# AMINO-TERMINAL AMINO ACID SEQUENCES AND THE EVOLUTION OF FROG (*RANA ESCULENTA*) TRYPSIN AND CHYMOTRYPSIN

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## 1. Introduction

At present it is unknown at what point during the molecular evolution of the serine proteases trypsin and chymotrypsin diverged from each other. Chymotrypsin has been found in all vertebrates that have been examined [1], whereas no chymotrypsin-like esterase activity is detectable in the digestive tract of the invertebrate crayfish, *Astacus fluviatilis* [2]. Crayfish trypsin [3] as judged by its amino-terminal sequence and the number and position of the disulfide bonds, appears to have evolved before trypsin and chymotrypsin diverged from a common ancestor, suggesting that the divergence occurred sometime before the appearance of the vertebrate species. Since amphibians are much closer to the origin of vertebrate evolution and hence to the trypsin/chymotrypsin divergence than are many other vertebrate species, we examined amphibian trypsin and chymotrypsin, assuming that these enzymes in lower vertebrates resemble to a greater extent the common ancestral serine protease from which trypsin and chymotrypsin diverged than do bovine trypsin and chymotrypsin. Here we report the amino terminal sequences of trypsin and chymotrypsin from the frog *Rana esculenta.*

## 2. Materials and methods

Bovine trypsin, *N*-acetylphenylalanine-*p*-naphtyl ester (APNE), *N*-benzoylarginine-*p*-nitroanilide (BANA) and ovomucoid were products of Sigma (St Louis). Bovine chymotrypsin, *N*-benzoylarginine ethyl ester (BzArgOEt) and soybean trypsin inhibitor (SBTI) were purchased from Merck (Darmstadt). CNBr-activated Sepharose 4B was obtained from Pharmacia (Uppsala), *N*-acetyltyrosine ethyl ester (AcTyrOEt) from Serva (Heidelberg) and Agarose from Behring-Werke (Marburg). Frogs of the species *Rana esculenta* were procured from R. Stein (Lauingen/Donau). The animals were killed by decapitation, the pancreas glands removed and homogenized in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.01 M $CaCl_2$. After centrifugation at 13 000 × *g* for 30 min at 4°C, the supernatant fluid was used for activation studies.

Enzyme assays for trypsin were performed spectrophotometrically in a Zeiss spectrophotometer at 253 nm, using a solution of 1 mM BzArgOEt in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.01 M $CaCl_2$ at room temperature. For measuring the activity of chymotrypsin, the hydrolysis of AcTyrOEt was monitored at 234 nm at room temperature, using a solution of 2 mM substrate in 0.05 M Tris–HCl buffer (pH 7.0) containing 0.02 M $CaCl_2$. One unit (U) of enzyme activity is defined as the quantity of enzyme capable of hydrolyzing 1 μmol substrate/min. Polyacrylamide electrophoresis was carried out according to [4]. Protein bands were stained with amidoblack or developed by use of the chromogenic substrate APNE [2]. Cellulose acetate membrane electrophoresis was performed in 0.06 M Veronal–HCl buffer (pH 8.0) at 2 mA/foil for 30 min.

Affinity chromatography columns were prepared by coupling 85 mg SBTI or ovomucoid to 5 g CNBr-activated Sepharose 4B, in 0.05 M Tris–HCl (pH 8.0) containing 0.01 M $CaCl_2$. Activated pancreatic extracts

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(20 ml) were applied to the affinity adsorption columns (5 ml each), followed by extensive washing of the adsorbant with buffer. The desired proteins were eluted with 3 M acetic acid.

Proteins were reduced and pyridylethylated [5] prior to sequence analysis on a Beckman Sequencer Model 980 B using the method of [6] as modified in [7]. Phenylthiohydantoin-amino acids were identified by gas chromatography and by high performance liquid chromatography [8]. Amino acid analyses were performed on a Durrum Model D-500 amino acid analyzer. Duplicate samples were hydrolyzed in 6 M HCl at 110°C for 24 h.

## 3. Results

### 3.1. *Activation*

In order to obtain from the small frog pancreas an amount of material sufficient for sequence studies, an affinity chromatography procedure was developed. For this purpose, the activation pattern of frog trypsinogen and chymotrypsinogen was studied. Frog pancreas glands (50) were homogenized in 20 ml 0.1 M Tris–HCl (pH 8.0) containing 0.01 M $CaCl_2$ and centrifuged at 13 000 × *g* for 30 min. Weak activity toward casein, BzArgOEt and AcTyrOEt developed when the supernatant fluid was incubated for 10 min at 30°C. Under these conditions, a maximum activity of 20 μmol AcTyrOEt . $min^{-1}$ . $ml^{-1}$ was reached in <75 min and was maintained for ⩾ 4 h.

### 3.2. *Affinity chromatography*

Frog trypsin and chymotrypsin were purified by a series of passages through SBTI and ovomucoid columns (fig.1). In the first step, trypsin and chymotrypsin were adsorbed together on a SBTI-column, separated from a considerable amount of accompanying material and eluted, as usual, by 3 M acetic acid, pH 3.0 (fig.1A).

Subsequently, both enzymes were separated on an ovomucoid column. Chymotrypsin appeared in the breakthrough peak without detectable traces of trypsin and the acetic acid eluted trypsin fraction did not contain any residual chymotrypsin activity (fig.1B).

The chymotrypsin fraction was applied once more to a SBTI column (fig.1C). The recovered material showed a high degree of purity as did the preparation of trypsin. Starting with 50 frog pancreas glands, ~10 mg of pure frog trypsin or chymotrypsin could be obtained with a single purification procedure.

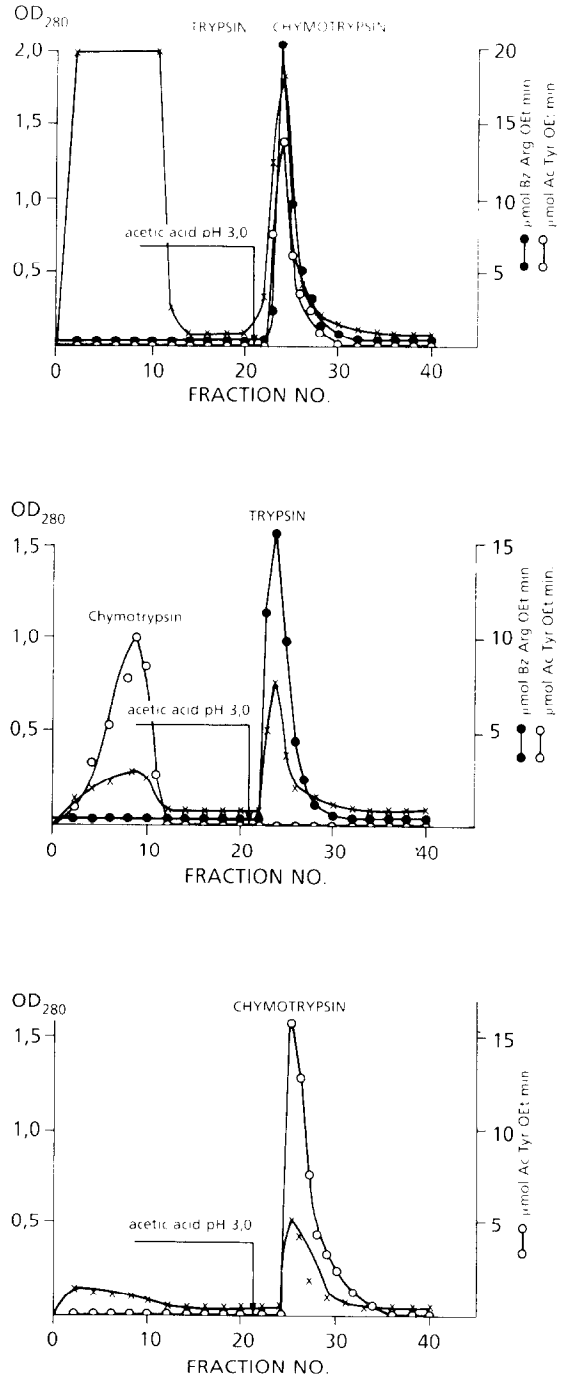



Fig.1. Affinity chromatography of frog trypsin and chymotrypsin. Top: Elution of frog trypsin and chymotrypsin from a SBTI–Sepharose 4B column. Center: Separation of frog trypsin and chymotrypsin on an ovomucoid–Sepharose 4B column. Bottom: Second purification step of frog chymotrypsin on a SBTI–Sepharose 4B column.

### 3.3. *Electrophoretic mobility*

Frog trypsin migrates as a single band toward the cathode upon electrophoresis on either cellulose acetate membrane or polyacrylamide, whereas frog chymotrypsin migrates in 3 bands (fig.2). All bands were enzymatically active against chromogenic substrates and no inactive impurities were detectable. On the basis of the electrophoretic patterns, frog trypsin and chymotrypsin are less basic than bovine trypsin and chymotrypsin.

### 3.4. *Autolysis, calcium-binding and pH-stability*

Trypsins from different sources differ significantly

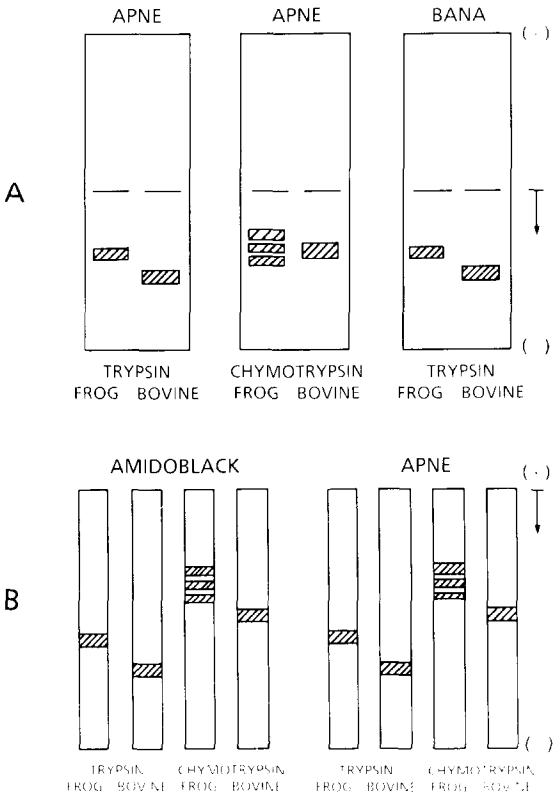



Fig.2. Electrophoretic mobility, multiple forms and purity of frog trypsin and chymotrypsin. (A) Cellulose acetate membrane electrophoresis, buffer: 0.06 M Veronal–HCl (pH 8.0), 1 mA/foil, 30 min. Bands developed with the chromogenic substrates APNE and BANA. (B) Polyacrylamide electrophoresis of frog trypsin and chymotrypsin. Stacking gel buffer: KOH–acetic acid (pH 6.8). Separation gel (15% acrylamide) buffer: KOH–acetic acid (pH 4.3). Electrode buffer: 0.035 M β-alanine–acetic acid, (pH 4.5), 6 mA/gel, 100 min. Bands stained with amido black or developed with the chromogenic substrate APNE.

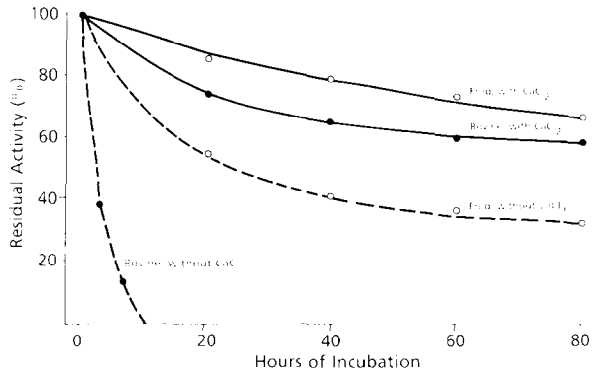



Fig.3. Frog and bovine trypsin. Different rates of inactivation by autodigestion, at pH 8.0 and 30°C.

in their rate of autodigestion. While bovine trypsin is known for its rapid self-inactivation, crayfish trypsin has been found to be remarkably stable even after several days [2]. Trypsin from dogfish, porcine, horse and sheep exhibit a graded stability which parallels the evolution of the respective herbivorous animals [9]. The stability of frog trypsin appears to reflect the evolutionary level of the amphibians since after 20 h incubation in the absence of $CaCl_2$ in the medium, ⩾50% residual activity could be found whereas bovine trypsin, under the same conditions, was completely inactivated. Frog trypsin seems to have a binding site for $CaCl_2$, too, because its stability could be enhanced considerably by the addition of 0.01 M $CaCl_2$ to the incubation mixture (fig.2).

While invertebrate trypsins, e.g., crayfish [2], are instantly and irreversibly denatured at pH 3.0, the stability of frog trypsin is similar to that of other vertebrate trypsins. After incubation of frog trypsin at pH 3.0 at 30°C for 80 h, 40% of the enzyme activity remained, whereas 80% of the initial activity of bovine trypsin was observed under these conditions.

### 3.5. *Amino acid composition*

Based on the relative composition (table 1) of 11 half-cystines of *Rana esculenta* trypsin, it is probable that the enzyme resembles other known trypsins of vertebrate origin in having 6 disulfide bonds. In this respect, frog trypsin is clearly distinguished from the trypsins of more primitively organized organisms, e.g., crayfish [10] and trypsin of *S. griseus* [11], which each have only 3 disulfide bonds. The 36 serine residues in frog trypsin represent the highest value found in trypsins. On the other hand, the (Arg + Lys)

Table 1
Amino acid composition of frog trypsin and comparison to bovine and dogfish trypsin (residues/molecule)

| | Frog[a] | Bovine [12] | Dogfish [13] |
|---|---|---|---|
| Cys/2 | 11 | 12 | 12 |
| Asp | 31 | 22 | 24 |
| Thr | 15 | 10 | 7 |
| Ser | 36 | 33 | 17 |
| Glu | 21 | 14 | 15 |
| Pro | 14 | 9 | 10 |
| Gly | 30 | 25 | 28 |
| Ala | 21 | 14 | 16 |
| Val | 13 | 17 | 18 |
| Met | 2 | 2 | 9 |
| Ile | 11 | 15 | 14 |
| Leu | 15 | 14 | 14 |
| Tyr | 6 | 10 | 12 |
| Phe | 3 | 3 | 1 |
| His | 3 | 3 | 8 |
| Lys | 5 | 14 | 5 |
| Arg | 2 | 2 | 7 |

[a] These are apparent values (24 h hydrolysates) rounded out to the nearest integer

content of frog trypsin is identical to that of the invertebrate crayfish trypsin, which is significant since the lysine concentration of bovine trypsin is 3-times greater than that of frog trypsin.

### 3.6. *N-terminal sequences*

The amino-terminal sequence analyses provided the first 30 residues of frog trypsin and the first 20 residues of frog chymotrypsin. These structures are compared to those of similar regions of bovine and dogfish trypsin, and also of bovine chymotrypsin (table 2). The partial sequences of frog trypsin and chymotrypsin clearly establish a homologous relationship of these amphibian enzymes to the known mammalian serine proteases.

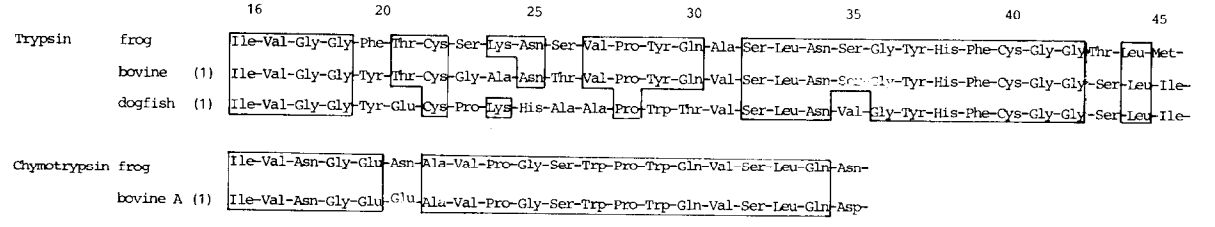
## 4. Discussion

The amino-terminal sequences reported herein for frog trypsin and chymotrypsin represent the first reported structures of amphibian serine proteases. It is evident from these partial structures (table 2) that there exists a high degree of homology between these enzymes and other known mammalian serine proteases. Interestingly, the amino-terminal substitution of Ile–Val–Asn–Gly present in all known chymotrypsins by Ile–Val–Gly–Gly– in all trypsins is already evident in the amphibian serine proteases, indicating that this structural alteration occurred before the appearance of the amphibians.

Whereas a comparison of the amino-terminal sequences of frog and bovine trypsin reveals four amino acid residue substitutions among the first 20 positions, there are only 2 amino acid residue substitutions in this region of frog and bovine chymotrypsin. Should this observation prove consistent with additional sequence regions, it may indicate different rates of evolution of 2 closely related enzymes which are produced by the same organ and are released into the same medium.

Do the structures of frog trypsin and frog chymotrypsin resemble each other to a greater extent than those of bovine trypsin and bovine chymotrypsin? Even from the limited data available it is clear that considerable differences in the primary structures exist between frog trypsin and chymotrypsin. However, they are not less than those between bovine trypsin and chymotrypsin, i.e., 12 substitutions among the first 20 amino-terminal residues. This observation suggests that the trypsin and chymotrypsin divergence occurred long before the appearance of amphibians and probably not in a vertebrate species.

Table 2
Amino-terminal sequences of frog trypsin and chymotrypsin – Comparison to homologous sequences of bovine trypsin and chymotrypsin and dogfish trypsin



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