Human Trypsin. Isolation and Physical–Chemical Characterization*

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ABSTRACT: The purification of human trypsin from activated extracts of human pancreas has been accomplished by a combination of salt fractionation, gel filtration on Sephadex G-75, and ion-exchange chromatography on SE-Sephadex C-25. The preparation was homogeneous by gel electrophoresis at pH 2.9 with a mobility comparable with bovine trypsin. At pH 4.3, disc electrophoresis indicated slight heterogeneity, although no other sharp bands could be detected. It is estimated that the purity of this preparation is greater than 95%. Ultracentrifuge studies indicated a single component with a molecular weight of 22,900. The amino acid composition of this molecule indicates it to be similar to other mammalian trypsins; however, only four disulfide bonds are possible, as compared with six for the other mammalian species, and only one methionine residue is present. Human trypsin is inhibited by L-1-tosylamido-2-phenylethyl chloromethyl ketone and diisopropylphosphorofluoridate but not by soybean trypsin inhibitor or by ovomucoid. The absence of inhibition by these macromolecular inhibitors may be related to a difference in tertiary structure of the human enzyme as compared with other trypsins. This difference may be related to the decrease in disulfide bonds.

Since the isolation of bovine trypsin by Northrup and Kunitz (1932), the molecular and catalytic properties of this molecule have been studied extensively (Green and Neurath, 1954; Neurath, 1964). These studies have culminated in the total sequence of bovine trypsinogen (Walsh and Neurath, 1964). In recent years, comparative studies have been performed on trypsins isolated from pig, turkey, sheep, dog, and rat (Travis and Liener, 1965; Ryan *et al.*, 1965; Travis, 1968; Marchis-Mouren *et al.*, 1961, 1963). These results suggest that despite the similarity in enzyme specificity of these mammalian trypsins, their physical and chemical properties may differ markedly.

Buck *et al.* (1962) were the first to report on the properties of a partially purified sample of human trypsin, and their results indicated a possible relationship between human and pig trypsins. Keller and Allan (1967) examined more closely the proteins of human pancreatic juice and found the presence of two trypsins and one chymotrypsin, as well as large amounts of pancreatic trypsin inhibitor.

In the present communication, we report the isolation and physicochemical properties of a homogeneous preparation of human trypsin.

Materials

Human pancreas were obtained at autopsy from St. Joseph's Hospital, Marshfield, Wis., and sharp frozen to prevent autolysis. Bovine trypsin and soybean trypsin inhibitor were obtained from the Worthington Biochemical Corp., while pork trypsin was prepared by the method of Travis and Liener (1965).

Sephadex G-75 and sulfoethyl Sephadex C-25 were products of Pharmacia Fine Chemicals Inc.

Benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester were purchased from Mann Research Laboratories. 1-Chloro-3-tosylamido-7-amino-2-heptanone and L-1-tosylamido-2-phenylethyl chloromethyl ketone were obtained from the Cyclo Chemical Corp.; DFP was a product of the Sigma Chemical Co.

Methods

Activity Measurements. Trypsin and chymotrypsin esterase activities were determined by the spectrophotometric procedure of Schwert and Takenaka (1955) using benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester, respectively, as substrates. All assays were carried out at pH 8.0 in 0.1 M sodium borate buffer, containing 0.05 M $CaCl_2$. The temperature was maintained constant at 23°. A unit of tryptic or chymotryptic activity was defined as an absorbance change of 1 unit/min and specific activity as the number of units per milligram of protein. Trypsin assays were read at 253 mμ and chymotrypsin at 237 mμ.

Protein Concentration. For enzyme purification, protein was determined spectrophotometrically by measuring absorption at 280 and 260 mμ, as described by Warburg and Christian (1942). For more purified preparations, a specific extinction coefficient of 13.5/1% solution, as determined for pork trypsin (Travis and Liener, 1965), was utilized.

Polyacrylamide Electrophoresis. Disc electrophoresis was carried out by the procedure of Reisfeld *et al.* (1962) for basic proteins. The concentration of acrylamide in the small pore gel was 7.5%.

Acid polyacrylamide slab electrophoresis was carried out by the method of Jordan and Raymond (1969) in the E-C vertical gel electrophoresis apparatus. The gels were made from a 12% solution of Cyanogum-41 (Fisher Chemical Co.) dissolved in 0.12 M Tris adjusted to pH 2.9 with citric acid. The polymerization was catalyzed with 0.1% ascorbic acid, 0.0025% ferrous sulfate, and 0.03% hydrogen peroxide. The electrode

* From the Department of Biochemistry, University of Georgia, Athens, Georgia (J. T.), and the Marshfield Clinic Foundation for Medical Research and Education, Marshfield, Wisconsin (R. C. R.). *Received August 19, 1968.* Supported in part by Research Grant AM 13169 from the National Institutes of Health and Grant No. T-444 from The American Cancer Society.

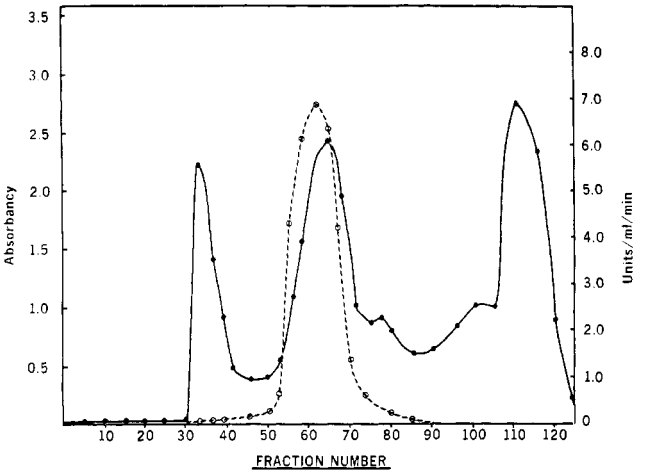

FIGURE 1: Sephadex G-75 chromatography of activated 0.2–0.8 saturation $(NH_4)_2SO_4$ salt fraction from extracts of human pancreas. A column of Sephadex G-75 (5 × 60 cm) was equilibrated with 0.2 M NaCl–0.05 M $CaCl_2$ (pH 2.6). Protein (800 mg) dissolved in 10 ml of the same solution was added, the flow rate was adjusted to 30 ml/hr, and 10-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (●——●), left ordinate; activity against benzoyl-L-arginine ethyl ester (○——○), right ordinate.

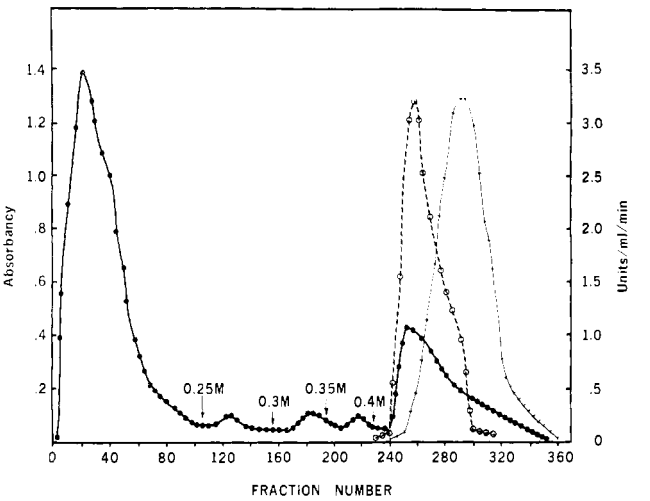

FIGURE 2: SE-Sephadex C-25 chromatography of active fraction obtained from Sephadex G-75 column. The column was equilibrated with 0.2 M NaCl–0.5 M $CaCl_2$ (pH 2.6) and stepwise elution with increasing NaCl solutions was applied at the positions indicated by the arrows. Column dimensions 1.5 × 20 cm; flow rate, 15–18 ml/hr; fraction size, 5 ml. Curves are designated as follows: optical density at 280 mμ (●——●), left ordinate; activity against benzoyl-L-arginine ethyl ester (○——○), right ordinate; activity against *N*-acetyl-L-tyrosine ethyl ester (▲——▲), right ordinate.

chambers contained 0.37 M glycine adjusted to pH 4.0 with citric acid.

Ultracentrifugal Analysis and Molecular Weight. A Spinco Model E ultracentrifuge equipped with an AN-H rotor, electronic speed control, and an RTIC temperature control unit was used to measure sedimentation rate and to determine a molecular weight by approach to equilibrium. All analyses were performed in 10^{-3} N HCl containing 0.1 M NaCl. The sedimentation coefficient was determined from measurements obtained with a microcomparator of the maximum ordinate on schlieren photographs. The sedimentation coefficient was corrected to 20° in H_2O (Schachman, 1957). No attempt was made to determine concentration dependence of the sedimentation coefficient.

The molecular weight was determined by the boundary depletion sedimentation equilibrium method of Yphantis (1964) in a double-sector interference cell equipped with sapphire windows. The equilibrium run was carried out on a 0.025% solution of human trypsin dissolved in 10^{-3} N HCl containing 0.1 M NaCl which had been dialyzed against the solvent for 36 hr. The equilibrium run was carried out at 20° for 15 hr.

The partial specific volume was obtained from density differences between a 0.3% solution of human trypsin and the solvent for 36 hr. The absolute protein concentration was determined by drying to constant weight. The density measurements were made with a Cahn RG electrobalance equipped with a density cell and glass plummet (Elgert and Cammann, 1967) at 20.0°. The partial specific volume was also determined from the amino acid composition by the method of McMeekin and Marshall (1952).

Amino Acid Analyses. The amino acid composition of human trypsin was obtained according to the procedure of Spackman *et al.* (1958) using a Beckman–Spinco Model 120B amino acid analyzer. Hydrolysis times of 12, 22, 72, and 96 hr in constant-boiling HCl were used in the standard determination. Cysteine and methionine residues were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (Hirs, 1956). Tryptophan was measured by the differential spectrophotometric procedure of Goodwin and Morton (1946).

Results

Purification of Human Pancreatic Trypsin. STEP I. EXTRACTION. Frozen human pancreas (6 lb) was allowed to thaw slightly and stripped of fat and connective tissue. They were then sliced into 1-in. thick sections and homogenized in twice their volume of 0.25 N H_2SO_4 in a large Waring Blender. As a precaution against foaming, 1 or 2 drops of Dow Corning Anti-foam B was routinely added to the solution prior to homogenization. This was found to have no adverse effect on subsequent procedures.

The homogenate was spun at low speed (1465*g*) in a centrifuge at 4° and the precipitate was reextracted as described above and recentrifuged. The supernatants were combined and centrifuged at 23,300*g* for 30 min and the precipitate was discarded. The final volume was approximately 3 l.

STEP II. AMMONIUM SULFATE FRACTIONATION. The extract obtained in step I was brought to 0.2 saturation with solid $(NH_4)_2SO_4$, centrifuged at 23,300*g*, and discarded. The supernatant was brought to 0.8 saturation with solid $(NH_4)_2SO_4$, and the precipitate obtained was redissolved in water and dialyzed exhaustively against 10^{-3} M HCl at 4°. A small amount of precipitate was obtained which was removed by centrifugation. The clear solution was then lyophilized. The yield was approximately 15 g of powder.

STEP III. ACTIVATION. The powder (1g) obtained from step II was dissolved completely in 25 ml of 0.1 M sodium borate–0.05 M $CaCl_2$ (pH 8.0) and the solution was incubated at 4°

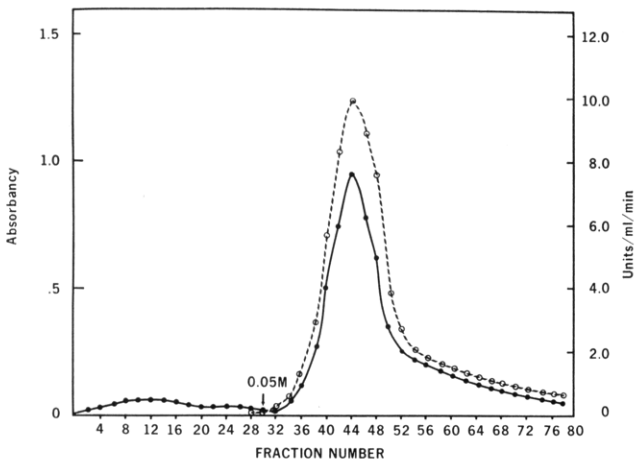

FIGURE 3: Rechromatography of peak fraction (from Figure 2) on SE-Sephadex C-25. The column was equilibrated with 0.05 M sodium citrate (pH 5.0)–0.05 M $CaCl_2$, and stepwise elution with 0.05 M NaCl begun at the position indicated by the arrow. Column dimensions, 1.0 × 60 cm; flow rate, 9–12 ml/hr; fraction size, 3 ml. Curves are designated as in Figure 1.

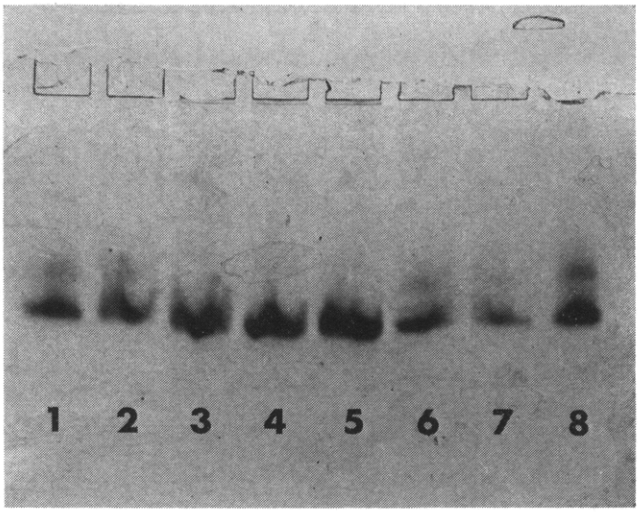

FIGURE 5: Acid polyacrylamide electrophoresis of human trypsin and beef trypsin. Samples 1, 2, 6, 7, and 8 are beef trypsin; samples 3, 4, and 5 human trypsin. Gel buffer Tris-citrate (pH 2.9), electrode buffers glycine–citrate (pH 4.0). Both proteins were dissolved in 10^{-3} N HCl at a concentration of 20 mg/ml. The patterns were stained with 1% Schwartz Amido Black 10B in 7.5% acetic acid.

for 12 hr. Activation for longer periods of time resulted in no further gain in enzyme activity. A precipitate gradually formed which was removed by centrifugation. The extract was then adjusted to pH 3.0 with 1 N HCl, dialyzed against 10^{-3} M HCl, and lyophilized. The yield was 800 mg of protein.

STEP IV. GEL FILTRATION. The protein obtained at step III was dissolved in 10 ml of 0.2 M NaCl containing 0.05 M $CaCl_2$ which had been adjusted to pH 2.6 with HCl and chromatographed at 4° on a column of G-75 Sephadex equilibrated against the same solvent (Figure 1). After passage of a yellow band through the column, the enzyme was eluted just prior to a second yellow band containing little protein as judged by absorption at 280 mμ. The active material was pooled (fractions 50–70) and utilized directly for ion-exchange chromatography. The yield, based on absorption at 280 and 260 mμ, was 200 mg of protein.

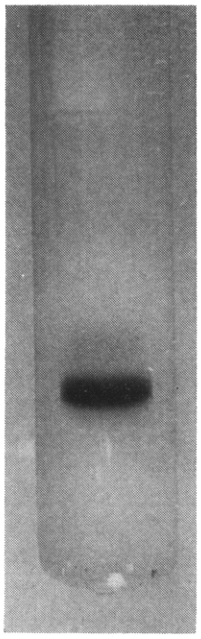

FIGURE 4: Disc electrophoresis of 2% solution of human trypsin; anode is at the top and cathode is at the bottom. Direction of migration is from top to bottom. Running time 60 min at 7 mA/tube. Patterns were stained with 1% Schwartz Amido Black 10B in 7.5% acetic acid.

STEP V. FIRST SE-SEPHADEX C-25 COLUMN CHROMATOGRAPHY. The solution obtained in step IV (205 ml) was pumped directly onto a column of SE-Sephadex C-25 also equilibrated with 0.2 M NaCl–0.05 M $CaCl_2$ at pH 2.6. As shown in Figure 2, a great deal of protein passed unretarded through the column. This material was completely inactive toward benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester. The column was washed with the same buffer until the absorption at 280 mμ had leveled. Then stepwise increases in the salt concentration of the eluting buffer to 0.25 M NaCl, 0.30 M NaCl, and 0.35 M NaCl were made. In each case, little or no protein was eluted. However, at 0.4 M NaCl, a peak containing both chymotrypsin and trypsin activity was eluted. Fractions 239–280 were collected, dialyzed against 0.05 M sodium citrate–0.05 M $CaCl_2$ (pH 5.0), and used directly for the next chromatographic step. The protein yield was approximately 90 mg.

STEP VI. SECOND SE-SEPHADEX C-25 CHROMATOGRAPHY. The active, dialyzed eluate (210 ml) was reapplied to a column of SE-Sephadex C-25 equilibrated with 0.05 M sodium citrate buffer–0.05 M $CaCl_2$ (pH 5.0). After washing the column with this buffer until the absorbance at 280 mμ was zero, stepwise elution was initiated with 0.05 M sodium citrate buffer, 0.05 M $CaCl_2$, and 0.05 M NaCl (pH 5.0). The results are shown in Figure 3 and indicated the complete absence of chymotrypsin-like activity, all of which was retained on the column. Furthermore, the specific activity of individual fractions was found to be essentially constant throughout the protein peak. Fractions 36–56 were pooled, dialyzed exhaustively *vs.* 10^{-3} M HCl, and lyophilized. The yield of dry protein was 38 mg. This material was utilized for the remainder of the studies described below.

TABLE I: Purification of Human Trypsin from 0.2 to 0.8 Saturated Salt Fraction.

Fractionation Step	Total Protein (mg)	Total Act. (units × 10^{-2})	Recov (%)	Sp Act. (units/mg)	Purifcn
Activation	1000	7.0	100	0.7	1
Centrifugation and dialysis	800	7.0	100	0.87	1.2
Sephadex G-75 column	200[a]	5.6	80	2.8	4
First SE-Sephadex C-25 column	90[a]	5.0	70	5.5	7.8
Second SE-Sephadex C-25	38	3.7	53	9.9	14.1

[a] Protein estimated by absorbance at 280 and 260 mμ.

A summary of the purification of human trypsin beginning with 1 g of salt-fractionated protein is shown in Table I.

Zone Electrophoresis. Disc electrophoresis of the final preparation at a concentration of 20 mg/ml dissolved in 10^{-3} M HCl is shown in Figure 4. A very diffuse, minor component is visible indicating the presence of small amounts of impurities.

Acid polyacrylamide gel electrophoresis was carried out on gel slabs to allow a direct comparison of the mobilities of beef and human trypsins. The results are shown in Figure 5. The human trypsin preparation showed a single component in this system with a mobility similar to that of the major component of commercial bovine trypsin.

Ultracentrifugal Analysis and Molecular Weight. Sedimentation velocity experiments indicated a single component with an $s_{20,w}$ of 2.68 S at a concentration of 2.5 mg/ml. The sedimentation pattern at 64 min is shown in Figure 6.

The results obtained from the boundary depletion equilibrium run are shown in the ln C *vs.* r^2 plot shown in Figure 7. The ln C values are expressed as ln Δy, where $\Delta y = y_r - y_o$; y_r = the ordinate of a fringe at distance r from the center of rotation and y_o = the ordinate of the same fringe in the meniscus region of the solution column. Each Δy value in Figure 7 represents the average Δy values for two fringes. No significant departure from linearity could be detected over the length of the ln C *vs.* r^2 plot. The slope of the least-squares line of regression was 1.88.

The partial specific volume determined by density measurements with the microelectrobalance was 0.717. The partial specific volume calculated from the amino acid composition was 0.715. The density of the solvent, determined with a 10-ml pycnometer, was 1.0022.

The apparent molecular weight of human trypsin was determined to be 22,900, using the slope from Figure 7 and the partial specific volume value 0.717. Since the initial protein concentration was low, 0.025%, this apparent molecular weight should closely approximate the actual molecular weight.

Amino Acid Composition. The results of the amino acid analysis of human trypsin are shown in Table II. In determining this composition, the assumption was made that 1 molecule of trypsin contained 13 residues of alanine; this was based primarily on the reasoning used in the determination of the amino acid composition of bovine carboxypeptidase A (Bargetzi *et al.*, 1963).

In Table III a comparison of the amino acid composition of several mamma ian trypsins is made. The most significant difference in these compositions appears to be in the low

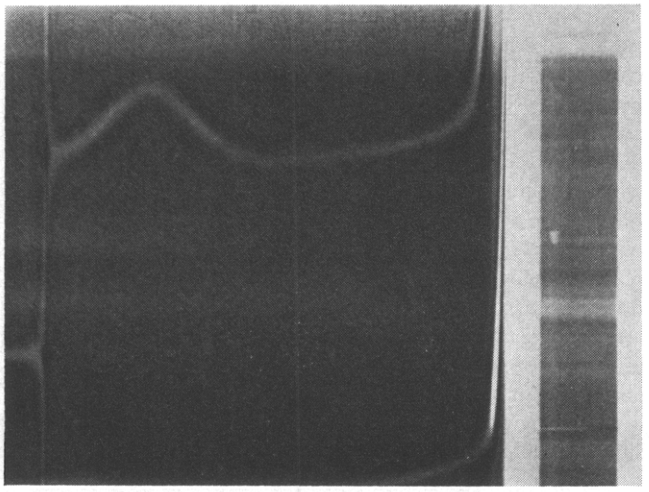

FIGURE 6: Sedimentation velocity pattern of a 0.25% solution of human trypsin at 64 min. Speed 60,000 rpm, bar angle 45°. Sedimentation is from left to right.

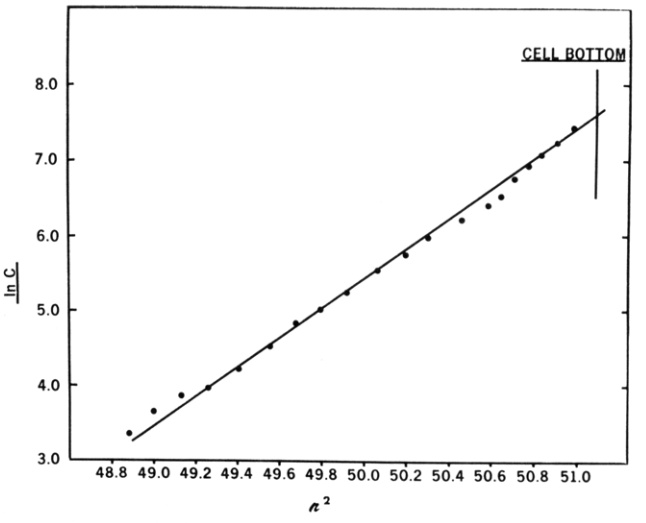

FIGURE 7: Plot of ln C *vs.* r^2 from data obtained from the sedimentation equilibrium run on a 0.025% solution of human trypsin. C is the concentration expressed in terms of ln Δy values. Δy is defined in the text.

TABLE II: Amino Acid Composition[a] of Human Trypsin.

Amino acid	Time of Hydrolysis (hr) 12	22	72	96	Value Taken	Nearest Integer
Lysine	11.2	11.4	11.4	11.2	11.3	11
Histidine	3.0	3.0	2.9	2.8	2.9	3
Arginine	6.0	5.9	5.6	5.7	6.1[b]	6
Aspartic acid	20.9	21.2	20.8	21.0	21.0	21
Threonine	8.7	7.9	7.3	6.0	9.7[b]	10
Serine	20.6	19.0	17.0	14.7	23.6[b]	24
Glutamic acid	21.0	21.0	21.0	20.0	20.8	21
Proline	9.1	9.0	8.9	9.0	9.0	9
Glycine	20.0	20.0	19.8	20.4	20.0	20
Alanine		(13)			13.0	13
Half-cystine					7.8[c]	8
Valine	10.6	13.7	14.7	15.6	15.6[b]	16
Methionine	1.2	1.2	1.1	1.1	1.1[c]	1
Isoleucine	9.0	10.9	11.6	11.9	11.9[b]	12
Leucine	12.0	12.3	12.4	12.0	12.2	12
Tyrosine	6.0	6.9	6.6	6.6	6.6	7
Phenylalanine	4.0	4.0	3.9	4.0	4.0	4
Tryptophan					3.2[d]	3

[a] Data are expressed as amino acid residues per molecule, assuming 13 residues of alanine/molecular weight of 22,900. [b] Threonine and serine values are based on a first-order approximation to zero time. Isoleucine and valines are from 96-hr hydrolysates. Arginine is based on a linear extrapolation to zero time. [c] Averages of 22-hr hydrolysates of oxidized samples. [d] Determined spectrophotometrically. The tyrosine value found by this procedure was 7.1.

TABLE III: Amino Acid Composition of Human and Other Mammalian Trypsins.

Amino Acid	Residues/Molecule Human[a]	Beef[b]	Pork[c]	Sheep[d]
Lysine	11	14	10	12
Histidine	3	3	4	3
Arginine	6	2	4	4
Aspartic acid	21	22	18	20
Threonine	10	10	11	15
Serine	24	33	24	26
Glutamic acid	21	14	17	14
Proline	9	9	10	9
Glycine	20	25	26	19
Alanine	13	14	16	17
Half-cystine	8	12	12	12
Valine	16	17	16	17
Methionine	1	2	2	2
Isoleucine	12	15	15	10
Leucine	12	14	16	14
Tyrosine	7	10	8	6
Phenylalanine	4	3	4	5
Tryptophan	3	4	6	

[a] From Table II. [b] Walsh and Neurath (1964). [c] Travis and Liener (1965). [d] Travis (1968).

values of the sulfur-containing amino acids in human trypsin.

Inhibition Studies. In order to determine whether human trypsin was inactivated by both chemical and naturally occurring trypsin inhibitors, studies were carried out on the effects of 1-chloro-3-tosylamido-7-amino-2-heptanone, L-1-tosylamino-2-phenylethyl chloromethyl ketone, DFP, and soybean trypsin inhibitor on enzyme activity. In he presence of a 10:1 inhibitor–enzyme molar ratio of either 1-chloro-3-tosylamino-7-amino-2-heptanone or DFP at pH 8.0 in 0.1 M sodium borate buffer–0.05 M $CaCl_2$, total loss of enzyme esterase activity occurred within 15 min. However in the presence of a 10 molar excess of L-1-tosylamino-2-phenylethyl chloromethyl ketone, no loss of trypsin esterase activity occurred. In contrast, with soybean trypsin inhibitor at inhibitor–enzyme molar ratios of 20:1, less than 10% inhibition took place in 30 min.

Discussion

Isolation procedures were utilized which would minimize losses in enzymatic activity. Thus, unlike many of the other mammalian trypsins isolated to date, no attempt was made to crystallize the human enzyme. Nevertheless, the final yield of human trypsin per gram of pancreas was low in comparison with the yields obtained from beef or pork pancreas. A possible explanation for this low yield is that many of the pancreata were atrophied and exhibited fatty replacement and could, therefore, not be considered as functioning efficiently. Attempts to isolate the zymogen rather than the active enzyme have not yet been successful, primarily due to the partial activation of the initial pancreatic extracts.

Previous communications regarding the properties of human pancreatic proteolytic enzymes have suggested that there are two trypsins present in human pancreatic juice (Keller and Allan, 1967) and that there was no apparent requirement for Ca^{2+} for enzyme stability (Buck *et al.*, 1962). Our results differ considerably in that we have found only one type of trypsin molecule which seem to require Ca^{2+} to retard autolysis. The relationship between the two anionic benzoyl-L-arginine ethyl ester esterases found by Keller and Allan in pancreatic juice and the benzoyl-L-arginine ethyl ester esterase fraction we isolated from pancreatic tissue is not known. The requirement of Ca^{2+} was observed by us in early attempts to purify human trypsin, when it was found that highly purified fractions lost activity rapidly in the absence of this ion. An attempt to compare these results with those of Buck *et al.* is unwarranted since their preparation was only about 50% pure and autolysis may have been retarded by the protective effect obtained by digestion of the contaminating proteins, or possibly by the presence of human pancreatic trypsin inhibitors.

Absolute proof that the benzoyl-L-arginine ethyl ester splitting enzyme isolated from human pancreatic tissue is the homologous enzyme to other mammalian trypsins must await elucidation of active site and the hydrolytic mechanism

involved. However, the information so far collected indicates that we are dealing with human trypsin. The enzyme is inhibited by DFP and 1-chloro-3-tosylamino-7-amino-2-heptanone, suggesting the presence of serine and histidine in the active site. The electrophoretic mobility at pH 2.9 is comparable with bovine trypsin, and the enzyme is active in the same pH range as other mammalian trypsins. The enzyme is the same size, and has a similar amino acid composition to the other trypsins studied.

The lack of inhibition of human trypsin by soybean trypsin is a surprising finding. Buck *et al.* reported that their human trypsin activity was not affected by ovomucoid. The facts that these two macromolecular trypsin inhibitors do not inhibit the esterase activity of human trypsin, but the low molecular weight inhibitors DFP and 1-chloro-3-tosylamino-7-amino-2-heptanone do inhibit the enzyme, suggest that the three-dimensional structure of the human enzyme differs from other mammalian trypsins. The finding of only eight half-cystines in the amino acid analysis, allowing only four disulfide bonds, instead of the six found in the other trypsins presented in Table III further suggests changes in the three-dimensional structure.

Indirect evidence of the reaction of human trypsin with soybean trypsin inhibitor has been inferred in experiments measuring serum "trypsin" activity. For example, Siegelman *et al.* (1962) briefly state in their discussion that serum "trypsin" is inhibited by soybean trypsin inhibitor. Directly contrary to this finding is the data presented by Rutkowski (1966), who shows that serum benzoyl-L-arginine ethyl ester esterase activity is only decreased by 20% by 30 μg of soybean trypsin inhibitor/ml. Plasma benzoyl-L-arginine ethyl ester esterase activity was inhibited to a much greater degree by soybean trypsin inhibitor than the serum activity. The role of serum α2-macroglobulin–trypsin complexes (Mehl *et al.*, 1964), which would still show trypsin esterase activity, but can no longer be inhibited by soybean trypsin inhibitor, must be considered in explaining these results. In addition, there are several enzymes in serum showing trypsin-like activity. These references illustrate the difficulties existing in measuring serum "trypsin" activity and describing properties to human trypsin from such measurements. The availability of pure human trypsin should make it possible to elucidate the role of trypsin in human "trypsin" activity.

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