# Human Pancreatic Enzymes. Characterization of Anionic Human Trypsin†

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ABSTRACT: An anionic form of human trypsin has been isolated from acetone powders of human pancreas. The purification procedure included salt fractionation followed by ion-exchange chromatography on SE-Sephadex C-25 and on DEAE-Sephadex A-50. The preparation was homogeneous by disc electrophoresis and by sedimentation equilibrium centrifugation studies. The latter resulted in an estimated molecular weight of 25,800 for this enzyme. Comparison of anionic trypsin with the cationic enzyme indicates a high degree of similarity in the amino acid composition of each protein. However, the anionic enzyme gives only a weak cross-reaction toward antibodies directed against the cationic enzyme, is much less stable than the cationic enzyme, and is inhibited by soybean trypsin inhibitor and, to a lesser extent, by porcine Kazal pancreatic inhibitor and ovomucoid, all three of which are poor inhibitors of cationic trypsin.

The presence of two forms of trypsinogen and trypsin in human pancreatic juice has been well documented by the results of Keller and Allan (1967), Figarella *et al.* (1969), and Robinson *et al.* (1970, 1972). In a previous report from this laboratory (Travis and Roberts, 1969) the isolation and properties of the major trypsin component, a cationic molecule, were described. At that time the detection of a second anionic form of human trypsin could not be made. Recently, however, improved procedures for purifying human pancreatic proteases have been made and these have allowed us to not only detect an anionic form of human trypsin but also to purify the protein to homogeneity and investigate its properties.

## Materials

Human pancreatic tissue was obtained quick-frozen at autopsy from either St. Joseph's Hospital, Marshfield, Wis., or Athens General Hospital, Athens, Ga. Pipes[1] was purchased from Calbiochem and BzArgOEt from Schwarz/Mann. SBTI, LBTI, chicken ovomucoid, and Kunitz pancreatic trypsin inhibitor were obtained from Worthington Biochemical Co., $iPr_2FP$ from Aldrich Chemical Co., and Tos-Lys$CH_2Cl$ and Tos-Phe$CH_2Cl$ from the Sigma Chemical Co. Porcine and bovine Kazal inhibitors were generously provided by Dr. Lewis Greene, Brookhaven National Laboratory, Upton, N. Y. Double immunodiffusion plates were supplied by Hyland Laboratories. DEAE-Sephadex A-50 and SE-Sephadex C-25 were from Pharmacia Fine Chemicals. $\alpha$-1-Antitrypsin fractions were obtained free of $\alpha_2$-macroglobulin by gel filtration of whole human plasma on Sephadex G-75. All other reagents were of analytical grade obtained from various commercial sources.

## Methods

*Enzyme Assays.* The esterolytic activity of trypsin was measured by the spectrophotometric method of Schwert and Takenaka (1955) using BzArgOEt as substrate. Assay conditions were at 25° in 0.1 M Tris-HCl, pH 8.0, containing 0.05 M $CaCl_2$. A unit of activity was defined as an absorbance change of 1 optical density unit/min at 253 nm. Specific activity was calculated as units of trypsin activity/milligram of protein. Protein concentration was determined spectrophotometrically by the method of Warburg and Christian (1942). For purified preparations, a specific extinction coefficient of 15.8 (1% solution), as determined in this paper, was utilized. Proteolytic activity was measured by casein hydrolysis using the method of Kunitz (1947).

*Inhibition Studies.* Inhibition experiments were carried out by mixing various quantities of inhibitor with enzyme in 0.05 M Tris-HCl, pH 8.0, containing 0.05 M $CaCl_2$. After incubation for 15 min at room temperature the samples were assayed for esterolytic activity as described above. Solutions containing Kunitz pancreatic trypsin inhibitor were allowed to incubate 60 min prior to assay because of the slow response of the inhibitor. Controls were performed before and after each assay to correct for autolysis.

*Polyacrylamide Electrophoresis.* Disc electrophoresis on polyacrylamide gels was performed in 7.5 and 15% acrylamide at pH 8.3 as described by Brewer and Ashworth (1969).

*Amino Acid Analysis.* The amino acid composition of anionic trypsin was obtained according to the method of Spackman *et al.* (1958) using a Beckman Model 120C amino acid analyzer. Samples were hydrolyzed *in vacuo* with 6 N HCl for 22, 48, and 72 hr. Cysteine and methionine residues were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (Hirs, 1956). Tryptophan was determined by amino acid analysis of samples hydrolyzed in the presence of 4% thioglycolate (Matsubara and Sasaki, 1969).

*Analytical Ultracentrifugation.* Analytical ultracentrifuge studies were performed with a Beckman Spinco Model E ultracentrifuge. The sedimentation coefficients ($s_{20,w}$) was ob-

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[1] Abbreviations used are: $iPr_2FP$, diisopropyl phosphorofluoridate; Tos-Lys$CH_2Cl$, 1-chloro-3-tosylamido-7-amino-2-heptanone; Tos-Phe$CH_2Cl$, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Pipes, piperazine-*N*,*N*-bis(2-ethanesulfonic acid) monosodium monohydrate; SBTI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; PSTI, pancreatic secretory trypsin inhibitor; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester.

TABLE I: Purification of Human Anionic Trypsin.

| Fractionation Step | Vol (ml) | Total Protein (mg) | Total Act. | Sp Act. (Units/mg) | Recovery (%) | Purification |
|---|---|---|---|---|---|---|
| Crude extract | 500 | 4025 | 2250 | 0.560 | 100 | 1 |
| Salt fractionation 0.2–0.8 | 1000 | 3360 | 2100 | 0.625 | 93 | 1.11 |
| SE-Sephadex C-25 | 430 | 104 | 300 | 2.88 | 13.3 | 5.15 |
| DEAE-Sephadex A-50 | 120 | 16.3 | 126 | 7.73 | 5.6 | 13.8 |

tained by sedimentation velocity (Schachman, 1957) and the molecular weight by the sedimentation equilibrium method of Yphantis (1964). Actual molecular weight calculations were done, in part, with the aid of a Nova computer (Data General) using a partial specific volume of the enzyme calculated from the amino acid composition (McMeekin and Marshall, 1952). The extinction coefficient of anionic trypsin was determined by the method of Babul and Stellwagen (1969) using interference optics.

## Results

*Purification of Human Anionic Trypsin.* INITIAL PROCEDURE. Experimental conditions for the initial isolation of human anionic trypsin fractions varied only slightly from those used for the purification of other proteases from human pancreatic tissue. Thus, 40 g of acetone powder, representing 1000 g of pancreatic tissue, was extracted at acid pH, salt fractionated between 0.2 and 0.8 with solid ammonium sulfate, and dialyzed at alkaline pH against 0.05 M Tris-HCl, pH 8.0, containing 0.05 M $CaCl_2$, to remove the salt and initiate activation of zymogens. The details of this procedure have been reported elsewhere (Coan *et al.*, 1971).

SE-SEPHADEX C-25 CHROMATOGRAPHY. In preliminary experiments it was noted that when the pH of an activated extract of human pancreatic tissue was adjusted to below pH 4.0, 15–20% of the trypsin esterase units were irreversibly lost. Therefore, the activated material obtained in the previous step was adjusted to pH 4.6 with 0.1 M acetic acid, diluted with water to an ionic strength equivalent to that of a buffer consisting of 0.005 M Pipes–0.025 M $CaCl_2$, pH 6.5 (buffer A), and applied to a column of SE-Sephadex C-25 (1.7 × 28 cm) equilibrated in the same buffer. The column was washed with 0.005 M acetic acid–0.025 M $CaCl_2$, pH 4.6, until the $A_{280}$ was less than 0.020. It was then washed with buffer A, causing the elution of a single sharp protein component containing approximately 15% of the applied trypsin esterase units but completely devoid of any chymotrypsin esterase activity. The active material eluted as a yellowish brown fraction. The insertion of this step had no effect on the elution of cationic trypsin and chymotrypsin (Coan *et al.*, 1971).

DEAE-SEPHADEX A-50 CHROMATOGRAPHY. The most active fractions of the SE-Sephadex C-25 column chromatography were pooled and applied immediately to a DEAE-Sephadex A-50 column (1.7 × 26 cm) equilibrated against buffer A. After the passage of unretarded, inactive protein, the anionic trypsin was eluted by initiation of a linear gradient from 0.025 to 0.125 M $CaCl_2$ both in 0.005 M Pipes, pH 6.5. The elution pattern is shown in Figure 1 and a summary of the purification scheme is given in Table I. The poor recovery reported (5.6%) is primarily due to the loss of cationic trypsin which represents 70% (see Discussion) of the BzArgOEt esterase activity in the starting material and which is still retained on the SE-Sephadex C-25 column under these conditions. It should be noted that the component eluted after anionic trypsin was also a protease. This enzyme is currently under investigation.

*Criteria of Homogeneity and Molecular Weight Studies.* The protein was found to autolyze rapidly after elution from the DEAE-Sephadex columns, and column chromatography of active fractions on either Sephadex G-75 or DEAE-Sephadex A-50 resulted in symmetrical peaks with a constant but lower specific activity than that obtained in the fractions initially isolated. This material, after analytical gel electrophoresis, separated into one major and several minor components. However, preparations of anionic trypsin treated with $iPr_2FP$

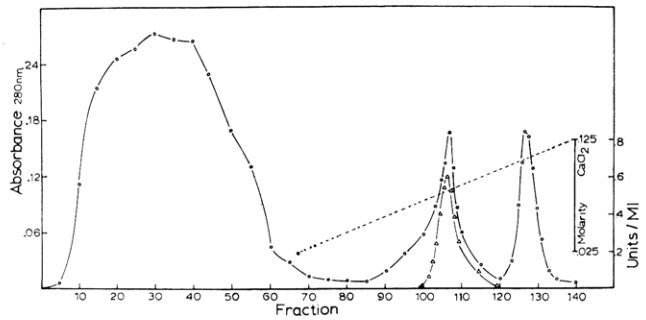


FIGURE 1: DEAE-Sephadex A-50 chromatography of active fractions from SE-Sephadex C-25 column chromatography. The column was equilibrated with 0.005 M Pipes-HCl, pH 6.5, containing 0.025 M $CaCl_2$, and eluted with a linear gradient to 0.125 M $CaCl_2$ as indicated. Column dimensions: 1.7 × 26 cm; flow rate, 20 ml/hr; fraction size, 5 ml. Curves are designated as follows: optical density at 280 nm (○), left ordinate; activity against BzArgOEt (△), right ordinate.

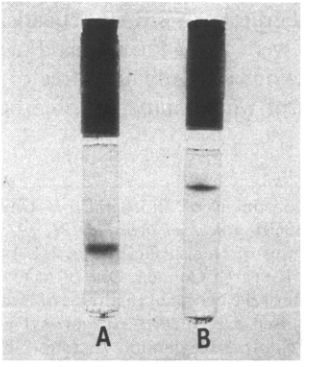


FIGURE 2: Polyacrylamide disc electrophoresis of $iPr_2P$ anionic human trypsin (100 μg). Direction of migration is from cathode (top) to anode (bottom). Patterns were stained with 1% Amido Schwarz in 7.5% acetic acid: (A) pH 8.3, 7.5% gel; (B) pH 8.3, 15% gel.

TABLE II: Amino Acid Composition[a] of Human Anionic Trypsin and Other Mammalian Trypsins.

| Amino Acid | Anionic Trypsin with Hydrolysis Time (hr) of | | | Value Taken | Nearest Integer | Other Mammalian Trypsins (Residues/Molecule) | | |
|---|---|---|---|---|---|---|---|---|
| | 22 | 48 | 72 | | | Human Cationic[e] | Bovine[f] | Porcine[g] |
| Lysine | 11.6 | 12.6 | 11.9 | 12.0 | 12 | 12 | 14 | 10 |
| Histidine | 5.5 | 5.3 | 4.9 | 5.2 | 5 | 3 | 3 | 4 |
| Arginine | 6.6 | 6.9 | 6.7 | 6.7 | 7 | 6 | 2 | 4 |
| Aspartic acid | 24.0 | 24.9 | 24.8 | 24.6 | 25 | 22 | 22 | 18 |
| Threonine | 10.2 | 9.1 | 8.9 | 10.6[b] | 11 | 11 | 10 | 11 |
| Serine | 18.5 | 16.5 | 12.5 | 19.8[b] | 20 | 25 | 33 | 24 |
| Glutamic acid | 24.5 | 24.7 | 23.5 | 24.2 | 24 | 22 | 14 | 17 |
| Proline | 13.0 | 11.1 | 11.1 | 11.7 | 12 | 10 | 9 | 10 |
| Glycine | 22.0 | 22.8 | 22.5 | 22.6 | 23 | 21 | 25 | 26 |
| Alanine | | (15) | | 15.0 | 15 | 14 | 14 | 16 |
| Half-cystine | | | | 8[c] | 8 | 8 | 12 | 12 |
| Valine | 14.2 | 14.8 | 15.7 | 14.9 | 15 | 17 | 17 | 16 |
| Methionine | 2.4 | 1.8 | 2.8 | 2.3[c] | 2 | 1 | 2 | 2 |
| Isoleucine | 12.0 | 12.4 | 13.1 | 12.5 | 13 | 13 | 15 | 15 |
| Leucine | 18.0 | 17.7 | 17.9 | 17.9 | 18 | 13 | 14 | 16 |
| Tyrosine | 8.2 | 7.1 | 7.1 | 7.6 | 8 | 7 | 10 | 8 |
| Phenylalanine | 7.0 | 6.9 | 6.6 | 6.8 | 7 | 4 | 3 | 4 |
| Tryptophan | 1.6 | 3.7 | 3.6 | 3.0[d] | 3 | 3 | 4 | 6 |
| | | | | No. of Residues | 228 | 212 | 223 | 219 |

[a] Data are expressed as amino acid residues/molecule, assuming 15 alanines/mol wt of 25,800. [b] Threonine and serine values are based on linear extrapolations to zero time. [c] Averages of 22-hr hydrolysates of oxidized samples. [d] Tryptophan determined by average of 22-hr hydrolysates in the presence of 4% thioglycolate. [e] Recalculation of Travis and Roberts (1969) (personal communication). [f] Walsh and Neurath (1964). [g] Travis and Liener (1965).

immediately after elution from DEAE-Sephadex A-50 gave only single bands after electrophoresis at pH 8.3 in either 7.5 or 15% gels (Figure 2) and this material was utilized for both ultracentrifuge studies and amino acid analysis.

Sedimentation velocity experiments run in 0.005 M Pipes–0.02 M $CaCl_2$, pH 6.5, at protein concentrations of 7.0 and 3.5 mg/ml migrated as symmetrical single components indicating that the enzyme was homogeneous. In comparison to human cationic trypsin which has a $s_{20,w}$ of 2.68 S (Travis and Roberts, 1969), the value for human anionic trypsin was 2.84 S. An extinction coefficient ($E^{1\%}_{1\,cm}$) of 15.8 was computed from ultracentrifuge studies using interference optics.

Sedimentation equilibrium experiments were performed on $iPr_2P$ anionic trypsin at an initial protein concentration of 0.21 mg/ml. Using an estimated partial specific volume of 0.722, calculated from the amino acid composition, a mol wt of 25,800 was determined. There was no significant deviation from linearity in ln $c$ *vs.* $r^2$ plots, indicative of the homogeneity of the enzyme preparation. The apparent mol wt of human anionic trypsin is significantly larger than that of 22,900 for human cationic trypsin (Travis and Roberts, 1969) when determined by this same method.

*Amino Acid Composition.* The results of the amino acid analysis of human anionic trypsin are summarized in Table II. The number of residues of each amino acid present was based on an assumed value of 15 residues of alanine/mol of protein. A mol wt of 24,660 was calculated from these data.

The composition of human anionic trypsin, compared with that of human cationic trypsin, bovine trypsin, and porcine trypsin, is also given in Table II. The amino acid compositions of these trypsins are remarkably similar, and obvious reasons for the differences in the ionic properties of the two human trypsins are not immediately discernible; differences in amide content of the two proteins, however, are presumably responsible for the anionic and cationic characters of the two proteins.

*Stability of Human Anionic Trypsin.* The pH optimum of anionic trypsin was determined over a wide range of pH values from pH 2.0 to 10.5. The buffers used were 0.1 M sodium citrate (pH 2.0–5.5), 0.1 M Pipes–HCl (pH 6.0–7.7), 0.1 M Tris-HCl (pH 7.2–8.0), and 0.1 M sodium carbonate (pH 7.7–10.5). The activity of the enzyme toward BzArgOEt was highest between pH 8.0 and 10.0. Controls were performed before and after each assay to correct for autolysis.

The effect of pH on the stability of the enzyme was determined by incubation of aliquots of the enzyme at several pH values ranging from 1.3 to 9.5 at 23°. The buffers used were identical with the above with the addition of 0.13 N HCl for pH 1.3. The incubation mixtures were assayed at pH 8.0 with BzArgOEt at various time intervals up to 24 hr. The results of the experiment are shown in Figure 3. The enzyme was found to autolyze in the entire range of pH values tested, losing from 50 to 90% of its activity over a 24-hr period. Addition of calcium ions (0.5 M) was found to have no effect in stabilizing the enzyme.

Disc electrophoresis patterns of aliquots of anionic trypsin incubated at various pH values for 24 hr indicate that this inactivation is probably a result of rapid autolysis since freshly prepared enzyme incubated for 1 hr at pH 6.5, followed by treatment with $iPr_2FP$, gave one major band as well as some minor components. In all of the other samples no single major component could be detected.

TABLE III: Effect of Some Naturally Occurring Inhibitors on Human Anionic,[a] Human Cationic, and Bovine Trypsins.

| Inhibitor | Molar Ratio Inhibitor: Enzyme | Incubation Time (min) | % Inhibition | | |
|---|---|---|---|---|---|
| | | | Human Cationic Trypsin | Human Anionic Trypsin | Bovine Trypsin |
| α-1-Antitrypsin | | 15 | 100[b] | 100 | 100[b] |
| SBTI | 1:1 | 15 | 20[b] | 100 | 98[b] |
| LBTI | 1:1 | 15 | 95[b] | 93 | 95[b] |
| Porcine PSTI | 1:1 | 15 | 6 | 65 | |
| Bovine PSTI | 1:1 | 15 | 5 | 12 | 96[c] |
| Chicken ovomucoid | 1:1 | 15 | 0[b] | 30 | 51[b] |
| Kunitz | 1:1 | 60 | 99[b] | 97 | 99[b] |

[a] Expressed as per cent inhibition of esterase activity as measured at pH 8.0 as described under Methods. [b] Coan and Travis (1971). [c] Green *et al.* (1969).

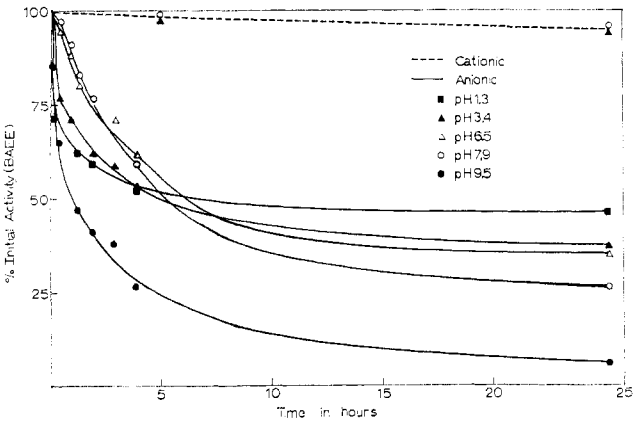


FIGURE 3: pH stability of human anionic trypsin (—) and human cationic trypsin (- - -). Samples of enzymes (0.3 mg/ml) were incubated in universal buffers, containing 0.1 M $CaCl_2$, at the pH values indicated.

*Effect of Inhibitors on Anionic Trypsin Activity.* In order to determine the similarity of anionic and cationic trypsins several chemical inhibitors were tested. An enzyme concentration of $10^{-5}$ M was routinely used. Using a mol wt of 25,800 derived above for human anionic trypsin, it was found that $iPr_2FP$ completely inhibited this protein in 15 min when present in an inhibitor–enzyme molar ratio of 10:1. Similarly, Tos-Lys$CH_2Cl$ at a molar ratio of 100:1 totally inhibited the enzyme within 90 min, whereas Tos-Phe$CH_2Cl$, a potent inhibitor of chymotrypsin, was ineffective even at a molar ratio of 100:1.

Anionic trypsin was also tested against a wide range of naturally occurring trypsin inhibitors including SBTI, LBTI, porcine Kazal pancreatic inhibitor, bovine Kazal pancreatic inhibitor, Kunitz bovine pancreatic inhibitor, chicken ovomucoid, and crude α-1-antitrypsin. The results of these experiments are given in Table III and are compared to results noted for human cationic trypsin and bovine trypsin. In contrast to human cationic trypsin (Travis and Roberts, 1969), anionic trypsin was rapidly and completely inactivated by SBTI[2] even at inhibitor–enzyme molar ratios as low as 1:1. Chicken ovomucoid and porcine Kazal pancreatic inhibitor which have little effect on cationic trypsin (Buck *et al.*, 1962, Coan and Travis, 1971) show intermediate inhibitory characteristics toward anionic trypsin. Comparison of these three trypsins shows that human anionic trypsin is certainly closer in inhibitory characteristics to bovine trypsin than to human cationic trypsin. It should be noted that Feeney *et al.* (1969) tested a number of naturally occurring inhibitors against an activated trypsinogen preparation obtained from Keller and Allan (1967). They found ovomucoid to be very weak or inactive as an inhibitor. Unfortunately, it is not known whether their material represented anionic trypsin, cationic trypsin, or possibly a mixture of both.

*Immunological Studies of Anionic Trypsin.* Using the Ouchterlony immunodiffusion technique, a weak but detectable cross-reaction between anionic trypsin and rabbit antibodies prepared to the cationic enzyme could be observed. However, anionic trypsin from humans gave no reaction against rabbit antibodies to porcine trypsin, bovine trypsin, or even to human chymotrypsin.

## Discussion

In earlier purification procedures used for the isolation of human cationic trypsin and chymotrypsins (Travis and Roberts, 1969; Coan *et al.*, 1971) no indication was ever observed of the presence of a second form of trypsin despite the obvious presence of two trypsinogens (Figarella *et al.*, 1969; Keller and Allan, 1967; Robinson *et al.*, 1970, 1972). The results presented here indicate that the lack of detection of anionic trypsin was most probably due to a combination of autolysis at alkaline pH and enzyme instability at low pH.

The isolated enzyme has many properties in common with trypsins from other species and with human cationic trypsin. These include similarities in molecular weight, amino acid composition, pH optimum, ability to digest casein, and inhibition by several synthetic and naturally occurring proteinase inhibitors. However, the anionic enzyme differs markedly from cationic trypsin in that (a) it autolyzes rapidly at neutral or alkaline pH, (b) calcium ions do not appear to stabilize the protein against autolysis, (c) the protein is rapidly inactivated by SBTI, and (d) significant inhibition by the porcine Kazal secretory inhibitor can be demonstrated. In addition, only a weak immunological cross-reaction between anionic trypsin and antibodies to cationic trypsin can be detected suggesting major differences in the antigenic sites on the two enzymes. A summary of the properties of the two human trypsins is given in Table IV.

[2] The effect of SBTI on anionic trypsin was first pointed out by Figarella (1972).

TABLE IV: Comparative Properties of Human Anionic and Human Cationic Trypsins.

| Property | Anionic Trypsin | Cationic Trypsin |
|---|---|---|
| Mol wt | 25,800 | 22,900[a] |
| $s_{20,w}$ (S) | 2.84 | 2.66[a] |
| Isoelectric point | 4.6–6.5 | >6.5[a] |
| pH optimum | 8–10 | 8–9[a] |
| $E^{1\%}_{280\,nm}$ | 15.8 | 14.5[c] |
| Sp act. (BzArgOEt) (units/mg) | 7.7 | 9.9 |
| Stability | | |
| Alkaline (no $Ca^{2+}$) | Unstable | Unstable[b] |
| Alkaline (0.5 M $Ca^{2+}$) | Unstable | Increased stability[b] |
| Acid | Unstable | Stable[b] |

[a] Travis and Roberts (1969). [b] Coan and Travis (1971). [c] Coan *et al.* (1971).

We have noticed that anionic human trypsin is not always present in activated acetone powder extracts from individual pancreas preparations. This confirms the results of Robinson *et al.* (1972) who reported that two trypsinogens could not always be detected during their analysis of duodenal and pancreatic juices. One of these zymogens, which was originally named trypsinogen II by Figarella *et al.* (1969) has been reported by Robinson *et al.* (1972) to comprise only approximately 10% of the potential trypsin present in pancreatic juice. This agrees with the value of 10–20% which we attribute to anionic trypsin in the crude activated extracts of the human pancreatic gland, and suggests that trypsinogen II, upon activation, yields anionic trypsin. Whether this low yield of anionic trypsin is due to prior autolysis cannot be determined without isolation of the zymogen, but its presence in much lower quantities than cationic trypsin seems certain.

The isolation of anionic trypsin could in part account for the low yield of human cationic trypsin previously reported by this laboratory (Travis and Roberts, 1969), but because of the small quantities present and its unstable nature the role of anionic trypsin in digestion seems to be highly questionable.

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