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# ISOLATION AND PROPERTIES OF SOME REPTILIAN AND FISH CHYMOTRYPSINS

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## SUMMARY

Chymotrypsinogens were isolated in highly purified form by a chromatographic procedure, from the pancreas of the turtles *Chelydra serpentina* and *Pseudemys elegans*. After activation with bovine trypsin, they both hydrolyze the substrate *N*-benzoyl-L-tyrosine ethyl ester about three times as rapidly as bovine chymotrypsin does.

The rates of the inactivations by chloromethyl ketone derivatives of L-phenylalanine and L-leucine were measured, on chymotrypsins from three reptiles and the tuna. From these, and from the relative activities on tyrosine and leucine substrates, a partial similarity to porcine chymotrypsin C (which shows high rates with leucine derivatives) was discerned.

It is therefore concluded that although the reactivity of the chymotrypsin active center is basically similar from the fish to the mammals, complex variations on the basic pattern in relation to the binding site are apparent in each species examined. A clear division into A and C types, as found in mammals, could not be made in the range of lower vertebrates investigated here. The alkylation and specificity evidence suggests that forms with combined features of both the A and C types have appeared in reptilian and fish species.

## INTRODUCTION

It has recently been reported[1,2] that enzymes similar to bovine chymotrypsin can be identified in the pancreas of many lower vertebrates. These enzymes all show a parallel specificity towards substrates with amide and ester bonds (casein, *N*-benzoyl-L-tyrosine ethyl ester (BTEE)) and towards irreversible, active center-directed inhibitors (DFP, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (ref. 3)).

Abbreviations: BAEE, *N*-benzoyl-L-arginine ethyl ester; BLEE, *N*-benzoyl-L-leucine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; TLeuCK, *N*-tosyl-L-leucine chloromethyl ketone; TLysCK, α-*N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

To establish molecular and kinetic properties of these enzymes in comparison with the well-known properties of chymotrypsins of higher vertebrates (cow and pig), we report here the isolation of two reptile chymotrypsins in highly purified form, and the examination of reaction rates on substrates and inhibitors in the cases of four reptiles and a teleost fish.

Methods are used which show rather subtle, and complex differences between the active centers of these enzymes, within the framework of a basically similar general pattern. Chemical characterization of an active center group in these enzymes is made in the associated paper[4].

## MATERIALS AND METHODS

### *Materials*

Pancreatic tissue was obtained as previously[1], and its identity and purity in each species checked by histological examinations. Bovine enzymes were commercial samples as used previously[1]. *N*-benzoyl-L-leucine ethyl ester (BLEE) and *N*-tosyl-L-leucine chloromethyl ketone (TLeuCK) were from the Cyclo Chemical Corp. CM-cellulose was Whatman CM-52 (microgranular) and was pretreated according to the manufacturer's directions. $(NH_4)_2SO_4$ was Enzyme grade (Mann Research Laboratories). Other materials were as specified previously[1].

### *Enzyme assays*

The activation buffer used in the trypsin activation of zymogens in these studies was always 0.05 M Tris–0.1 M KCl–0.02 M $CaCl_2$ (pH 7.5) at 4°. Trypsin activity was assayed with *N*-benzoyl-L-arginine ethyl ester (BAEE) and chymotrypsin activity with BTEE, as described previously[1]. Assays on BLEE were by the method of FOLK AND SCHIRMER[5].

### *Other methods*

Protein concentration was determined by a quantitative biuret method[6] or (where shown) by $A_{280\ m\mu}$. Inactivation of enzymes with specific inhibitors, and other methods not specified, were as described previously[1]. Electrophoresis was in the Beckman microzone apparatus, using Gelman Sepraphore III cellulose–acetate membranes, and staining with naphthol blue–black (Allied Chemical Corp.) in 3% trichloroacetic acid–methanol.

### *Partial purification of chymotrypsins*

Extracts were always prepared by homogenizing the pancreatic tissues at 0° in 0.02 M sodium acetate (pH 5.0; 3–6 ml/g tissue). The homogenate was readjusted to pH 5 with 1 M HCl and, after stirring for 30–45 min at 0°, centrifuged at 20 000 × *g* for 30 min at 0°. The extracts of the reptile species contained the chymotrypsins and trypsins exclusively in the form of their zymogens, as shown by the complete absence of reaction on BTEE or BAEE. To determine the content of enzyme, aliquots were activated at 4° by adding bovine trypsin until an excess of about 70 μg/ml could be measured by BAEE assay. In the cases of the turtle *Pseudemys elegans* and the cayman, this required 3–4 times the theoretical amount of trypsin, showing that these tissues

are rich in a trypsin inhibitor. Activation in these conditions was then maximal in about 6 h.

For partial purification, tuna extract (using pyloric ceca[1]) was subjected to acetone precipitation and $(NH_4)_2SO_4$ fractionation as described previously[1].

The extract of the turtle *Chelydra serpentina* (about 20 mg protein/ml) was also first subjected to acetone precipitation, by adding to the extract, at 0°, 2 vol. of acetone at between 0° and −4° with rapid stirring. The precipitate was centrifuged at once and thoroughly extracted with half the original volume of 0.02 M sodium acetate (pH 5) in a glass homogenizer. Insoluble material was removed by centrifugation and the supernatant dialyzed against the same buffer. A heavy precipitate (containing no activity) formed during dialysis and was removed by centrifugation. This solution (12–15 mg protein/ml) was fractionated with $(NH_4)_2SO_4$ at 0°, retaining the fraction precipitating at 20–45% saturation. This precipitate was dissolved in

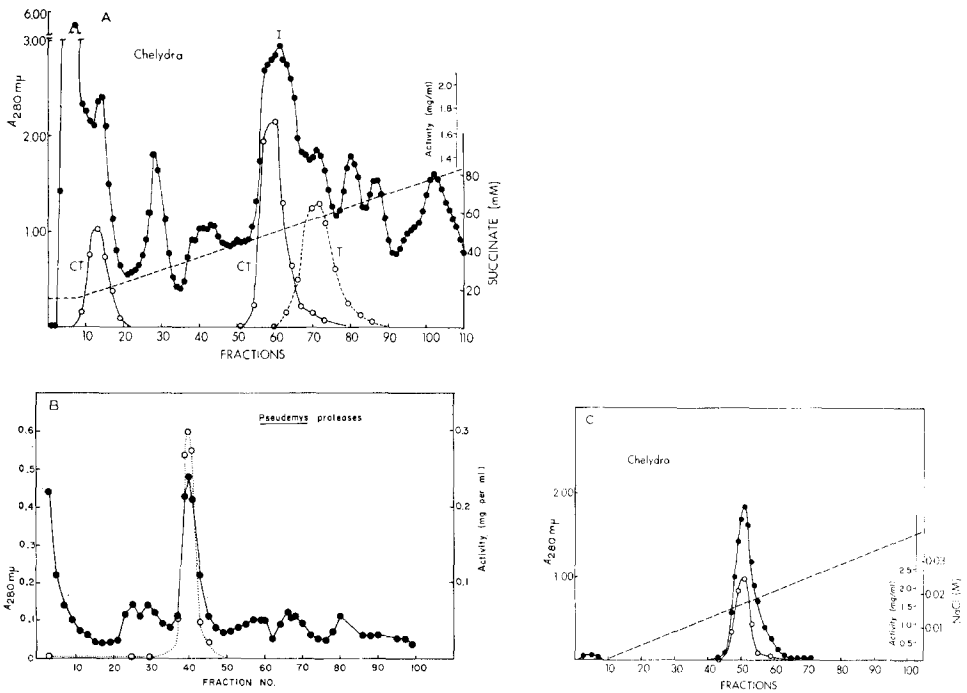


Fig. 1. (A) Chromatography of *C. serpentina* chymotrypsinogen (partly purified preparation) at 4° on a column (18 cm × 2 cm) of CM-cellulose, using a concentration gradient of sodium succinate (pH 5.0) in 0.001 M EDTA (— — —). The protein peaks obtained are shown by $A_{280\,m\mu}$ (●—●). Fractions of 7.5 ml were collected. The activity on BTEE (chymotrypsin, CT) (○—○), and on BAEE (trypsin, T) (○— — —○), were measured after tryptic activation of samples, and expressed as the equivalent weight (in mg) of bovine chymotrypsin per ml, after 1:1 dilution. (B) Similar chromatography of *P. elegans* chymotrypsinogen (partly purified) but on a 25 cm × 0.9 cm column and 3-ml fractions. The gradient was from 0.015 M to 0.150 M sodium succinate (pH 5.0) in 0.001 M EDTA. Activity was located and expressed as in Fig. 1A. ○ · · · · ○, activity on BTEE. No additional activity on BAEE was found in the region shown. (C) Second rechromatography of *C. serpentina* chymotrypsinogen at 4° on a column (25 cm × 0.9 cm) of CM-cellulose. The gradient was from 0.015 M sodium succinate (pH 5.0) to 0.015 M sodium succinate–0.06 M NaCl (pH 5.6), in 0.001 M EDTA. Fractions of 4 ml were collected. Other conditions as in Fig. 1A. Peak I consists of Fractions 55–65 in Fig. 1A.

0.015 M sodium succinate–0.001 M EDTA (pH 5), and dialyzed against two changes of the same buffer.

The *P. elegans* extract was taken through the same procedure with the omission of the initial acetone precipitation. In the cases of the other reptiles, the initial extracts were not purified further, due to scarcity of material.

*Final purification of C. serpentina and P. elegans chymotrypsins*

The partly purified materials obtained as above were chromatographed on CM-cellulose at 4°. The columns were pre-equilibrated with 0.015 M sodium succinate–0.001 M EDTA (pH 5). Elution was by a linear salt gradient from 0.015 to 0.10 M sodium succinate, all in 0.001 M EDTA at pH 5. The total volume of the eluent was 20 column-volumes, and the flow rate was 1 column-volume/h (see Figs. 1A and 1B). The fractions containing chymotrypsinogen (determined by activating aliquots of the fractions with 100 $\mu$g of bovine trypsin/ml for 6 h at 4°, and assaying with BTEE) were combined, and dialyzed against 0.015 M sodium succinate–0.001 M EDTA at pH 5 (for *C. serpentina*) or pH 5.5 (for *P. elegans*). The location of trypsin in the chromatogram was determined after a similar activation of aliquots (but for 24 h) and assay on BAEE.

These chymotrypsinogen preparations were similarly rechromatographed (twice, in the case of *C. serpentina*) on CM-cellulose, using elution gradients in 0.001 M EDTA of 0.015 M sodium succinate (pH 5) to 0.015 M sodium succinate–0.06 M NaCl (pH 5.5) (for *C. serpentina*), and 0.015 M sodium succinate (pH 5.5) to 0.05 M sodium succinate (pH 6) (for *P. elegans*).

## RESULTS

*Purification of turtle chymotrypsinogens*

The pancreatic extract of *C. serpentina* was fractionated by acetone and $(NH_4)_2SO_4$ to yield the chymotrypsinogen-containing fraction. This was purified by chromatography on CM-cellulose in a succinate gradient. Two chymotrypsinogens were separated, both of which are anionic at pH 7. The major one (Peak I in Fig. 1A)

TABLE I

PURIFICATION OF *C. serpentina* CHYMOTRYPSINOGEN

The extract was from 50 g pancreas. Activity is expressed as $\mu$moles of BTEE split per min under standard conditions[1], after activation with bovine trypsin. The first and third CM-cellulose chromatography stages are indicated.

| *Procedure* | *Protein (mg)* | *Activity (units)* | *Specific activity (units/mg protein)* | *Yield (%)* |
|---|---|---|---|---|
| Extract | 2590 | 23 300 | 9.1 | 100 |
| Acetone | 1495 | 19 700 | 13.2 | 85 |
| $(NH_4)_2SO_4$ | 760 | 14 000 | 18.4 | 60 |
| CM-cellulose (first) | 105 | 5 480 | 52.5 | 23.5 |
| CM-cellulose (third) | 18 | 1 990 | 121.0* | 8.5 |

* This specific activity was found in Fractions 47–50 (Fig. 1C); the average specific activity over the whole peak was 110 units/mg protein.

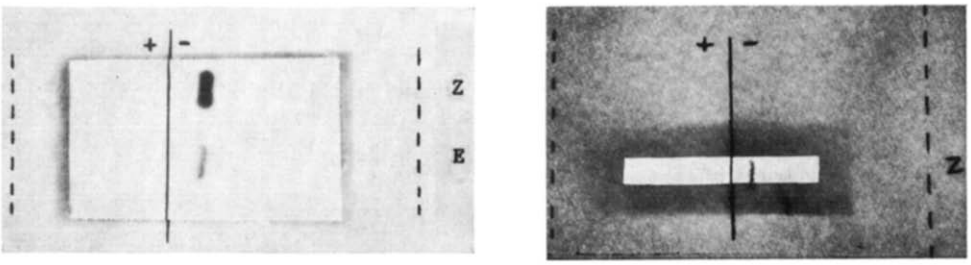


Fig. 2. Cellulose acetate electrophoresis in 0.025 M imidazole/HCl–0.001 M EDTA–0.3 M sucrose (pH 6.2). 20 min, 3 mA, 300 V, room temperature. Left: *C. serpentina* chymotrypsinogen (Z) and activated chymotrypsin (E). Right: *P. elegans* chymotrypsinogen. After staining and drying, strips carrying the sole band present were cut out and photographed in transmitted light, in a mask of paper showing the origin, polarity and path length available (between broken lines).

was further purified by rechromatography in a salt/pH gradient, yielding an essentially pure protein (Fig. 1C), as shown by electrophoresis on cellulose–acetate (Fig. 2). The maximal specific activity of this enzyme was, after activation, 121 units/mg protein. The purification is summarized in Table I. Preliminary experiments in this laboratory (M. DERECHIN AND W. MÖCKEL, unpublished results) show that the molecular weight of this chymotrypsinogen is very close to that of the bovine A enzyme, indicating that the turnover number is almost three times as high as that of the bovine chymotrypsin.

The pancreatic extract from *P. elegans* was similarly fractionated (Fig. 1B). A single anionic chymotrypsinogen was isolated in an essentially pure form upon rechromatography. It has, after activation, 115 units/mg protein. Of the potential chymotrypsin activity applied to the column, 65% was recovered in the main peak of the first chromatography. The final chymotrypsinogen preparations of both species were free of trypsin and trypsinogen.

The zymogens from both species were activated by bovine trypsin at about the same rate as, or faster than, bovine chymotrypsinogen, and the chymotrypsin thus obtained in each case was homogeneous on electrophoresis (Fig. 2). These chymotrypsins migrated as cations at pH 6.1 (imidazole buffer) as bovine chymotrypsin does, but much more slowly. Their zymogens were also cationic under the same conditions, but in 0.02 M sodium citrate buffer (pH 6.1) they migrated as anions (in contrast to bovine chymotrypsinogen A). Hence, the isoelectric point of these proteins appear to be close to pH 6.

These zymogens were not stable at pH 3 or pH 9 even at 4°, where their potential activity declined, and electrophoresis under a wider range of conditions gave unsatisfactory results for this reason.

In both turtle species, a trypsinogen was also present in considerable amounts and was separated from the chymotrypsinogen. In each case, this zymogen could be activated by bovine trypsin and the trypsin was inhibited by the specific alkylating agent $\alpha$-*N*-tosyl-L-lysine chloromethyl ketone (TLysCK) (ref. 7), in the conditions previously described[2].

In the case of *P. elegans*, the trypsinogen was readily separated, since it did not travel in the chromatographic conditions used for the chymotrypsinogen (Fig. 1B). The trypsinogen of *C. serpentina*, however, always showed very similar behavior to the chymotrypsinogen in chromatography and electrophoresis. It was just separable from

the chymotrypsinogen in appropriate chromatographic conditions (Fig. 1A) and was absent on rechromatography of the latter peak (Fig. 1C). In electrophoresis it always migrated very close to the chymotrypsinogen; for example, at pH 6.1, 20 mM citrate, both slowly moved to the cathode, the trypsinogen very slightly faster. Hence, the charge in the two zymogens is almost the same in the case of *C. serpentina*, and care is necessary in the chromatographic isolation of either.

*Reactions with specific inhibitors*

The chromatographed (Peak I, Fig. 1A) *C. serpentina* chymotrypsin was used; for *P. elegans*, both the $(NH_4)_2SO_4$-fractionated preparation and the chromatographed protein were used. TPCK and TLeuCK, applied at very low concentration, each inactivated the reptilian chymotrypsins (Fig. 3). The initial rates with TLeuCK were

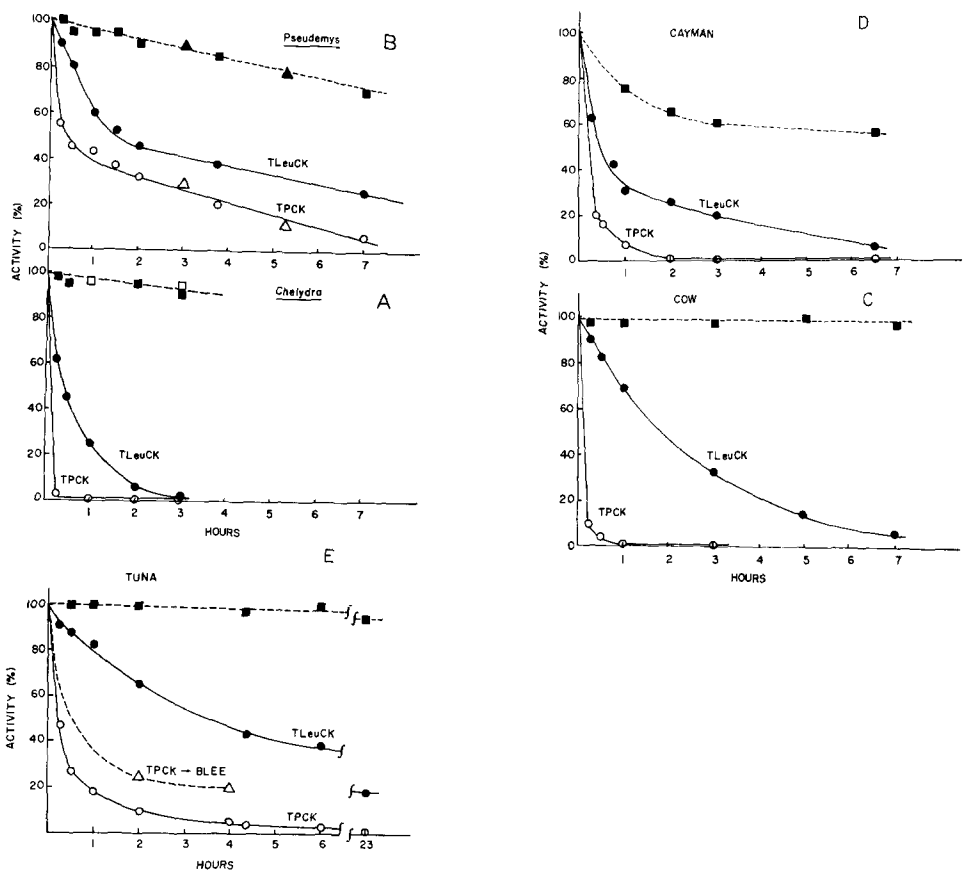


Fig. 3. (A–E) Inactivations of chymotrypsins, in 0.1 M Tris–0.05 M $CaCl_2$–0.05 M NaCl, (pH 7.3), at 25° (see also Table II). For each species shown, the enzyme was incubated alone as a control (■— —■), or with 0.3 mM TPCK (○—○), or with 0.3 mM TLeuCK (●—●). For *P. elegans*, the chromatographed material was also used in TPCK (△) and control (▲) incubations. For *C. serpentina* 1 mM TLysCK (□) was also applied (another point, not shown, at 16 h, also showed no more activity loss than in the parallel control incubation). All solutions, including the controls, contained 3% methanol (v/v). Activity was always on BTEE, except that in the tuna (E) the same TPCK-reacted sample was also assayed (△) with BLEE.

TABLE II

REACTION OF CHYMOTRYPSINS WITH ALKYLATING REAGENTS AT 0.3 mM

Conditions are as in Fig. 3, at pH 7.3 (except for tuna, at pH 7.2). The enzyme concentration (based arbitrarily on the specific activity of bovine chymotrypsin) was 1/15 of inhibitor concentration (but 1/60 in the case of the cayman): n.d., not determined; $\infty$, no significant reaction over a period of at least 3 h; TLysCK concn. was 1 mM.

| *Species* | $t_{\frac{1}{2}}$ *of inactivation reaction (min)** | | |
|---|---|---|---|
| | *TPCK* | *TLeuCK* | *TLysCK* |
| Turtles | | | |
| *Chelydra serpentina* | 9 | 25 | $\infty$ |
| *Pseudemys elegans* | 16 (+ a very slow phase) | 78 (+ a very slow phase) | $\infty$ |
| Cayman | | | |
| *Caiman crocodilus* | 12 | 28 | n.d. |
| Tuna | | | |
| *Thunnus secundodorsalis* | 15 | 210 | $\infty$ |
| Cow (chymotrypsin A) | 8 | 110 | $\infty$ |

* Half-time of reaction, determined from semi-logarithmic first-order plots of the reactions (and corrected by similar plots of the spontaneous inactivation, if any). Where the reaction is not pseudo-first order ,the rate for the initial fast phase is noted.

distinctly faster than that for bovine chymotrypsin A (Fig. 3; Table II). The TPCK rate was comparable for all, including those from the tuna and the cow (Table II), except that the chymotrypsin of the turtle *P. elegans* reacts in a complex manner with TPCK (Fig. 3B), confirming the previous observation[2] on a crude extract from this species. This reaction shows a second, very slow phase. The *P. elegans* preparation showed a similar biphasic reaction with TLeuCK; the initial rate is the same as, or faster, than that with bovine chymotrypsin A, while in the later phase it is much slower than the latter (Fig. 3B). Since points obtained with the chromatographed material lie on the curve for the TPCK reaction obtained using the less-pure preparation, it appears that these biphasic responses do not reflect the presence of two chymotrypsins with very different reactivities, but rather a non-first-order behavior of a single enzyme.

The pancreatic extract from the cayman, *Caiman crocodilus*, also showed a tendency towards the behavior shown by *P. elegans*, but this was less pronounced (Fig. 3D). The reptile chymotrypsins all showed some spontaneous loss of activity at pH 7.3 at 25° (Fig. 3). While this was slow enough not to interfere with alkylation studies, it was in contrast to the complete stability of bovine chymotrypsin (Fig. 3C), and may denote a greater susceptibility to self-digestion. In the cayman extract this auto-inactivation was initially fairly high, and the initial apparent alkylation rates needed correction for this.

Two other reptiles, the snake *Bungarus fasciatus* and the lizard *Iguana iguana*, were also examined, and shown to possess pancreatic chymotrypsinogen (Table III). Due to scarcity of material a preparation for analysis could not be made.

The tuna chymotrypsin (purified as previously)[1] gave the fast reaction with TPCK (confirming the previous report[2]), but underwent an exceptionally slow reaction with TLeuCK (Fig. 3E and Table II). The latter reaction did not go beyond 80% inactivation even after exceptionally long treatment with the alkylating agent.

The specific inhibitor of bovine trypsin, TLysCK (ref. 7), at a similar concentration gave no inhibition whatsoever with any of these enzymes (Table II). It completely inhibited the trypsin derived from the same extracts.

*Activity on specific substrates*

The chymotrypsins were defined by their activity on BTEE (Table III and ref. 1). Each was shown also to have a high protease activity with casein[1] as substrate. The leucine ester, BLEE, was also tested as a substrate in six species, and the rate (under standardized conditions) compared with the rate on BTEE in each case (Table III).

With the tuna chymotrypsin preparation, it was noted that about 20% of the activity on BLEE persisted when the activity on BTEE had been essentially eliminated by TPCK reaction (Fig. 3E). The simplest interpretation of this dual behavior is that two enzymes are present in this preparation, the minor one being an esterase that hydrolyses BLEE but otherwise does not resemble chymotrypsin.

The mean level of chymotryptic activity in the pancreas from each species is also noted (Table III). The actual content of each enzyme cannot be stated, because the specific activities can differ significantly from bovine chymotrypsin A and the contribution of each type is not yet known. Hence the contents were measured in terms of the equivalent amount of the bovine enzyme.

All the enzymes were present in the pancreas on initial extraction as their zymogens, except for the tuna pyloric ceca, as discussed previously[1].

TABLE III

ACTIVITIES OF CHYMOTRYPSINS

For the contents, active chymotrypsin was measured by the activity on BTEE and expressed as the equivalent weight of thrice-crystallized bovine chymotrypsin A having the same activity, per gram of wet weight of pancreas. Values from crude extracts prior to and after activation by bovine trypsin are shown. For *C. crocodilus* only two animals were used, for *B. fasciatus* and *I. iguana* only one, whereas the other values are the means for tissue from many individuals; n.d., not determined.

| *Species* | *Content in pancreas* | | *Activity on BTEE* / *Activity on BLEE* Ratio* |
|---|---|---|---|
| | *Before activation* | *After activation* | |
| Cow A | 0 | 6.4 | 80 |
| Pig A | | | 75 |
| Pig C | | | 1 |
| *C. serpentina* | 0 | 11 | 57 |
| *P. elegans* | 0 | 16 | 29 |
| *C. crocodilus* | 0 | 3.8 | 9 |
| Snake *(B. fasciatus)* | 0 | 1.2 | n.d. |
| Lizard *(I. iguana)* | 0.3 | 9.9 | 15.5 |
| Tuna | 0.18 | 0.18 | 24 |

* Activities of the purified preparations, measured at 0.5 mM BTEE or 2 mM BLEE at 25° in 0.04 M Tris–0.05 M $CaCl_2$ (pH 7.8), containing 28% methanol (v/v). Kinetic constants for these two substrates with bovine chymotrypsin A and porcine chymotrypsins A and C in nearly identical conditions have been reported[5]. Taking those constants and the simple kinetic scheme to which they refer, the values of the ratio of activities (at our substrate concentrations) have been calculated for porcine chymotrypsins A and C; these are the values shown for Pig A and Pig C chymotrypsins.

## DISCUSSION

Chymotrypsinogens have been obtained from the two turtle species examined in these studies in an apparently completely pure form. The characterization of these proteins, while still incomplete, is sufficient to show that basically the molecules are similar to that of bovine chymotrypsinogen. Further evidence in this and the associated paper[4] on the completely selective TPCK reaction with the chymotrypsins from these reptile and fish species supports the conclusion previously proposed from other evidence[1], that the active center of chymotrypsin is similar throughout the vertebrates.

Within this overall similarity, individual differences can be discerned. When a number of the chymotrypsins from diverse vertebrate species were examined[2], that from one reptile species, *P. elegans*, was found to be much slower in the reaction with TPCK than any other chymotrypsin tested (although still showing a reactivity much higher than that for a "normal" histidine). This suggested to us that a substrate specificity difference is present. Differences in specificity within the previously characterized chymotrypsins (A, B and C) from the cow and pig have been described[5,8]. Porcine chymotrypsin C has a much higher $v_{max}$ in the hydrolysis of BLEE than bovine or porcine chymotrypsin A, while for BTEE the $v_{max}$ values are all similar[5]. Kinetic constants have not yet been derived in the present case, since we have isolated in pure form only two of these chymotrypsins. The ratio of activities on BLEE and BTEE in arbitrary conditions is, nevertheless, a guide to distinct specificity differences. This test is sufficient to show that bonds adjacent to leucine are split more readily by the chymotrypsins from four reptile and one fish species (Table III), than by bovine chymotrypsin A. This difference can become quite substantial.

Three of the reptile species also showed a reactivity toward TLeuCK that is significantly greater than that of bovine chymotrypsin A (Table II). This inhibitor was introduced by TOBITA AND FOLK[9] as an agent that rapidly alkylates porcine chymotrypsin C. In the latter case, both the TLeuCK and TPCK reactions occurred at the active center histidine[9]. It seems a reasonable assumption that the one histidine observed[4] to be alkylated by TPCK is also the site of the specific TLeuCK reaction we have seen.

The tuna chymotrypsin preparation shows a particularly complex behavior. The leucine substrate is, as noted above, hydrolyzed relatively faster by this enzyme than by bovine chymotrypsin A; the reaction with TLeuCK is, paradoxically, unusually slow (Fig. 2E). In addition, 20% of the activity on the leucine substrate persists after BTEE activity has virtually vanished. Probably two enzymes are being detected here. One of these has a much greater preference for the leucine substrate than bovine chymotrypsin A, but is much slower in reaction with the leucine-alkylating reagent than chymotrypsin C. A second enzyme appears to be present which is also active on the leucine ester substrate, but is unreactive (or extremely slowly reactive) toward TPCK and TLeuCK. This latter esterase (which would account for about 20% of the BLEE activity) cannot yet be classified as a chymotrypsin.

Only a few arbitrarily selected reptiles were examined. Even within this small number a multiplicity of chymotrypsin types is apparent from the tests we have applied. The reaction rates with TLeuCK are all as fast as, or faster than, that found[9] (after correction for concentration differences) for porcine chymotrypsin C. They each show some enhancement of the relative rate on leucine substrates compared to bovine

chymotrypsin A. There are minor quantitative peculiarities when series are set up based on each parameter tested. The anomalous kinetics found for the later phase of the TPCK and TLeuCK reactions with *P. elegans* chymotrypsin are not understood, and further analysis of this situation is currently being made.

In conclusion, the evidence shows that while the general form of the active center of chymotrypsin is similar across these wide phyletic distances in the vertebrates, many individual variations can occur. These variations must be in the binding site region and/or in its steric relation to the catalytic functional groups of serine and histidine. The use of both tyrosine and leucine substrates, as in one example here, when combined with the application of TPCK and TLeuCK, provides a test for differences in the active center of these homologous enzymes. The difference between chymotrypsins A and C, which was defined by specificity when two mammalian species (cow and pig) were analyzed, does not show a generally applicable or very sharp distinction in the reptiles. It seems preferable to discuss these types more in terms of a specificity range. If other vertebrate species and other binding groups of substrates were to be examined, many other minor types of chymotrypsin would doubtless be recognized.

There is evidence[5] for considerable differences in molecular structure between bovine chymotrypsinogen A and porcine chymotrypsinogen C, which has a molecular weight of 6700 more than the former protein. A chymotrypsinogen from an elasmobranch fish, *Squalus acanthias* also has a molecular weight very close to that of the bovine A form[10]. If the present indications are confirmed at the protein structural level, the reptilian chymotrypsins may offer an opportunity to study the ancient divergence of the chymotrypsinogen molecule into these two types of structure.

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