Pancreatic Trypsinogen from the African Lungfish*

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ABSTRACT: The purification and characterization of a pancreatic trypsinogen from the African lungfish, *Protopterus aethiopicus*, is reported. The purified zymogen has been characterized by molecular weight, amino acid composition, amino-terminal sequence, and by activation. The activation peptide of lungfish trypsinogen has the sequence Phe-Pro-Ile-Glu-Glu-Asp-Lys- and differs from all previously known trypsinogens in lacking a tetraaspartyl sequence. A possible scheme for the evolutionary changes in the structure of the activation peptide of trypsinogen is presented. The autoactivation of lungfish trypsinogen is promoted by calcium ions in a manner similar to that of bovine trypsinogen. Lungfish trypsin has also been isolated and characterized. Its functional properties are similar to those of the bovine enzyme.

The last few years have witnessed the discovery and characterization of trypsin-like enzymes from lower vertebrates, invertebrates, and microorganisms. Those which have been characterized include pancreatic trypsin from the dogfish (Tye, 1970), trypsin-like enzymes from the starfish (Winter and Neurath, 1970; Camacho *et al.*, 1970), shrimp (Gates and Travis, 1969), sea pansy (Coan and Travis, 1970), horseshoe crab,[1] crayfish (Zwilling and Tomasek, 1970), moth (Kafatos *et al.*, 1967), and a bacterium, *Streptomyces griseus* (Jurasek *et al.*, 1969). The amino acid compositions and molecular weights of these proteins are similar to those of bovine trypsin (23,000). These similarities assume added significance in light of the substantial homology in amino acid sequence in certain regions of bovine trypsin and the trypsin-like enzyme from *Streptomyces griseus*. This finding raises the possibility that the sequences of many, if not all, of the trypsins listed are homologous to those of the vertebrate trypsins.

We have previously presented a chromatographic analysis of the major enzymes and zymogens in the pancreas of the African lungfish, *Protopterus aethiopicus* (Reeck *et al.*, 1970). Three separate chromatographic fractions containing trypsinogen were identified. In the present study one of these trypsinogen fractions was purified and characterized and related to the evolutionary pattern of trypsins.

Experimental Section

Materials

Tissue was obtained and stored as described previously (Reeck *et al.*, 1970).

Substrates for Enzymatic Assays. N^{α}-Benzoyl-L-arginine ethyl ester and *p*-tosyl-L-arginine methyl ester were purchased from Cyclo Chemical Corp. Benzoylglycyl-L-arginine was obtained from Fox Chemical Co. and carbobenzoxyglycyl-L-phenylalanine from Cyclo Chemical Corp.

DE-52 cellulose was purchased from Whatman Reeve Angel and Sephadex G-100 from Pharmacia Fine Chemicals.

The proteins used as standards in sodium dodecyl sulfate gel electrophoresis were all obtained from Worthington Biochemical Corp. except myoglobin, which was purchased from Mann Research Laboratories.

Soybean trypsin inhibitor (chromatographically prepared) and chicken ovomucoid were purchased from Worthington Biochemical Corp.

DFP was obtained from the Pierce Chemical Co. The reagent was diluted to 1 M in water-free isopropyl alcohol stored over molecular sieves (Matheson Coleman & Bell).

Guanidine·HCl (spectrophotometric grade) was obtained from Heico, Inc.; β-mercaptoethanol from Baker Chemical Co.; and *p*-nitrophenyl *p'*-guanidinobenzoate was synthesized by R. A. Kenner of this laboratory following the procedure of Chase and Shaw (1967).

Sodium dodecyl sulfate was a product of Mallinckrodt Chemical Works; N^{α}-tosyl-L-lysine chloromethyl ketone was from Cyclo Chemical Corp.; and tris(hydroxymethyl)aminomethane was from Signa Chemical Co. and Mann Research Laboratories.

Methods

Amino acid Composition. A Spinco Model 120 amino acid analyzer was used for all analyses. Amino acid compositions of trypsinogen and trypsin were derived from duplicate hydrolyses in 6 N HCl at 110° for 24, 48, 72, and 96 hr. Individual analyses were placed on a common molar basis by adjusting all alanine values to a constant number. The reported values for serine and threonine are extrapolations to zero time of hydrolysis assuming first-order kinetics of destruction. Isoleucine and valine values are the average of 96-hr hydrolyses unless these amino acids were fully liberated after 72-hr hydrolysis, in which case the 72- and 96-hr results were averaged and reported. Half-cystine and methionine were determined after performic acid oxidation as cysteic acid and methionine sulfone (Hirs, 1967). Tryptophan was determined by the method of Edelhoch (1967).

Analytical Polyacrylamide Disc Gel Electrophoresis. Proteins were subjected to acrylamide disc gel electrophoresis at pH 8.3 following the general method of Davis (1964) except that the separating gels were poured before the sample gels and the concentrating gel was omitted since its inclusion did

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[1] M. R. Moore and J. Travis (1970) as quoted by Walsh (1970).

not affect the resulting electrophoresis. Gels were run in an apparatus purchased from Hoeffer Scientific Instruments. Distilled water at 4° was used for the preparation of the reservoir buffer and the gels were run at room temperature.

Gels were stained with 1% Amido-Schwarz in 7% acetic acid for at least 1 hr. They were destained using a quick gel destainer of the Canalco Co.

Column Chromatography and Gel Filtration. All columns were developed in the cold room (4°). Columns were packed with media as directed by the manufacturers. Ion-exchange cellulose and Sephadex were stored in 0.03% toluene to prevent microbial growth.

Dialysis Tubing. Visking dialysis tubing was cut into appropriate lengths and immersed in 0.1 M $NaHCO_3$. The tubing was heated on a steam cone for 8 hr and the water replaced with a fresh solution of $NaHCO_3$. After heating for another 8 hr, the tubing was rinsed several times with distilled water and stored for later usage in 0.03% toluene.

Enzymatic Assays. *p*-Tosyl-L-arginine methyl ester was employed to assay spectrophotometrically for trypsin activity (Hummel, 1959). *N*-Benzoyl-L-tyrosine ethyl ester was the substrate for the spectrophotometric assay of chymotrypsin according to the method of Hummel (1959) except that the substrate was dissolved directly in 0.04 M Tris (pH 7.8) containing 0.05 M $CaCl_2$, by heating and shaking vigorously (Horbett, 1970). No methanol was added to dissolve the substrate.

Exopeptidase activities were determined with carbobenzoxyglycyl-L-phenylalanine and benzoylglycyl-L-arginine as substrates for carboxypeptidases A and B, respectively. The hydrolysis of carbobenzoxyglycyl-L-phenylalanine was followed by measuring the decrease in absorbance at 225 nm of a 1 mM solution dissolved in 5 mM Tris (pH 7.5) containing 0.10 M NaCl (Whitaker *et al.*, 1966). Activity toward benzoylglycyl-L-arginine was measured in a spectrophotometric assay as described by Folk *et al.* (1960).

Zymogen preparations to be assayed for activity toward tosyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, carbobenzoxyglycyl-L-phenylalanine, or benzoylglycyl-L-arginine were routinely activated by adding 40 μg of bovine trypsin, 50 μl of 1 M $CaCl_2$, and 50 μl of 1 M Tris (pH 8.0) to 0.5 ml of sample. Activities were followed until a maximum was reached. This required less than 0.5 hr in every case except that 4 hr was allowed for complete activation of trypsinogen. In the initial DE-52 chromatography, the trypsin assays were performed after incubation of 0.5 ml of the appropriate fractions with 50 μl of an extract of Rhozyme 41 (Rohm and Haas) and 100 μl of 0.5 M sodium citrate (pH 3.5) for 10 min at room temperature. Rhozyme 41 is a crude protease preparation from *Aspergillus oryzae* from which an acid protease can be prepared which will activate bovine trypsinogen at acidic pH's (N. C. Robinson, unpublished experiments). This method of activating lungfish trypsinogen samples was advantageous since the activation was fast and the activating extract showed no activity toward tosyl-L-arginine methyl ester, the substrate for the trypsin assay. In comparison to values obtained after activation with bovine trypsin, the activation by Rhozyme 41 was incomplete.

Routine spectrophotometric assays were performed with the Gilford Model 2000 spectrophotometer. A Cary Model 16 spectrophotometer was used for kinetic analyses and determination of specific activities.

High-Voltage Paper Electrophoresis. Whatman No. 3MM paper was used for electrophoresis at 2000 V for 90 min as described by Naughton *et al.* (1960).

Sodium Dodecyl Sulfate Gel Electrophoresis. The general procedures outlined by Weber and Osborn (1969) were used with the following modifications. The protein samples were immersed in boiling water for 1 min and incubated for 2 hr at 37° in 0.01 M sodium phosphate (pH 7.0) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. The amount of bromophenol blue suggested by Weber and Osborn (1969) was increased tenfold for adequate visualization of the dye bands. No protein bands were seen on the gels unless the proteins, dissolved in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, were immersed in a boiling water bath for 1 min. In plotting logarithms of the molecular weights of several standard proteins against the mobilities of the proteins nonlinearity was consistently observed in high and low molecular weight regions.

Ultracentrifugation. High-speed sedimentation equilibrium analyses were performed at 32,159 rpm using the six-channel centerpiece of Yphantis (1964). The optimum speed for centrifugation was estimated by eq 12 of Teller *et al.* (1969) and the sample centrifuged for 24 hr to achieve sedimentation equilibrium. Photographs of the Rayleigh interference patterns at equilibrium were taken with Kodak II-G photographic plates. After photographs were taken of the equilibrium pattern and the cell thoroughly shaken, baseline interference patterns were photographed at 4000 rpm. Fringe displacement was measured as a function of position in the cell with a modified Nikon microcomparator (Teller, 1967). The data were analyzed by a computer program of Teller *et al.* (1969) which calculates the molecular weight moments, M_n, M_w, and M_z, as functions of protein concentration. Monomer molecular weights were calculated by quadratic extrapolation of $2M_n - M_w$ and $2M_w - M_z$ to zero protein concentration. A partial specific volume of 0.736 was assumed. The solvent was 0.01 M potassium phosphate (pH 8.0) containing 0.2 M KCl, and the temperature, 10.8°.

Ultrafiltration. Protein samples were concentrated by ultrafiltration in cells of the Amicon Corp. (65- or 400-ml size). UM-10 Diaflo membranes, also produced by Amicon, were used in all cases. Membranes and cells were handled as recommended by the manufacturer.

Results

Purification. The initial chromatographic step in the purification of lungfish trypsinogen was based on the system used for the analytical-scale separation of the anionic proteins in the lungfish pancreatic extract (Reeck *et al.*, 1970). For large-scale preparation it was necessary to increase the column size to accommodate the proteins from 100 g of acetone powder. Thus a DE-52 cellulose column (50 cm in length and 7.5 cm in diameter) was utilized to chromatograph the protein sample from 100 g of powder. The powder was extracted for 4 hr with 1000 ml of water at 4°. The slurry was adjusted to 1 mM DFP initially, and after 2 hr, by the addition of 1 M DFP in isopropyl alcohol. After 4 hr of extraction the slurry was centrifuged for 30 min at 10,400*g* and the supernatant adjusted to 70% saturation by the addition of solid ammonium sulfate. The precipitated proteins, collected by centrifugation for 30 min at 10,400*g*, were dissolved in 315 ml of water and dialyzed overnight against 48 l. of 0.005 M potassium phosphate (pH 8.0) containing 0.005 M NaCl. Before and after dialysis the sample was adjusted to 1 mM DFP by addition of the appropriate volumes of 1 M DFP in isopropyl alcohol. Immediately before application of the sample to the DE-52 column, a small amount of precipitate was removed by centrifugation for 15 min at 30,000*g*.

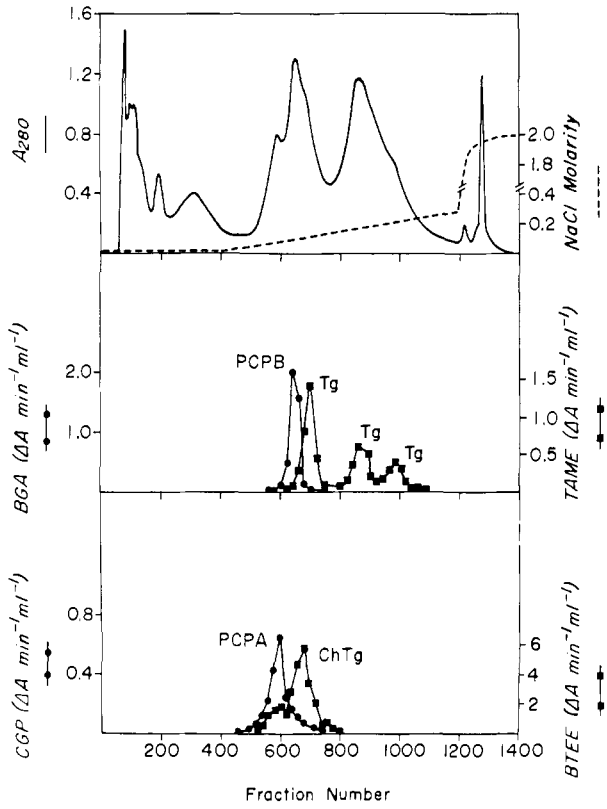

FIGURE 1: Chromatography on DE-52 cellulose of the aqueous extract of 100 g of lungfish pancreas acetone powder. Fraction volume was 25 ml. Other details are given in the text.

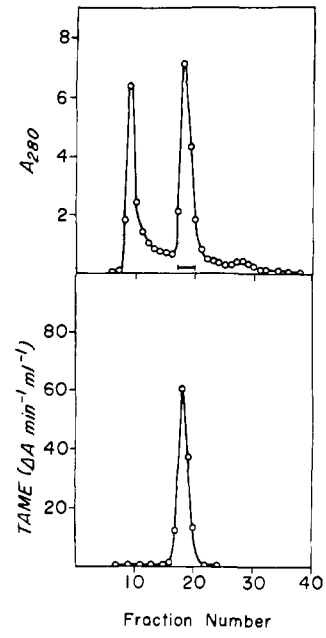

FIGURE 2: Gel filtration of lungfish trypsinogen. Preparation of the sample is described in the text. Fraction volume was 20 ml.

The column, equilibrated with 0.005 M potassium phosphate (pH 8.0) containing 0.005 M NaCl, was developed at a flow rate of 1000 ml hr^{-1} using a Milton Roy pump. Figure 1 shows the elution profile and assays from this chromatography. At fraction 400 a linear gradient from 0.005 to 0.3 M NaCl in a total volume of 20 l. of phosphate buffer was applied. The resulting chromatography was, in all essential respects, comparable to the small-scale chromatography previously described (Reeck *et al.*, 1970). Procarboxypeptidase A, however, tended to separate incompletely from procarboxypeptidase B on the larger scale system. The trypsinogens were usually better resolved than is shown in this chromatogram (Figure 1).

The third trypsinogen fraction eluted from the initial chromatography on DE-52 cellulose was the starting material for further purification. The appropriate fractions were combined, concentrated to 200 ml on the Amicon TC ultrafiltration system, and adjusted to pH 3.0 with 1 N HCl while stirring the protein solution. The sample was then dialyzed overnight against 0.01 M sodium citrate and lyophilized. The lyophilized trypsinogen samples from two such chromatographies, constituting 400 mg of protein, were dissolved in 16 ml of water, the pH adjusted to 8.0 with 1 M NaOH, and 15 μl of 1 M DFP in isopropyl alcohol was added. The sample was then applied to a 2.5 × 100 cm column of Sephadex G-100 equilibrated with 0.01 M potassium phosphate (pH 8.0). The trypsinogen fractions from this gel filtration (Figure 2) were applied directly to a 1.5 × 20 cm column of DE-52 cellulose which had been equilibrated with the same phosphate buffer. A linear salt gradient from 0.0 to 0.05 M NaCl in a total volume of 700 ml of 0.01 M potassium phosphate (pH 8.0) was applied to elute the trypsinogen, and then 0.3 M NaCl was applied to elute all remaining protein. The resulting elution profile is shown in Figure 3. The trypsinogen fractions indicated by the bar in Figure 3 were combined, adjusted to 1 mM DFP by the addition of 250 μl of 1 M DFP, concentrated to 62 ml by ultrafiltration, and lyophilized.

From 200 g of acetone powder, 125 mg of pure trypsinogen was obtained. In subsequent preparations the precautionary

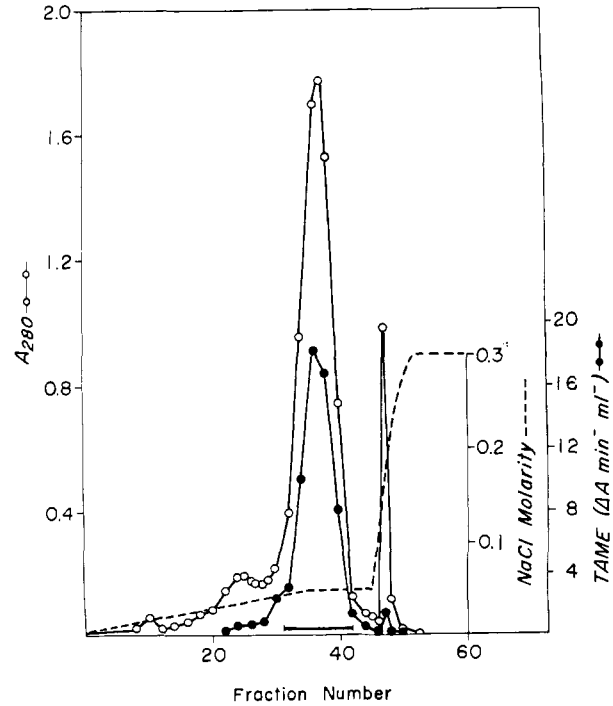

FIGURE 3: Chromatography of trypsinogen on DE-52 cellulose. The sample was fractions 17–20 of the gel filtration shown in Figure 2. Details of the chromatography are given in the text. Sample volume was 20 ml.

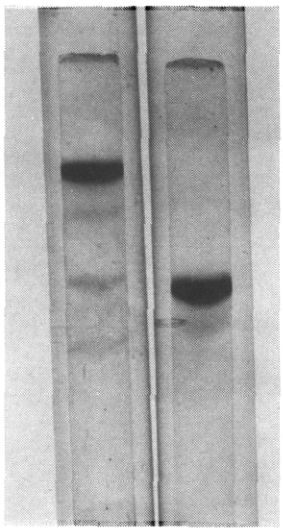

FIGURE 4: Analytical disc gel electrophoresis of trypsinogen and trypsin. Trypsinogen is on the right and trypsin on the left. Migration was from top to bottom (cathode to anode). Gels were severed at the dye front before staining.

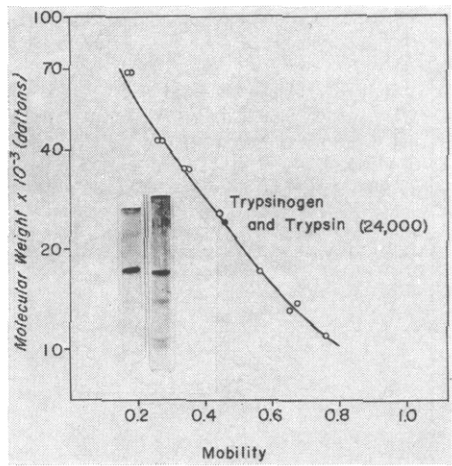

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of lungfish trypsinogen and trypsin. Standard proteins were: bovine serum albumin (68,000), ovalbumin (43,000), glyceraldehyde phosphate dehydrogenase (36,000), chymotrypsinogen (25,700), myoglobin (17,200), ribonuclease (13,700), and the two chains of chymotrypsin (13,000 and 11,000). In the insert trypsinogen is on the left and trypsin on the right.

lowering of the pH of the trypsinogen solution after the initial chromatography was in fact unnecessary. Furthermore, the large TC ultrafiltration system was not used in the later preparation because some denaturation of protein seemed to occur during the concentration process. Instead the solution was concentrated by ultrafiltration in a 400-ml Amicon cell. This procedure was rapid enough to avoid autoactivation.

Physicochemical Characterization of Lungfish Trypsinogen. The essential homogeneity of the trypsinogen thus prepared was demonstrated by electrophoresis on polyacrylamide gels at pH 8.3. As can be seen in Figure 4, the zymogen displayed one major band and a very faint band of slightly greater mobility. As supporting evidence for the homogeneity of lungfish trypsinogen and particularly for the absence of high molecular weight contaminants, a sample of the lyophilized protein was dissolved in water to a protein concentration of 3.8 mg ml^{-1}, and immediately run in the ultracentrifuge at 67,988 rpm. The sedimentation of the protein was followed with schlieren optics. A photograph taken after 152 min of centrifugation is shown in Figure 5. The symmetrical nature of the peak after this extended period of centrifugation is evidence for the hydrodynamic homogeneity of the preparation. A sedimentation constant, $s_{20} = 2.82$ S, was calculated from this experiment.

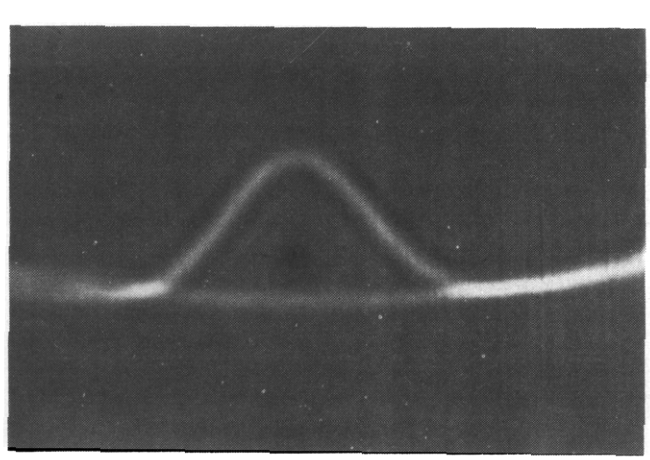

FIGURE 5: Schlieren optical pattern of lungfish trypsinogen. The picture was taken after 152-min centrifugation at 67,988 rpm and 3.1°. The protein concentration was 3.8 mg ml^{-1}. The bar angle was 60°. Sedimentation was from left to right.

As further evidence for the homogeneity of the trypsinogen and as an estimate of its molecular weight, the protein was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. The gel shown in Figure 6 reveals a single band corresponding to a molecular weight of 24,000.

The molecular weight was also determined by high-speed sedimentation equilibrium analysis. Protein solutions of concentrations 0.24, 0.46, and 0.68 mg ml^{-1} in 10 mM potassium phosphate (pH 8.0) containing 0.2 M KCl were examined. Molecular weight moments increased as a function of protein concentration in the ultracentrifuge cell. Coupled with the evidence from sedimentation velocity analysis, this finding indicates that trypsinogen undergoes self-association. A monomer molecular weight of 24,000–25,000 was calculated from the computer program.

The amino acid composition of lungfish trypsinogen, determined as outlined in Methods, is presented in Table I.

In separate analyses the extinction coefficient of trypsinogen was determined to be 1.99 using the procedure of Walsh and Brown (1962).

Activation of Lungfish Trypsinogen. KINETICS OF AUTOACTIVATION. In light of the unique structure of the activation peptide of lungfish trypsinogen (see below), it was of interest to examine the effect of calcium and urea on the activation of this zymogen. The time course of the autoactivation of lungfish trypsinogen was examined in the presence and absence of calcium chloride and urea. All measurements were performed at a protein concentration of 5 mg ml^{-1} in 10 mM Tris (pH 8.0). In each case, the very small amount of trypsin present in the preparation (estimated to be 0.2% by activity measurements) was allowed to initiate the activation; no exogenous trypsin was added.

As can be seen in Figure 7, calcium chloride is extremely effective in promoting the activation of the zymogen, as has been observed previously for the bovine (MacDonald and Kunitz, 1941) and dogfish (Tye, 1970) zymogens. It should be

TABLE I: Amino Acid Compositions of Lungfish Trypsinogen and Trypsin.

	Trypsin (Residues/ 24,000 Daltons)	Trypsinogen (Residues/ 25,000 Daltons)	Trypsinogen (Nearest Integral Value)
Asp	23.0	25.0	25
Thr	8.9	9.3	9
Ser	30	30	30
Glu	16.6	18.9	19
Pro	10.4	10.8	11
Gly	24.5	24.6	25
Ala	11.7	11.7	12
Cys/2	11.3	11.6	12
Val	18.6	17.4	17
Met	2.87	2.97	3
Ile	14.6	15.4	15
Leu	13.8	13.7	14
Tyr	11.8	13.2	13
Phe	2.55	2.96	3
Trp	5.3	5.9	6
Lys	6.57	7.20	7
His	6.17	6.22	6
Arg	5.42	4.95	5
Total residues	224.1	231.8	232

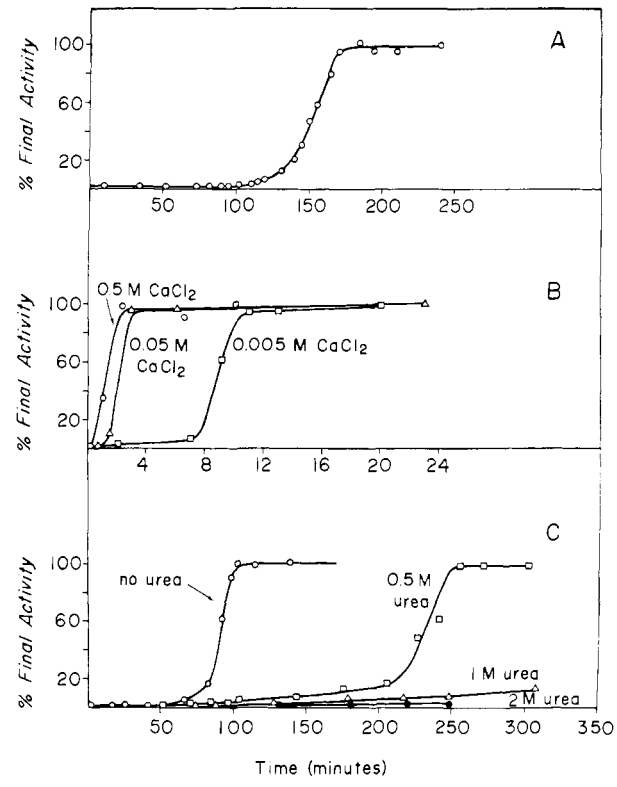

FIGURE 7: The kinetics of autoactivation of lungfish trypsinogen. All studies were done at a trypsinogen concentration of 5 mg ml^{-1} in 0.01 M Tris (pH 8.0). (A) Activation at 37° in the absence of calcium chloride. (B) Activation at 37° in the presence of 0.005, 0.05, and 0.5 M $CaCl_2$. (C) Activation at 0° in the presence of 0.1 M $CaCl_2$ and 0.0, 0.5, 1.0, and 2.0 M urea.

noted that the activation of lungfish trypsinogen differs from that of bovine trypsinogen in that the same level of activity is achieved in the presence and absence of calcium. In each instance, the final activity toward tosyl-L-arginine methyl ester was 120 ΔA min^{-1} ml^{-1}. This is 80% of the theoretical value for quantitative conversion of the zymogen to the enzyme. As in the case of the activations of bovine and dogfish trypsinogens (Radhakrishnan *et al.*, 1969a; Tye, 1970), relatively low levels of urea inhibit the activation of lungfish trypsinogen (Figure 7C).

ISOLATION OF THE ACTIVATION PEPTIDE. The activation peptide (defined operationally as a low molecular weight peptide released from the amino-terminal region of trypsinogen during its activation) was isolated to confirm its structure as suggested by the sequenator data (Hermodson *et al.*, 1971). The peptide was isolated by high-voltage paper electrophoresis at pH 6.5, adapting procedures previously utilized by N. C. Robinson (unpublished experiments). The appearance of a peptide in proportion to the appearance of enzymatic activity was first demonstrated as follows.

Trypsinogen (5 mg ml^{-1}) in 0.01 M *N*-ethylmorpholine acetate at pH 8.0 was allowed to activate spontaneously at 37°. At times corresponding to 0, 15, 35, 70, and 100% activations, 100-μl aliquots were removed from the activation mixture and added to 100 μl of glacial acetic acid to stop the reaction. The samples were lyophilized and dissolved in 25 μl of 50% acetic acid and subjected to high-voltage paper electrophoresis at pH 6.5. Figure 8 shows the electropherogram. The most prominent feature is the appearance, with increasing times of reaction, of an increasingly strong staining spot migrating toward the anode 0.42 times as fast as cysteic acid.

In a separate experiment, 1 ml of 5 mg ml^{-1} of lungfish trypsinogen in 0.01 M *N*-ethylmorpholine acetate was warmed to 37° and allowed to autoactivate. The sample was then lyophilized and dissolved in 0.5 ml of water. Proteins and peptides in the sample were separated by gel filtration on a 1.5 × 20 cm column of Sephadex G-25 equilibrated with 0.05 M formic acid and monitored at 250 nm. The peptide fractions were lyophilized, dissolved in 100 μl of 50% acetic acid, and subjected to high-voltage paper electrophoresis at pH 6.5, using paper which had been washed for 3 days by descending paper chromatography. Guided by the electropherogram derived from about 10% of the sample, the portion of the paper corresponding to the activation peptide was excised and the peptide eluted with 50% acetic acid. Half of the eluate was subjected to amino acid analysis after 24-hr hydrolysis at 110° in 6 N HCl.

The amino acid composition of this material, given in Table II, agrees well with the composition of the amino-terminal heptapeptide sequence of lungfish trypsinogen (Hermodson *et al.*, 1971). The amino acid composition of the peptide after one Edman degradation is also given in Table II. The loss of one residue of phenylalanine established that the amino terminal residue of the peptide was phenylalanine and indicated the purity of the peptide. Amino acid compositions of the ninhydrin-positive material migrating slightly toward the cathode and of the very faintly visible material migrating 0.16 times as fast as cysteic acid toward the anode (Figure 8) indicate that the former is apparently protein and the latter a peptide seemingly unrelated to the activation peptide. These data establish that the activation of lungfish trypsinogen is accompanied by the release of the heptapeptide Phe-Pro-Ile-Glu-Glu-Asp-Lys from the amino terminus of the zymogen,

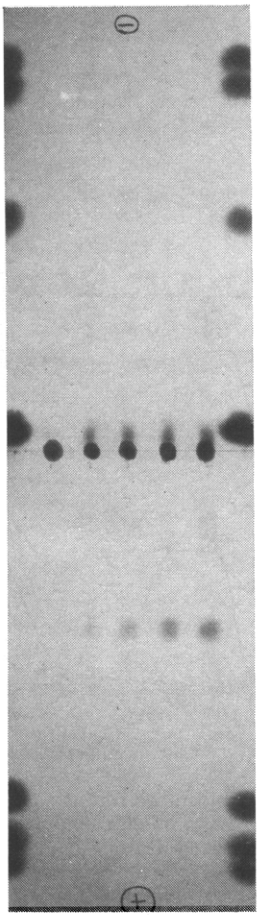

FIGURE 8: High-voltage paper electrophoresis of the activation mixture of lungfish trypsinogen. The seven samples were, from left to right: a standard mixture of amino acids; aliquots withdrawn from the activation mixture after 0, 15, 35, 70, and 100% activation; and another standard mixture of amino acids.

as was suggested by the sequenator data (Hermodson *et al.*, 1971).[2]

Characterization of Lungfish Trypsin. PHYSICOCHEMICAL PROPERTIES. Purified trypsin was prepared from lungfish trypsinogen by allowing the zymogen to undergo spontaneous activation and then dialyzing it against an appropriate buffer (0.01 M Tris, pH 8.0) to remove the activation peptide. The enzyme was nearly pure when examined by polyacrylamide disc gel electrophoresis (Figure 4).

The molecular weight of the enzyme was determined by sodium dodecyl sulfate gel electrophoresis (Figure 6). A value of 24,000 was obtained for the major band. The minor bands were of higher mobilities and therefore lower molecular weights, suggesting that some autolysis had occurred. The acid amino composition of lungfish trypsin is presented in Table I. Inclusion of norleucine in the samples for amino acid analyses allowed determination of the extinction coefficient of the protein (Walsh and Brown, 1962). From these analyses the protein concentration of the solution of trypsin was determined to be 2.50 mg ml^{-1}. The absorbance at 280 nm was 4.99 and thus $A^{1cm}_{0.1\%} = 1.98$.

ENZYMATIC PROPERTIES. The specific activities of lungfish trypsin toward 1 mM tosyl-L-arginine methyl ester and 10 mM

[2] In collaboration with Drs. Christoph de Haën and M. A. Hermodson we have determined that the trypsin sequence begins as expected with Ile_8 of the zymogen.

TABLE II: Amino Acid Composition of Trypsinogen Activation Peptide.

	Paper Eluate Residues/1.0 Asp	After Edman Degradation Residues/1.0 Asp
Asp	1.00[a]	1.00[b]
Ser	0.10	0.00
Glu	1.92	1.75
Pro	0.85	0.70
Ile	0.84	0.88
Tyr	0.17	0.00
Phe	0.84	*0.05*
Lys	0.93	0.72

[a] Absolute amount of Asp was 0.0229 μmole. [b] Absolute amount of Asp was 0.0195 μmole.

TABLE III: Enzymatic Properties of Three Trypsins.

Source of Trypsin	Substrate: N^{α}-Bzl-L-ArgOEt (min^{-1})	Substrate: *p*-Tosl-L-ArgOMe (min^{-1})
Lungfish[a]	1.7×10^3	4.1×10^3
Dogfish[b]	1.6×10^3	2.5×10^3
Cow[b]	1.6×10^3	6.0×10^3

[a] Specific activities, expressed as turnover numbers, are based on concentrations of active trypsin, determined with the active site titrant of Chase and Shaw (1967). Temperature was 25° for *p*-tosyl-L-arginine methyl ester and 23° for N^{α}-benzoyl-L-arginine ethyl ester measurements. Solvent for *p*-tosyl-L-arginine methyl ester (1 mM): 0.04 M Tris (pH 8.1), containing 0.01 M $CaCl_2$. Solvent for N^{α}-benzoyl-L-arginine ethyl ester (10 mM): 0.01 M Tris (pH 7.8), containing 0.05 M $CaCl_2$–0.1 M KCl. [b] From Tye (1970).

N-benzoyl-L-arginine ethyl ester are given in Table III, as are previously determined specific activities of dogfish trypsin and bovine β-trypsin. The molar ratio of the number of active sites per molecule (as determined by titration with *p*-nitrophenyl *p'*-guanidinobenzoate) varied from 0.8 to about 0.9. In a typical determination a protein solution having an absorbance at 280 nm of 0.26 (5.25 μM) gave an initial burst at 410 nm of 0.077, corresponding to a concentration of active sites of 4.82 μM (Chase and Shaw, 1967) or a molar ratio of 0.92.

Lungfish trypsin activity was stable at pH 8.0 (0.01 M Tris). No change in activity toward tosyl-L-arginine methyl ester could be observed for 12 hr at 37° (5 mg of protein/ml). The trypsin activity was also stable for 48 hr at 0° in 0.1 M sodium acetate (pH 3.0). The enzyme was rapidly inhibited by DFP. A solution (1 mg ml^{-1}) of the enzyme lost 50% of its activity in less than 0.5 min after addition of 10^{-2} M DFP.

N^{α}-Tosyl-L-lysine chloromethyl ketone inhibited lungfish trypsin (1 mg ml^{-1}) with a first-order rate constant of 0.16 min^{-1} at 1.25×10^{-4} M N^{α}-tosyl-L-lysine chloromethyl ketone in 0.05 M Tris (pH 7.0) containing 0.02 M $CaCl_2$. Under iden-

TABLE IV: Amino Acid Compositions[a] of Three Trypsinogens.

	Lungfish[b]	Dogfish[c]	Cow[d]
Asp	25.0	27.9	26
Thr	9.3	7.3	10
Ser	30	16.5	33
Glu	18.9	15.5	14
Pro	10.8	11.3	9
Gly	24.6	27.8	25
Ala	11.7	17.0	14
Cys/2	11.6	11.9	12
Val	17.4	17.4	18
Met	3.0	8.1	2
Ile	15.4	13.5	15
Leu	13.7	14.6	14
Tyr	13.2	11.5	10
Phe	3.0	1.2	3
Trp	5.9	5.4	4
Lys	7.2	6.3	15
His	6.2	7.6	3
Arg	5.0	6.5	2

[a] Residues per molecule. [b] From Table I. [c] Bradshaw *et al.* (1970). [d] Walsh and Neurath (1964).

TABLE V: N-Terminal Sequences of Various Trypsinogens.[a]

Species	N-Terminal Sequence
Cow	Val-Asp-Asp-Asp-Asp-Lys-
Sheep, goat	Val-Asp-Asp-Asp-Asp-Lys-
	Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys-
Pig	Phe-Pro-Thr-Asp-Asp-Asp-Asp-Lys-
Horse	Ser-Ser-Thr-Asp-Asp-Asp-Asp-Lys-
Dogfish	Ala-Pro-Asp-Asp-Asp-Asp-Lys-
Lungfish	Phe-Pro-Ile-Glu-Glu-Asp-Lys-

[a] Except for the lungfish peptide, these data were taken from Walsh (1970). Red deer and roe deer trypsinogens have been studied by Bricteux-Gregoire *et al.* (1971). In each case a pair of activation peptides was found, identical with the pair in the sheep.

tical conditions bovine trypsin was inhibited with a first-order rate constant of 0.091 min^{-1} (Shaw and Glover, 1970). The enzyme was fully inhibited by equimolar amounts of soybean trypsin inhibitor and chicken ovomucoid after incubation of the enzyme (1 mg ml^{-1}) with the inhibitor at pH 8.0 (0.01 M Tris) for 1 hr.

Discussion

The trypsinogen purified in this study is similar in molecular weight and amino acid composition to trypsinogens from other species (Table IV). The finding that the amino-terminal sequence of lungfish trypsinogen is homologous to the amino-terminal sequences of bovine and dogfish trypsinogens (Hermodson *et al.*, 1971) can be taken as preliminary evidence for homology throughout the molecule.

The importance of the tetraaspartyl sequence in the activation peptide of bovine trypsinogen (Radhakrishnan *et al.*, 1969b; Abita *et al.*, 1969) and its occurrence in all trypsinogens studied (Table V) had led to the view that this sequence was a general characteristic of the activation peptides of trypsinogens. Such, in fact, is not the case since in lungfish trypsinogen the sequence is Phe-Pro-Ile-Glu-Glu-Asp-Lys. Yet this zymogen is subject to the same promotion of activation by calcium ions as are bovine and dogfish trypsinogens. Thus it seems that only three acidic residues are needed for the response to calcium, a result which is consistent with the conclusions of Radhakrishnan *et al.* (1969b).

Lungfish occupy an intermediate position on the evolutionary tree (Romer, 1962) between the invertebrates and mammalian species from which other trypsins have been isolated. It is interesting that on the molecular level lungfish trypsinogen also seems to be intermediate in some properties between the invertebrate trypsins and the cationic mammalian trypsins. Thus lungfish trypsin, while being anionic and stable at neutral pH in the absence of calcium, like most invertebrate trypsins, is also stable at pH 3.0, like most mammalian trypsins.

Figure 9 shows a possible scheme for the changes in the structure of the activation peptide of trypsinogens in the course of evolution. This scheme is based on the assumption yet to be tested that the various trypsinogens are products of homologous gene loci. The evolutionary tree is derived from classical phylogenetic relationships and the ancestral activation peptides determined by minimizing the number of mutations required to fit the known sequences of the activation peptides to the tree. The existence of the tetraaspartyl sequence in all trypsinogens, with the exception of that from the lungfish, strongly suggests that the common precursor of the dogfish and lungfish, thought to be a member of the extinct class of Placodermi, possessed the tetraaspartyl sequence in its trypsinogen and so did the primitive amphibians. Thus the modification of this

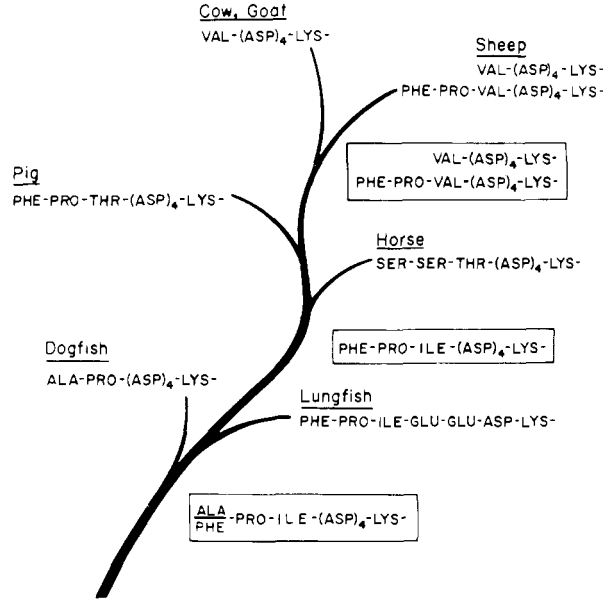

FIGURE 9: Evolution of the activation peptide of trypsinogen. Hypothetical ancestral sequences appear in boxes. Using the procedure described in the text, a choice cannot be made between Ala and Phe at the amino-terminal position of the lowest sequence.

sequence probably occurred in the Dipnoi (lungfish) after their divergence from the line leading to amphibians and other higher vertebrates (Bricteux-Gregoire *et al.*, 1971).

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