Isolation and Amino-Terminal Sequence Analysis of a New Pancreatic Trypsinogen of the African Lungfish *Protopterus aethiopicus*†

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ABSTRACT: The purification and characterization of three pancreatic trypsinogens A_1, A_2, and A_3, from the African lungfish, *Protopterus aethiopicus,* is reported. These zymogens are activated by trypsin, by enterokinase, by an acid protease from *Aspergillus oryzae,* and by autoactivation. The three trypsinogens contain the same amino-terminal amino acid sequence, beginning with the activation peptide Leu-Pro-Leu-Glu-Asp-Asp-Lys-. Like the activation peptide of the previously characterized trypsinogen B [Reeck, G. R., & Neurath, H. (1972) *Biochemistry 11,* 503] of the same organism, it lacks the tetraaspartyl sequence characteristic of other vertebrate trypsinogens. Two of the corresponding lungfish trypsins were found to have identical amino-terminal sequences for at least 27 residues. These data suggest that the three enzymes are allelic variants. In contrast, the amino acid sequences differ sufficiently from that of trypsinogen B of the same organism to indicate that trypsinogens A and B are the products of different gene loci.

The pancreatic serine proteases trypsin, chymotrypsin, and elastase have pronounced sequence similarities which place them in a class of proteins eminently suited for studies on the evolution of protein function (Hartley et al., 1972; Neurath et al., 1973; de Haën et al., 1975). Whereas for the active serine proteases an evolutionary tree could be elaborated (de Haën et al., 1975), the evolutionary history of those portions of these proteins found only in the zymogen forms is fragmentary. The evolution of these "activation peptides" has been discussed both in terms of independent addition of three different "prefixes" to the active enzyme chains of trypsin, chymotrypsin, and elastase (Neurath et al., 1973), and in terms of a common ancestor (de Haën et al., 1975). Recent sequence analysis of activation peptides of a trypsinogen (Hermodson et al., 1971; Reeck & Neurath, 1972) and of two proelastases (A and B) from the African lungfish *Protopterus aethiopicus* (de Haën & Gertler, 1974) revealed new aspects of these problems. First, trypsinogen activation peptides may not be as invariable as had been thought. Second, it was found that the amino-terminal sequence of elastase B closely resembled that of porcine elastase, and that the activation peptide of lungfish proelastase B shared with porcine proelastase the total number of amino acid residues and the lack of cysteine. Surprisingly, however, the amino-terminal sequences of lungfish elastase A and the activation peptide of the corresponding zymogen were more closely related to chymotrypsinogen C than to the other proelastases. Thus, the substrate specificity of the enzyme and the type of activation peptide need not necessarily go hand in hand. Indeed, substrate specificity could have changed several times in the course of evolution, while the type of activation peptide was maintained.

Several chromatographic fractions with potential trypsin activity have been isolated from African lungfish pancreas (Reeck et al., 1970). One of these trypsinogens has been characterized (Reeck & Neurath, 1972) and partial amino acid sequences have been reported (Hermondson et al., 1971; Reeck & Neurath, 1972; de Haën et al., 1975). We now report the isolation of three other trypsinogens from the same organism and compare their activation peptides with those of other zymogens.

Experimental Procedure

Materials. The collection and preparation of the lungfish tissue used in this work was carried out by Tiburon Biomarine Preparations, Edmonds, Wash., under the direction of Mrs. Susan Brown. The fish were netted in Uganda, Africa. The internal viscera, including the black pancreas in the gut wall (Laguesse, 1890), were removed and shipped frozen. Acetone powder of pooled glands was prepared by Dr. William P. Winter as described by Reeck et al. (1970).

Bovine trypsin (2× crystallized) was obtained from Worthington Biochemical Corp. and was further purified on a column of chicken ovomucoid-Sepharose according to Robinson et al. (1971). *Aspergillus oryzae* acid protease was carried through the first chromatographic step of purification described by Robinson et al. (1973) and stored frozen as a solution (A_{280} = 4).

Partially purified porcine enterokinase (lot 10) was a product of Miles Laboratories Inc. and had an activity of about 15 EKU/mg.

N^{α}-Tosyl-L-arginine methyl ester, N^{α}-benzoyl-D,L-arginine-β-naphthylamide, and Fast Garnet GBC salt (*o*-aminoazotoluene, diazonium salt) were products of Cyclo Chemical Corp. 4-Vinylpyridine (J.T. Baker Chemical Co.) was vacuum distilled before use. A stock solution of diisopropyl phosphofluoridate[1] (approximately 1 M) was prepared by dissolving 1 g of DFP (Pierce Chemical Corp.) in isopropyl alcohol previously dried over activated "Linde" molecular sieve 4A (Matheson Coleman and Bell).

Preswollen DEAE-cellulose (DE-52) was purchased from Whatman Inc., Clifton, N.J. After use it was regenerated by first partially hydrolyzing adsorbed proteins with 2 N NaOH under nitrogen for 2 days and then washing and acid-base cycling as prescribed by the manufacturers. Phosphoric acid

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[1] The abbreviations used are: DFP, diisopropyl phosphofluoridate; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

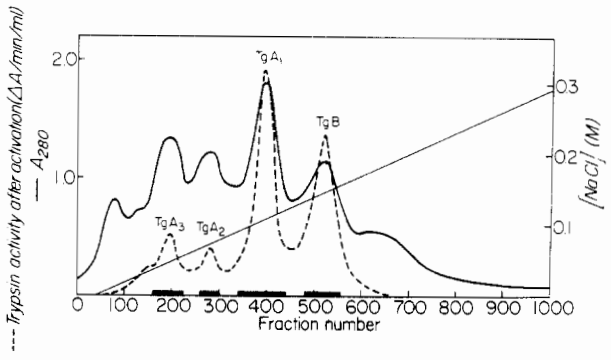

FIGURE 1: Chromatography on a 5 × 90 cm column of DEAE-cellulose of an aqueous extract of 100 g of acetone powder of lungfish pancreas. The conditions were analogous to those described by Reeck & Neurath (1972). The batch of acetone powder was selected for demonstration of four trypsinogen peaks, as explained further in the text. The fraction volume was 20 mL. ■ indicates pooled fractions.

was used instead of HCl in order to minimize degradation. The column material was used for more than ten preparations without evident loss of performance. Amicon UM-10 ultrafiltration membranes were stored in a solution initially 1 mM in DFP at 4 °C. All containers and ultrafiltration equipment were washed with 0.1 mM DFP before use.

Enzymatic Assay for Monitoring Columns. To 200 μL of trypsinogen solution were added 25 μL of 1 M $CaCl_2$, 25 μL of 0.2 M ammonium formate buffer (pH 3.2), and 25 μL of partially purified *Aspergillus oryzae* acid protease (A_{280} = 4) solution (Reeck & Neurath, 1972; Robinson et al., 1973). After 30 min at room temperature, 25 μL of this mixture was assayed in the spectrophotometer according to Hummel (1959), using a substrate concentration of 0.5 mM. The enzymatic activity of the activation mixture remained stable for several hours, but was usually only about 70% of that obtainable by autoactivation of trypsinogen. Trypsin activity after activation was expressed as ΔA_{247} per min per mL of trypsinogen solution ($\Delta A\ min^{-1}\ mL^{-1}$). Specific activity was obtained by dividing the trypsin activity after activation by the absorbance (280 nm) of the sample. Specific activity is reported in terms of ΔA_{247}/min for the activity of 1 mL of solution corresponding to A_{280} = 1.

Disc Gel Electrophoresis and Activity Staining. Proteins were subjected to acrylamide disc gel electrophoresis at pH 9.5 according to Davis (1964). Zymogen samples were prepared by dissolving lyophilized trypsinogen in sample buffer containing 10 mM DFP. Protein was detected with Coomassie brilliant blue R250 and trypsin activity was detected according to Garner et al. (1971). For trypsinogen detection the method was adapted in the following manner. After electrophoresis the gels were soaked for 10 min in 0.1 M $CaCl_2$ and 0.2 M ammonium formate (pH 3.2) to bring the external pH close to the pH optimum of *Aspergillus oryzae* acid protease. Gels were exposed for at least 2 min to a mixture (1:10) of a solution of partially purified acid protease (A_{280} = 4) and 0.1 M $CaCl_2$, 0.2 M ammonium formate (pH 3.2). The gels were then washed with 0.1 M Tris-HCl (pH 8), 0.1 M $CaCl_2$ and tryptic activity was detected as described by Garner et al. (1971). Whereas trypsin controls stained as a disk on the gels, trypsinogen stained only as a ring at the outer edge of the disc.

Sodium dodecyl sulfate gel electrophoresis was performed as detailed by de Haën & Gertler (1974).

Sequence Analysis. The proteins were reduced and S-pyridylethylated as described earlier for proelastase (de Haën & Gertler, 1974). Amino-terminal sequences were determined by automated Edman degradation using a Beckman Sequencer Model 890 A. The methods were those of Edman & Begg (1967) as modified by Hermodson et al. (1972). Stepwise yields exceeded 95%. On a molar basis the first step yielded 60 to 80% of the weighed protein sample.

Determination of the Extinction Coefficient. Trypsin (2.5 mg) was dissolved in 0.5 mL of 0.15 M acetic acid. The true protein concentration (4.275 mg/mL) was determined according to the procedure of Babul & Stellwagen (1969) in a Beckman Model E ultracentrifuge equipped with Raleigh optics. Weighed aliquots of this solution were then diluted by adding them to weighed amounts of water, and the absorbances were measured at 280 nm.

Amino Acid Composition. A Spinco Model 120 amino acid analyzer was used for all analyses. The amino acid composition is the average of two independent series of analyses, one of them on performic acid oxidized protein (Hirs, 1967). Each series consisted of duplicate hydrolyses in 6 N HCl at 110 °C for 24, 48, and 96 h. Individual analyses were placed on a common molar basis by adjusting all leucine values to 15.0 residues/molecule. The reported values for serine and threonine are extrapolations to zero time of hydrolysis, assuming first-order kinetics of destruction. Isoleucine and valine contents are 96 h results. Parallel analyses on bovine trypsin were used to check the reliability of the procedure.

Results

Purification of Lungfish Trypsinogen. Pancreatic acetone powder (100 g) from five or more pooled pancreas glands was extracted with water, and the proteins in the extract were precipitated with ammonium sulfate, redissolved, dialyzed, and chromatographed on a 5 × 90 cm column of DEAE-cellulose according to Reeck & Neurath (1972). Two or more trypsinogen peaks were observed; the major ones are identified as TgA_1 and TgB (Figure 1).[2] A third peak, A_3, also described by Reeck & Neurath (1972) was often seen, and a fourth peak, A_2, was occasionally seen (Figure 1). The relative size of the peaks varied widely from one batch of pancreas acetone powder to another. The purification of trypsinogen B has already been described by Reeck & Neurath (1972). Peak fractions of trypsinogens A_1, A_2, and A_3 were pooled separately, adjusted to 0.1 mM DFP, concentrated to 200 mL by ultrafiltration (using an Amicon UM-10 membrane) and lyophilized.

The material containing trypsinogen A_1 was dissolved in 20 mL of ammonium acetate 0.01 M, pH 8, containing 10 mM DFP and applied to a column of Sephadex G-100 (2.5 × 100 cm). Undissolved salt was applied to the column together with the protein and gradually dissolved as the elution proceeded. Figure 2 illustrates the elution profile. The yield of trypsinogen activity from this step (50–90%) was found to be inversely dependent on the concentration of DFP and the time of exposure of the zymogen during the preceding steps. Morgan et al. (1972) have shown that DFP inactivates not only enzymes, but also zymogens, although at a much slower rate. After lyophilization of the peak fractions containing activatable trypsinogen A_1, the material was dissolved and applied to a column (2.5 × 40 cm) of DEAE-cellulose (DE-52) previously equilibrated with 0.005 M sodium phosphate, pH 8 at 4 °C. The protein was eluted with 4 L of a mixture of buffers forming a linear gradient of 0 to 0.3 M NaCl (Figure 3). Fractions with maximal potential specific activity were pooled, dialyzed against 1 mM HCl, and lyophilized. Specific activities were somewhat variable from batch to batch, presumably due to

[2] The nomenclature is chosen to distinguish products of different gene loci (A and B) from apparent allelic variants (A_1–A_3).

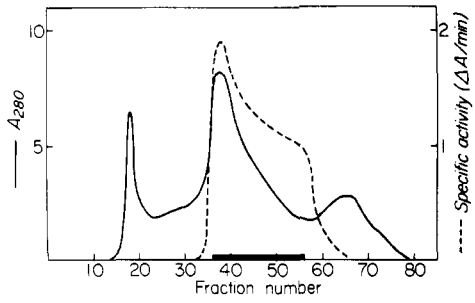

FIGURE 2: Chromatography of trypsinogen A_1 on a 2.5 × 100 cm column of Sephadex G-100. The sample contained fractions 340–440 of the ion-exchange chromatography shown in Figure 1. Preparation of the sample and the conditions are given in the text. The fraction volume was 10 mL. ■ indicates pooled fractions. Specific activity is the activity contained in 1 mL of a solution with A_{280} = 1.

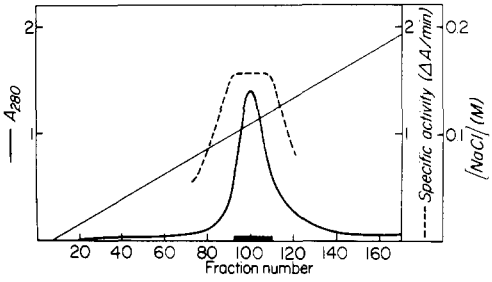

FIGURE 3: Chromatography of trypsinogen A_1 on a 2.5 × 40 cm column of DEAE-cellulose. The sample contained fractions 36–56 of the gel filtration shown in Figure 2. Details of the chromatography are given in the text. The fraction volume was 22 mL. ■ indicates pooled fractions. Specific activity is the activity in 1 mL of a solution with A_{280} = 1.

inactivation by DFP, but they were constant across the peak. Yields of this chromatographic step averaged approximately 50%.

These procedures yielded between 25 and 100 mg of pure trypsinogen A_1 from 100 g of acetone powder. The material displayed a single band on $NaDodSO_4$ gel electrophoresis corresponding to a molecular weight of about 24 000. Homogeneity of the preparations was confirmed by amino-terminal sequence analysis (see below). Disc gel electrophoresis at pH 9.5 showed a single major protein but some preparations contained also a slower moving minor protein. Both protein bands stained for trypsinogen, but only the minor band stained for trypsin. The minor protein comigrated with authentic trypsin A_1, indicating that some activation of trypsinogen had occurred in the initial phases of disc gel electrophoresis despite the presence of DFP in the sample buffer. Exactly the same sequence of purification steps also yielded trypsinogen A_2 (0–5 mg) and A_3 (0–15 mg) in comparable purity.

Purification of Trypsin. Occasionally during the preparation of these trypsinogens, spontaneous activation occurred despite the precautionary addition of DFP. Trypsin was purified from such preparations by affinity chromatography on chicken ovomucoid–Sepharose columns, prepared and eluted as described by Robinson et al. (1971) for bovine trypsin. Alternatively pure trypsinogen was allowed to undergo autoactivation in Tris-HCl buffer 0.01 M, pH 8.0, 0.05 M $CaCl_2$ at room temperature. When activation had reached 90% of the potential maximum, ovomucoid–Sepharose was added, the solution shaken mildly at 4 °C overnight and poured into a column, and the protein eluted as described by Robinson et al. (1971). Trypsin emerged as a symmetrical peak. Peak fractions were pooled and lyophilized. The materials showed a single band on disc gel electrophoresis at pH 9.5 and on $NaDodSO_4$ gel electrophoresis. Trypsins A_1 and A_3 displayed identical single amino-terminal amino acid sequences on automated Edman degradation (see below). Trypsin A_2 was not obtained in sufficient amounts for sequence analysis.

TABLE I: Amino Acid Composition of Trypsin A_1 of African Lungfish.

	Trypsin A_1 (residues/ molecule)	Trypsin B[a] (residues/ molecule)
Asp	27.0	23.0
Thr	7.6	8.9
Ser	25.6	30
Glu	18.0	16.6
Pro	8.6[b]	10.4
Gly	23.7	24.5
Ala	16.4[b]	11.7
Cys	11.7	11.3
Val	13.9	18.6
Met	2.0	2.9
Ile	17.9	14.6
Leu	15.0	13.8
Tyr	11.9	11.8
Phe	3.1	2.6
Trp	5.1	5.3
Lys	5.5[b]	6.6
His	4.0	6.2
Arg	5.5[b]	5.4
Total residues	222.5	224.2

[a] For purposes of comparison taken from Reeck & Neurath (1972). [b] The sum of Ala and Pro as well as of Lys and Arg values gives integral values, possibly as a consequence of allotypic replacements at certain positions. Sequenator analysis indicated Ala/Pro microheterogeneity at position 20 (Table II) but Ala was definitively predominant (Ala:Pro = 9:1).

TABLE II: Amino-Terminal Amino Acid Sequence of African Lungfisl Trypsinogen A.[a]

Leu-Pro-Leu-Glu-Asp-Asp-Lys-[b] Ile-Val-Gly-Gly-Tyr-Glu-Cys-Gln-
(10)
Lys-Asn-Ser-Val-Ala[c]/Pro-Tyr-Ile-Ala-Ser-Leu-Asn-Ile-Gly-Tyr-His-
(20) (30)
Phe-Cys-Gly-Gly-Ser-Leu-Ile-Asn-X[d]-X[d]-Trp-Val-Val-Ser-Ala-
(40)
Ala-His-Cys

[a] The first 11 residues were identical in trypsinogens A_1, A_2, and A_3 Trypsins A_1 and A_3 were identical for 41 residues (positions 8–48), excep for positions 35, 44, and 48, that could only be identified in trypsin A_3 [b] Point of activation. [c] Position 20 showed microheterogeneity Ala:Pr = 9:1. [d] There is a weak indication that the two residues are Ser-Gln.

The amino acid composition of trypsin prepared from trypsinogen A_1 is shown in Table I. The composition clearly differs from trypsin B of the same organism described previously (Reeck & Neurath, 1972). The absorption of trypsin A_1 at 280 nm was $A_{0.1\%}^{1cm}$ = 1.83.

Activation Studies. The lungfish trypsinogens of the A series activated spontaneously just as lungfish trypsinogen B. After reaching full activation in the presence of 3 mM NaN_3, the activity remained stable for at least 2 days at 37 °C. $CaCl_2$ added to the activation mixture accelerated autoactivation as described previously for other trypsinogens (Reeck & Neurath, 1972). No attempt was made to separate the specific effect of Ca^{2+} from the effect of ionic strength alone. Trypsinogen A was also activated by *Aspergillus oryzae* acid protease and by

TABLE III: Amino Acid Sequence Alignment and Comparison of the Activation Peptide of Trypsinogens.[a]

Val-Asp-Asp-Asp-Asp-Lys-	Cow (*Bos taurus*) cationic	Davie & Neurath (1955)
Phe-Pro-Ser-Asp-Asp-Asp-Asp-Lys-	Cow (*Bos taurus*) anionic	Louvard and Puigserver (1974)
Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys-	Sheep (*Ovis aries*)	Bricteux-Grégoire et al. (1966), Schyns et al. 1969)
Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys-	Goat (*Caprus hircus*)	Bricteux-Grégoire et al. (1971a)
Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys-	Roe deer (*Capreolus capreolus*)	Bricteux-Grégoire (1970)
Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys-	Red deer (*Cervus elaphus*)	Bricteux-Grégoire et al. (1971b)
Val-Pro-Ile-Asp-Asp-Asp-Asp-Lys-	Dromedary (*Camelus dromedarius*)	Bricteux-Grégoire et al. (1971c)
Phe-Pro-Thr-Asp-Asp-Asp-Asp-Lys-	Pig (*Sus scrofa*) anionic	Louvard & Puigserver (1974)
Phe-Pro-Thr-Asp-Asp-Asp-Asp-Lys-	Pig (*Sus scrofa*) cationic	Charles et al. (1963)
Phe-Pro-Ile-Asp-Asp-Asp-Asp-Lys-	Lesser rorqual (*Balaenoptera acutorostrata*)	Bricteux-Grégoire et al. (1975)
Phe-Pro-Thr-Asp-Asp-Asp-Asp-Lys-	Elephant seal (*Microunga leonina* L.)	Bricteux-Grégoire et al. (1974)
Ser-Ser-Thr-Asp-Asp-Asp-Asp-Lys-	Horse (*Equus caballus*)	Harris & Hofmann (1969)
Ala-Pro-Phe-Asp-Asp-Asp-Asp-Lys-	Man (*Homo sapiens*) 2	Guy et al. (1976)
Leu-Pro-Leu-Glu-Asp- - -Asp-Lys-	African lungfish (*Protopterus aethiopicus*) A	This work
Phe-Pro-Ile-Glu-Glu- - -Asp-Lys-	African lungfish (*Protopterus aethiopicus*) B	Hermodson et al. (1971)
Ala-Pro- - -Asp-Asp-Asp-Asp-Lys-	Spiny pacific dogfish (*Squalus acanthias*)	Bradshaw et al. (1970)

[a] To optimize the sequence alignment gaps have been introduced into the last three sequences.

porcine enterokinase as described in Experimental Procedure, but, since these enzyme preparations were crude, further characterization of these activation processes did not seem indicated.

Sequence Analyses. Eleven cycles of automated Edman degradation of trypsinogens A_1, A_2, and A_3 gave identical sequences, namely:

Leu-Pro-Leu-Glu-Asp-Asp-Lys-Ile-Val-Gly-Gly-

This sequence included the peptide identified below as the point of activation (Lys^7-Ile^8). In some but not all of the preparations, the major sequence was accompanied by a minor sequence (about 0–30%) identical with the major one, but lacking the amino-terminal leucine.

Amino-terminal sequence analyses of trypsins A_1 and A_3 each yielded single sequences starting with Ile-Val-Gly-Gly-. This demonstrated that the point of activation is indeed Lys^7-Ile^8. The two sequences were identical through 27 cycles of automated Edman degradation (Table II). Some residues further into the sequence could also be identified, some of them only in one or the other of the two proteins. In view of the identity of the sequences of trypsin A_1 and A_3, and the identity of the activation peptide sequences of trypsinogens A_1, A_2, and A_3, we propose that the three trypsinogens are allotypic gene products distinguishable chromatographically because of some differences in charged amino acid residues further toward the carboxy terminus.

Discussion

Reeck et al. (1970) described multiple peaks of trypsinogen on ion-exchange chromatography of extracts of the pancreas of the African lungfish. The most anionic peak fraction, trypsinogen B, has been purified and most of the amino acid sequence has been determined (Hermodson et al., 1971; Reeck & Neurath, 1972; de Haën et al., 1975). The isolation of the other trypsinogens (A_1, A_2, A_3) reported here, their amino-terminal amino acid sequences, and, in one case, the amino acid composition of the corresponding trypsin (A_1) indicate that the chromatographic heterogeneity was due in part to expression of different genes, rather than to allotypic replacements of charged amino acids in the previously characterized trypsinogen B. In contrast, the identity of amino-terminal amino acid sequences among the three trypsinogens of the A series strongly suggests that these three proteins are allelic gene products, differing in charged residues in areas of the sequence beyond those investigated. The simultaneous presence of allelic products may best be explained by the pooling of pancreata and arbitrary sampling of the animal population. This explanation is consistent with the batch-to-batch variability in the distribution of the three forms of trypsinogen A.

The activation peptide of lungfish trypsinogen A is unusual, as was that of lungfish trypsinogen B. Although these two activation peptides differ from each other by three out of seven amino acid residues, the really striking observation is the substitution of three acidic residues (Glu, Asp) for the four aspartyl residues present in all other known vertebrate trypsinogens (Table III). The reduction to three acidic residues in both zymogens could have resulted as independent responses to a common selective pressure. Enterokinase, the physiological activator of trypsinogen, could provide such a common constraint. However, porcine enterokinase that is adapted to the tetraaspartyl sequence was shown to activate lungfish trypsinogen. This observation suggests that the loss of one acidic residue of the activation peptide occurred in a common ancestor of trypsinogen A and B present in a forebear of the present day African lungfish. This interpretation would mean that trypsinogens A and B in that species are not the products of the same gene duplication event(s) that lead to the multiple trypsinogens in other mammals (e.g., Louvard & Puigserver, 1974; Rinderknecht & Geokas, 1972/73). Thus in the course of vertebrate evolution, the gene for trypsinogen duplicated more than once. In view of this finding, geneaologic trees of trypsinogen activation peptides must be constructed with caution.

In some preparations, pure trypsinogen A was found to have a heterogeneous amino terminus, as if it had been partly modified by aminopeptidases. A similar situation was encountered in the case of lungfish proelastase A (de Haën & Gertler, 1974). Some artiodactyl trypsinogens (goat, sheep, deer) contain both an eight-residue and a six-residue activation peptide (Bricteux-Grégoire et al., 1966; Schyns et al., 1969; Bricteux-Grégoire, 1970; Bricteux-Grégoire et al., 1971a,b). It is possible that these activation peptides belong to different gene products. Alternatively, the action of proteolytic enzymes [aminopeptidases, proline dipeptidases, and "post-proline" endopeptidases (Koida & Walter, 1976)] in the course of enzyme isolation may be responsible for these sequence variations (Bricteux-Grégoire et al., 1971; Louvard & Puigserver, 1974). It is possible also that there is some ambiguity in the site of

cleavage of the so-called "signal peptide" (Devillers-Thiery et al., 1975) from the nascent chain.

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