutes at 37° C and the reaction stopped by the addition of 1.0 ml of 0.25 N HCl. Absorbance units were read on the Beckman DU spectrophotometer at 383 m μ against a blank of buffer and substrate treated like the sample. A serum control was established by diluting serum to which no trypsin was added and treating it as was the sample. 2) Starch block electrophoretic fractions; 1 ml of the eluates of serum protein fractions was incubated with 40 μ g of trypsin and 600 μ g of α_1 -globulin trypsin inhibitor. This amount of trypsin inhibitor is sufficient to neutralize 95 per cent of the added trypsin. Residual tryptic activity was assayed, with TAME as the substrate in the automatic titrator.

7. *Determination of chymotrypsin-binding protein of serum by starch block electrophoresis.* One ml of the

eluate from the fractions of the starch block electrophoresis of 4 ml of serum containing 3.36 mg of chymotrypsin was assayed for enzymatic activity, with the following substrates used: casein, BAPNA, ATEE, and TAME.

RESULTS

1. *Paper electrophoresis.* When trypsin was added to normal serum in varying amounts (50 to 2,000 μg) and the protein fractions were separated by electrophoresis, the following results were obtained. After spraying the electrophoretic strip with BAPNA, a yellow band was detected in the α_2 -globulin region, indicating the presence of trypsin-like activity. Crystalline trypsin alone migrates toward the opposite electrode—i.e., the cathode—when subjected to electrophoresis under similar conditions (Figure 3).

Electrophoretic strips of mixtures of trypsin and serum (800 and 1,400 μg of trypsin per ml of serum) were cut into 1-cm sections, the protein fractions eluted, and tryptic or tryptic inhibitory activity determined in each section; the results are depicted in Figure 4. Fifty μl of serum, or the mixture of serum and trypsin, was applied to the paper strips. Tryptic inhibitory activity or tryptic activity was determined in a fraction of the

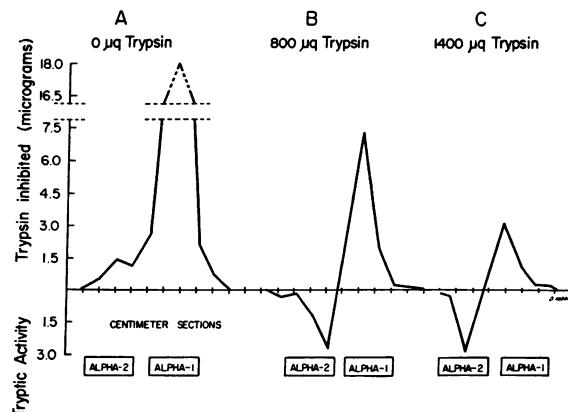


FIG. 4. TRYPTIC ACTIVITY OR TRYPTIC INHIBITORY ACTIVITY IN THE ELUATES FROM ELECTROPHORETIC PAPER STRIPS OF MIXTURES OF TRYPSIN AND HUMAN SERUM. Tryptic-like activity was found in the α_2 -globulin zone, and inhibitory activity was found in the α_1 -zone. Note that increasing the amount of trypsin added to serum from 800 to 1,400 μg did not increase the amount of trypsin-like activity in the α_2 -globulin zone. As would be expected, the additional trypsin decreased the remaining amount of α_1 -globulin trypsin inhibitor. Note that the difference between the trypsin inhibitors present in graphs A and B (720 μg trypsin inhibited per ml serum) plus the amount of tryptic activity present in the α_2 -globulin zone in B (116 μg per ml) closely approximates the 800 μg of trypsin added to 1 ml of serum.

TABLE I
The effect of partially purified α_1 - and α_2 -globulin trypsin inhibitors on crystalline trypsin and on the trypsin-protein esterase *

Crystalline trypsin	α_1 -Globulin inhibitor	α_2 -Globulin inhibitor	Tryptic activity	Tryptic inhibitory activity
μg	μg	μg	A units/80 min†	A units/80 min
1	0	0	0.320	
1	20	0	0.015	0.305
1	40	0	0.002	0.318
1	60	0	0.011	0.309
1	0	10	0.186	0.134
1	0	20	0.039	0.281
1	0	30	0.010	0.310
Trypsin-protein esterase (500 μg trypsin per ml serum diluted 1:25 with Tris)				
ml				
0.4	0	0	0.380	0
0.4	20	0	0.390	0
0.4	40	0	0.387	0
0.4	60	0	0.395	0
0.4	0	10	0.380	0
0.4	0	20	0.395	0
0.4	0	30	0.390	0

* Note that crystalline trypsin is effectively inhibited by the two inhibitors, but that the trypsin-protein esterase is unaffected.

† At 383 $\text{m}\mu$.

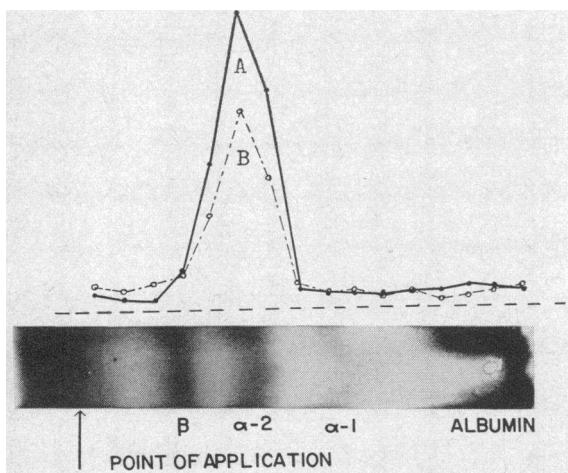


FIG. 5. IN GRAPH A TRYPSIN, FOLLOWED BY PARTIALLY PURIFIED α_2 -GLOBULIN TRYPSIN INHIBITOR, WAS ADDED TO THE ELUATES OF PROTEINS FROM CENTIMETER SECTIONS OF AN ELECTROPHORETIC STRIP OF NORMAL HUMAN SERUM. SUFFICIENT α_2 -GLOBULIN-TRYPSIN WAS ADDED TO COMPLETELY NEUTRALIZE ALL OF THE ADDED TRYPSIN. IN B, THE α_2 -GLOBULIN TRYPSIN INHIBITOR WAS ADDED FIRST AND FOLLOWED BY TRYPSIN, THE AMOUNTS REMAINING THE SAME AS IN THE FOREGOING EXPERIMENT. In both instances, tryptic activity was recorded only in the α_2 -globulin zone, indicating that the trypsin-binding protein is an α_2 -globulin. The substantial amount of tryptic activity in B indicates the greater affinity for trypsin of the α_2 -globulin-binding protein than of the α_2 -globulin-inhibiting protein. The area above the baseline represents tryptic activity.

eluates representing 40 μ l of serum. The area above the baseline is tryptic inhibitory activity, the area below is tryptic activity. It is noted in graph A of Figure 4 that normal serum without the addition of trypsin contains two trypsin inhibitors. The larger fraction migrated with the α_1 - and the smaller with the α_2 -globulin proteins. The amount of trypsin inhibited by the α_1 - and α_2 -globulin trypsin inhibitors, represented by the area under graph A, was 38.3 μ g per 40 μ l of serum (960 μ g per ml). Graph B shows that trypsin-like activity was found in the α_2 -globulin zone when 800 μ g of trypsin was added to 1 ml of serum. It is also noted that trypsin activity present in the α_2 -globulin zone was the equivalent of 4.6 μ g of trypsin per 40 μ l of serum (116 μ g per ml). The amount of trypsin inhibited by the remaining α_1 -globulin trypsin inhibitor, represented by the area under the curve, was 9.41 μ g per 40 μ l of serum (256 μ g per ml). The differ-

ence between the trypsin inhibitors in graphs A and B is 28.9 μ g per 40 μ l (720 μ g per ml). In graph B the sum of the trypsin inhibited (720 μ g per ml) and the tryptic activity measured in the α_2 -globulin zone (116 μ g per ml) is 836 μ g per ml of serum. It is apparent that this sum corresponds to the 800 μ g of trypsin added to the 1 ml of serum. Graph C shows that the addition of 1,400 μ g of trypsin to the serum did not increase the tryptic activity assayed in the α_2 -globulin zone.

The following results were obtained when trypsin, followed by partially purified α_2 -globulin trypsin inhibitor, was added to the protein eluates from the centimeter sections after electrophoresis of serum alone. α_2 -Globulin trypsin inhibitor was added in amounts sufficient to completely inhibit the added trypsin (Table I). Trypsin-like activity was recorded only in the α_2 -globulin zone (Figure 5, graph A). When the α_2 -globulin trypsin inhibitor was added to the eluates and followed by the addition of trypsin, trypsin-like activity again was recorded only in the α_2 -globulin region, but approximately one-third less in amount (graph B).

Increasing amounts of trypsin (100 to 1,000 μ g in increments of 100 μ g) were added to 1 ml of normal serum. The serum and trypsin mixture was diluted 1:25 with Tris buffer and 0.4 ml was assayed for trypsin-like activity. Increasing trypsin-like activity was recorded until a plateau was reached at approximately the 500 μ g level

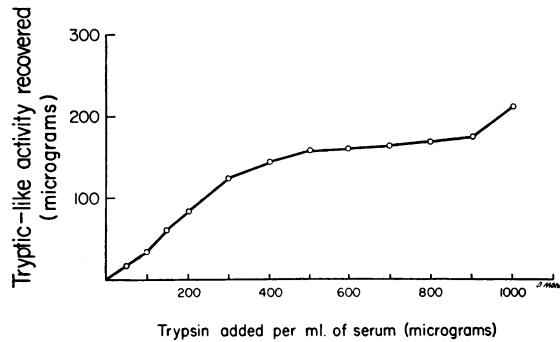


FIG. 6. INCREASING AMOUNTS OF TRYPSIN (100 TO 1,000 μ G) WERE ADDED TO 1 ML OF NORMAL SERUM. Observe that increasing trypsin-like activity is recorded from the mixture until a plateau is reached at approximately the 500 μ g level. As more trypsin was added only a slight increase in tryptic activity was recorded until the addition of more than 900 μ g, at which point tryptic inhibitory activity of the serum was exceeded and again large increases in tryptic activity were recorded.

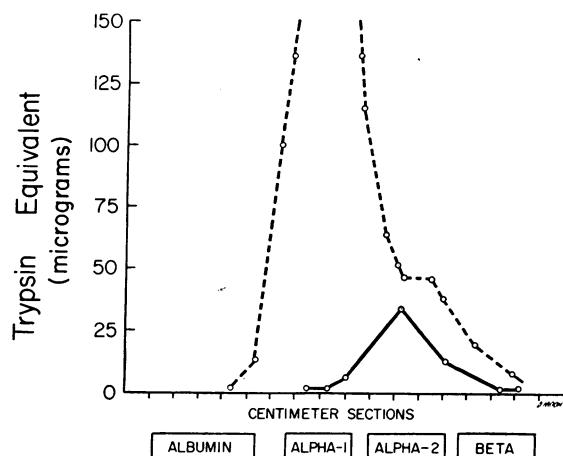


FIG. 7. THE DASHED LINE REPRESENTS THE SERUM α_1 AND α_2 -GLOBULIN TRYPSIN INHIBITORS IN THE ELUATES FROM STARCH BLOCK ELECTROPHORESIS OF SERUM. The solid line represents trypic activity when trypsin followed by α_1 -globulin trypsin inhibitor was added to the starch block eluates. Sufficient α_1 -globulin trypsin inhibitor was added to neutralize almost all of the added trypsin. Tryptic activity was recorded only in the α_2 -globulin zone. The amount of trypic activity recovered from the α_2 -globulin zone was the equivalent of 92 μ g crystalline trypsin for 0.4 ml serum.

(Figure 6). As more trypsin was added, no further substantial increase in trypic activity was noted until the addition of 900 mg, at which point serum trypic inhibitory activity was exceeded, and again increased trypic activity was recorded. The results illustrated in Figure 6 are representative of four similar studies.

2. *Starch block electrophoresis.* The following results were obtained when trypsin, followed by α_1 -globulin trypsin inhibitor, was added to the serum protein eluates. Trypsin-like activity was seen only in the α_2 -globulin zone (Figure 7). Sufficient α_1 -globulin trypsin inhibitor was added to neutralize 95 per cent of the added trypsin. It is noted, however, from Figure 7 that there was a large amount of normally occurring trypsin inhibitor in the α_2 -globulin fraction, in addition to the added α_1 -globulin trypsin inhibitor. The amount of trypsin-like activity recovered from the α_2 -globulin zone was the equivalent of 92 μ g of crystalline trypsin for 0.4 ml of serum; 240.0 μ g of trypsin had been added to the eluates of α_2 -globulin fraction.

When the eluates from the mixture of serum and chymotrypsin were assayed for enzymatic ac-

tivities on different substrates, the following results were obtained. 1) With ATEE as a substrate, chymotrypsin activity was noted only in the α_2 -globulin fraction (Figure 8). The amount of chymotrypsin activity recovered from the α_2 -globulin zone was the equivalent of 103 μ g of crystalline chymotrypsin per 4 ml of serum; 3.36 mg of crystalline chymotrypsin was added to the 4 ml of serum prior to electrophoresis. 2) With casein as the substrate, once again enzymatic activity was noted only in the eluates from the α_2 -globulin zone (Figure 8). 3) With BAPNA and TAME as the substrates, no enzymatic activity was detected in the α_2 -globulin fraction, nor in any of the other protein fractions (Figure 8). 4) When 20 μ g of trypsin was added to 1 ml of the

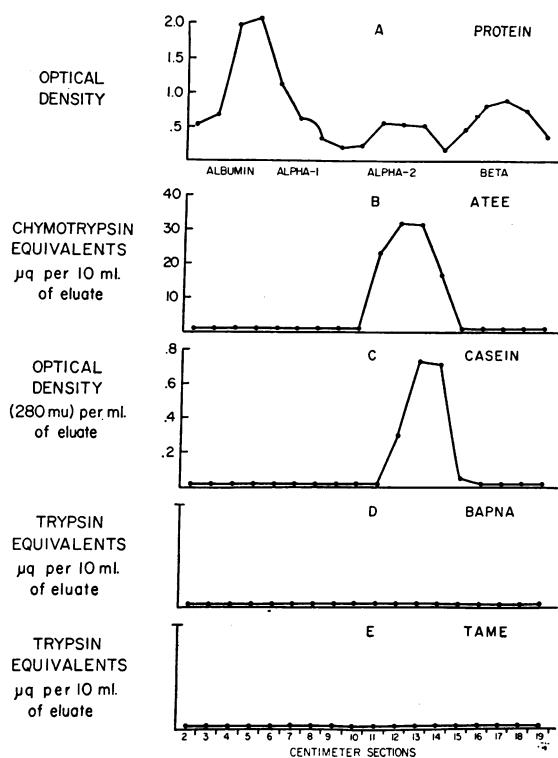


FIG. 8. GRAPH A REPRESENTS THE ELECTROPHORETIC PROTEIN FRACTIONS OF THE MIXTURE OF SERUM AND CHYMOTRYPSIN. B REPRESENTS THE ENZYMATIC ACTIVITY OF THE PROTEIN FRACTIONS USING THE SUBSTRATE N-ACETYL-L-TYROSINE ETHYL ESTER. NOTE THAT ONLY THE α_2 -GLOBULIN ZONE CONTAINS ENZYMATIC ACTIVITY. C REPRESENTS THE ENZYMATIC ACTIVITY OF THE ELUATE FRACTIONS USING CASEIN AS THE SUBSTRATE. D AND E REPRESENT THE ENZYMATIC ACTIVITY OF THE ELUATE FRACTIONS USING BAPNA AND TAME AS THE SUBSTRATE.

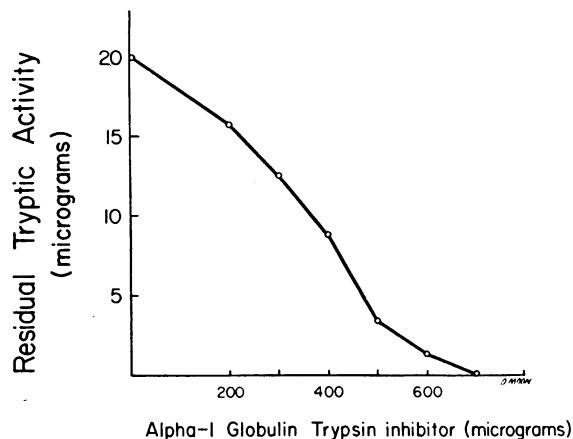


FIG. 9. THE EFFECT OF PARTIALLY PURIFIED α_1 -GLOBULIN TRYPSIN INHIBITOR ON THE ACTIVITY OF TRYPSIN, WITH TAME. Note that complete inhibition of 20 μg of crystalline trypsin is accomplished by 700 μg of the inhibitor.

eluates of the chymotrypsin and serum mixture, followed by the addition of 800 μg of α_1 -globulin trypsin inhibitor, no enzymatic activity was noted in any of the fractions using TAME as the substrate. It is noted from Figure 9 that 800 μg of the α_1 -globulin trypsin inhibitor is more than sufficient to neutralize 20 μg of crystalline trypsin.

It became important to determine whether known trypsin and plasmin inhibitors inhibit the trypsin-protein esterase activity found in the α_2 -globulin zone. Tryptic activity in the α_2 -globulin zone was obtained by two methods: by adding trypsin to serum in amounts less than the trypsin-inhibiting levels of the serum and by elution of the α_2 -globulin zone from electrophoretic strips containing the fractionated proteins of a mixture of trypsin and serum. It was demonstrated that neither the purified serum α_1 - nor the α_2 -globulin trypsin inhibitor suppressed the tryptic activity from either of the aforementioned sources (Table I). Soybean and colostrum trypsin inhibitors also did not inhibit the trypsin-protein esterase.

DISCUSSION

It is apparent that when trypsin or chymotrypsin is added to serum in an amount less than that necessary to saturate the inhibitors, proteolytic enzymatic activity is detected in the α_2 -globulin zone. The character of the activity is of interest, since it becomes important to determine whether

the enzymatic activity is a result of the activation of a naturally occurring precursor substance in blood or is a result of the binding of the added enzyme to an α_2 -globulin protein with the complex retaining enzymatic activity.

Of the different proteolytic enzyme systems in blood, the activation of plasminogen to plasmin must be considered as a possibly responsible factor for the α_2 -globulin enzymatic activity when trypsin is added to serum. It is known that plasminogen activation can occur spontaneously by precipitation of the globulin fraction, by tissue activators, by urinary activators, by trypsin, and by plasmin. The reaction in each case involves liberation of a peptide moiety from plasminogen (7-9). Several factors indicate, however, that the activation of plasminogen to plasmin is not responsible for the enzymatic activity in the α_2 -globulin fraction after addition of trypsin to serum. Norman has shown that the plasminogen in human plasma migrates with the β -globulin fraction upon electrophoresis in starch (10). Studies in this report have demonstrated that the only fraction of human serum proteins that contains proteolytic or esterolytic activity when trypsin and a trypsin inhibitor are added is the α_2 -globulin fraction. Also, the enzymatic activity in the α_2 -globulin zone is not inhibited by the α_2 -globulin trypsin inhibitor or soybean trypsin inhibitor, both of which suppress plasmin. Another fact against the hypothesis of plasminogen activation is the observation that, when chymotrypsin is added to serum and the proteins of the mixture are separated by electrophoresis, enzymatic activity again is noted only in the α_2 -globulin zone. This activity will hydrolyze the chymotrypsin substrate N-acetyl-L-tyrosine-ethyl ester, but not trypsin or plasmin substrates (BAPNA or TAME). Also, it will hydrolyze casein, which is a substrate for trypsin as well as chymotrypsin.

Another proteolytic enzyme present in serum, which must be considered in attempting to account for the enzymatic activity in the α_2 -globulin zone when trypsin is added to serum, is the esterase derived from the first component of complement. Donaldson (11) has shown that the extraction of serum with ether resulted in the separation of a fraction rich in esterase activity. The activity could not be identified with plasmin. The ether-esterase digested synthetic amino acid esters simi-

lar to those digested by esterase prepared from a partially purified preparation of the first component of complement (C'1-esterase). The substrates digested by both of these esterases include N-acetyl-L-tyrosine ethyl ester, N-acetyl-L-tryptophan ethyl ester, benzoyl-L-arginine ethyl ester, and *p*-toluene sulfonyl-L-arginine methyl ester. The properties of the ether-esterase suggest its identity with an esterase derived from the first component of complement (11). Although the electrophoretic mobility of the ether-esterase on paper was similar to an α_1 -globulin at pH 8.6 and ionic strength 0.15, it is unlikely that the ether-esterase is identical with the trypsin or chymotrypsin protein esterase for the following reasons: 1) the trypsin-protein esterase digests casein but does not hydrolyze N-acetyl-L-tyrosine ethyl ester; 2) the chymotrypsin-protein esterase does not digest BAPNA or TAME.

The more suitable explanation for the α_2 -globulin enzymatic activity is that trypsin or chymotrypsin can be bound to a component of the α_2 -globulin of serum. The fact that, when trypsin is added to serum, the sum of the inhibited trypsin plus the tryptic activity of the trypsin-protein esterase equals the amount of trypsin added, is further evidence favoring this hypothesis. The trypsin- α_2 -globulin complex retains its caseinolytic or esterolytic properties despite the fact that it migrates electrophoretically in the direction opposite to that of crystalline trypsin. However, the complex is not affected by the potent trypsin inhibitors found in soybean, colostrum, and in the α_1 - and α_2 -globulin fractions of human serum. The trypsin- α_2 -globulin complex is an active proteolytic enzyme with some properties different from crystalline trypsin. The type of combination that occurs between the trypsin and α_2 -globulin fraction that permits the complex to travel with the α_2 -globulin is not clear. It is logical to conclude, however, that the active tryptic site remains unmasked in the complex. With this in mind, the ineffectiveness of the usual trypsin inhibitors to the protein-bound trypsin, which can digest casein, is difficult to explain at present; it can only be stated that the complex has characteristics different from crystalline trypsin.

The properties of this protein-bound proteolytic enzyme may be a factor in explaining phenomena that previously have been enigmatic. Reports in

the literature have been concerned recently with "serum trypsin" levels in acute pancreatitis and pancreatic cancer (12, 13). The substances that have been used for the measure of "serum trypsin" for the most part have been benzoyl-L-arginine compounds. In addition to the fact that thrombin and plasmin hydrolyze these substrates, it is necessary to confirm the reported elevated "serum trypsin levels" in pancreatic disease by more precise methods. However, the fact that trypsin can be bound to an α_2 -globulin fraction and maintain its tryptic activity, which is unaffected by the serum inhibitors, presents a mechanism whereby serum may contain both tryptic activity and tryptic inhibitory activity simultaneously. The possibility that other proteolytic enzymes may be bound to proteins that occur in blood is of considerable interest. It has been established already, that chymotrypsin can be bound to an α_2 -globulin protein in a fashion similar to that of trypsin. The question naturally arises whether or not thrombin and plasmin (both of which are proteolytic enzymes resembling trypsin in many respects), ether-esterase, and chloroform-esterase have similar relationships with a serum globulin.

The basic principles involved in the binding of trypsin become important in a consideration of the mechanism involved in acute pancreatitis. It is known that human pancreatic juice obtained by catheter drainage of the duct of Wirsung has no spontaneous tryptic activity (4). It does, however, contain substantial amounts of trypsin inhibitor. Likewise, pancreatic tissue extracts contain substantial amounts of trypsin inhibitory substances. The etiology of pancreatitis remains obscure, but it is agreed by many that an important factor in this disease is the digestive action of the pancreatic proteolytic enzymes. The enzymes that are likely to be directly concerned with this action are trypsin and chymotrypsin. It appears that one of the keys to the production of pancreatitis is the transformation of trypsinogen to trypsin and that the newly formed trypsin be permitted by its chemical environment within the pancreas to maintain proteolytic activity. The binding of trypsin to a protein that is active in the presence of trypsin inhibitors presents a mechanism whereby small amounts of trypsin in or about the pancreas may break down tissue despite the presence of large amounts of substances inhibitory to trypsin.

This raises the important question of effective inhibitors to this complex. Many investigators in the past have administered various trypsin inhibitors to animals with induced pancreatitis, or to humans with pancreatitis, with equivocal results. It is likely that many, if not all, of these substances were ineffective in inhibiting the trypsin or the chymotrypsin-protein complex, despite the effective inhibition to the unbound enzymes. It is reasonable to suggest that, if proteolytic enzyme inhibitors are to be effective at all in the therapy of pancreatitis, they must be effective in inhibiting the trypsin-protein complex or protein complexes of other proteolytic enzymes found in the pancreas.

SUMMARY

1. Human serum contains an α_2 -globulin protein fraction which combines with trypsin. The trypsin- α_2 -globulin complex migrates with the α_2 -globulin fraction upon electrophoresis, while uncombined trypsin migrates toward the cathode. The protein-bound trypsin maintains its proteolytic and esterolytic activity, which was demonstrated by lysis of casein as well as the synthetic substrates benzoyl-L-arginine-paranitroanilide, and *p*-toluene-sulfonyl-L-arginine methyl ester.

2. Purified α_1 - and α_2 -globulin trypsin inhibitors isolated from human serum by elution from diethylaminoethyl cellulose columns do not inhibit the trypsin α_2 -globulin complex. These substances have been shown to be potent inhibitors to unbound crystalline trypsin. Other inhibitors to unbound crystalline trypsin and plasmin, such as colostrum and soybean inhibitor, likewise had no effect on the protein-bound trypsin.

3. It was demonstrated by starch block electrophoresis that chymotrypsin also may be bound to an α_2 -globulin. The protein-bound chymotrypsin will hydrolyze casein and N-acetyl-L-tyrosine ethyl ester, but not *p*-toluene-L-sulfonyl methyl ester.

4. From the results of this study it is reasonable to suggest that, if proteolytic enzyme inhibi-

tors are to be effective in the therapy of pancreatitis, they must be effective in inhibiting the trypsin-protein complex or protein complexes of other proteolytic enzymes found in the pancreas.

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