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# ISOLATION AND PROPERTIES OF TWO CHYMOTRYPSINS FROM THE TURTLE *PSEUDEMYS ELEGANS*

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## SUMMARY

From the pancreas of the turtle *Pseudemys elegans* two chymotrypsinogens, I and II, were purified by a procedure including two chromatographic separations on CM-cellulose columns. They were homogeneous by chromatographic and electrophoretic criteria. After activation by bovine trypsin, the specific enzymic (esterase) activity of each was distinctly greater than that of bovine chymotrypsin A.

The chymotrypsins I and II are each inactivated by *N*-tosyl-L-phenylalanine chloromethyl ketone at an essential histidine residue, but at rates 20 and 9 times (respectively) slower than that found with bovine chymotrypsin A. This lowered reactivity, and some increase observed in the activity on leucine relative to tyrosine esters, suggest that the active center has some features similar to those of the mammalian C, as well as the A, type of chymotrypsin.

The products of tryptic activation were isolated. The zymogen I releases no free peptide or amino acids in forming the fully active enzyme. The zymogen II releases a 14-residue peptide, a tripeptide, ($Arg_2$, Phe), and a free amino acid, tyrosine.

## INTRODUCTION

We have recently reported[1,2] the identification and isolation of chymotrypsinogens from a variety of lower vertebrate species. After activation with bovine trypsin, chymotrypsins were obtained which had specificities toward amide and ester substrates and toward the inhibitors, DFP, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (*cf.* ref. 3) and *N*-tosyl-L-leucine chloromethyl ketone (TLeuCK) (*cf.* ref. 4), that are very similar to the specificity of bovine chymotrypsin A.

During inactivation with either TPCK or with TleuCK, one of these chymotrypsin preparations, isolated from the pancreas of the turtle *Pseudemys elegans*,

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, $\alpha$-*N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

showed a biphasic reaction in each case, with a fast initial phase and a very slow later phase. This behavior demanded further investigation. We have now been able, by improved chromatographic procedures, to separate the chymotrypsinogen fraction of *P. elegans* into two chymotrypsinogens. We describe here the purification of these two zymogens, their activation behavior and the specificity features of the enzymes toward substrates and inhibitors.

## MATERIALS AND METHODS

### *Materials and standard methods*

*P. elegans* turtles were obtained from E. Steinhilber (Oshkosh, Wisc.) and were further maintained in tanks at 26°, feeding on horsemeat *ad libitum* for 3 weeks to elevate[1] their chymotrypsinogen content. Pancreatic tissue was removed from the freshly killed animals and at once frozen by spreading on dry ice. The frozen material was stored at −20° until used. All other materials were as specified previously[1,2] as were the activation of zymogens, enzyme assays, inactivation reactions, electrophoresis on cellulose acetate membranes and other methods not specified here. Protein hydrolyses and amino acid analyses (on a Beckman amino acid analyzer) followed the procedures noted elsewhere[5].

### *Isolation and purification of chymotrypsinogens*

Extraction of the pancreatic tissue and fractionation of the extract with $(NH_4)_2SO_4$ were carried out as described for the turtle *Chelydra serpentina* in a preceding paper[2]. The fraction precipitating at 20–45% saturation, which contained (after activation) the chymotrypsin activity, was further purified by chromatography on CM-cellulose (microgranular CM-52 at 4°). A column, 20 cm × 2 cm, was used for an amount of this protein derived from 50 g of pancreas. The column was equilibrated with 0.01 M sodium succinate–0.001 M EDTA (pH 5.0) and elution was by a salt gradient at pH 5.0 (Fig. 1). The total volume of the eluant was 1200 ml, the flow rate was 50 ml/h and 7.5-ml fractions were collected. Trypsinogen and chymotrypsinogens

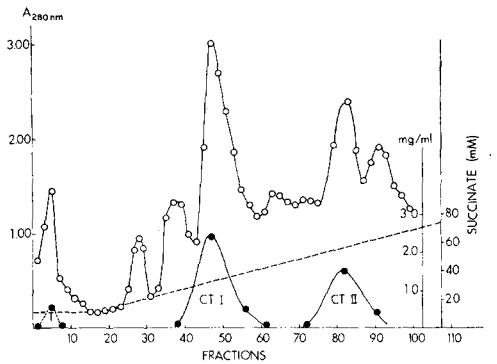


Fig. 1. Chromatography on CM-cellulose of *P. elegans* proteases (partly purified preparation) at 4°, using a concentration gradient (— — —) of sodium succinate in 0.001 M EDTA (pH 5.0). Protein is shown by $A_{280\ nm}$ (○—○); activities on BTEE (●—●); and BAEE (●— — —●) were measured after activation of samples and are expressed (mg/ml) as the equivalent of the pure bovine enzyme. T, trypsinogen; CT, chymotrypsinogen.

were located in the chromatogram by tryptic activation of aliquots[2] of the fractions and assays with *N*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE).

Final purification of the chymotrypsinogens was achieved by rechromatography on CM-cellulose. Fractions 44–53 from the first chromatography (Fig. 1) were combined (chymotrypsinogen I), as were Fractions 77–86 (chymotrypsinogen II). Each protein solution was dialyzed against 0.005 M sodium succinate–0.001 M EDTA (pH 5.4, 4°) and adsorbed on a CM-cellulose CM-52 column (17 cm × 1.5 cm), equilibrated with the same buffer. Elution was by combined salt and pH gradients: from 0.005 M sodium succinate (pH 5.4) to (for chymotrypsinogen I) 0.005 M sodium succinate–0.080 M NaCl (pH 6.0) or (for chymotrypsinogen II) 0.010 M sodium succinate–0.080 M NaCl (pH 6.0), all in 0.001 M EDTA. In each case, the total eluant volume was 600 ml, the flow rate was 20 ml/h and fractions of 5 ml were collected.

*Isolation of products of zymogen activation*

The solutions of chymotrypsinogens I and II were dialyzed against 0.005 M sodium succinate–0.001 M EDTA (pH 5.4, 4°). For activation, each sample was mixed with its own volume of 0.2 M Tris–HCl–0.1 M KCl–0.1 M $CaCl_2$ (pH 7.3). Bovine trypsin (0.005 mg/mg chymotrypsinogen) was added, and the mixture was incubated at 4° for 20 h. Assays on BTEE showed that complete activation was then achieved. The chymotrypsins were immediately inactivated by addition of TPCK (as 0.01 M solution in methanol) to these solutions. 0.15 mg TPCK per mg protein, applied in 4 portions over a period of 30 h at 25°, inactivated both chymotrypsins completely. (Assuming molecular weights of 25 000, this was an enzyme to inhibitor ratio of 1:10). $\alpha$-*N*-tosyl-L-lysine chloromethyl ketone (ref. 7) was also added at the same time, in the same ratio, inactivating all the trypsin present. The solutions of inactivated chymotrypsins, each reduced to 3 ml by evaporation, were then gel filtered (Fig. 3) and Fractions 22–33, containing the low-molecular-weight material, were combined in each case for further treatment.

## RESULTS

*Purification of P. elegans chymotrypsinogens*

Pancreatic tissue of the turtle *P. elegans* was extracted and fractionated with $(NH_4)_2SO_4$. A fraction containing the chymotrypsinogens (determined by assay on BTEE after activation with bovine trypsin[1,2]) was obtained, while no chymotrypsin activity was present prior to activation. This fraction was further purified by chromatography on CM-cellulose in a succinate gradient. Two chymotrypsinogens in nearly equal amounts and one trypsinogen were separated by this method (Fig. 1). The trypsinogen was very low in amount, most of the high content[1] of this zymogen being removed in the $(NH_4)_2SO_4$ fractionation. Both the chymotrypsinogens were finally purified by rechromatography on CM-cellulose in salt/pH gradients (Fig. 2). The zymogen II (Fig. 2B) was obtained thus in a completely homogeneous peak, while the zymogen I (Fig. 2A) was just separated from another protein peak which contained no chymotrypsin or zymogen. The fractions free from overlap (Fractions 73–78, Fig. 2A) were therefore taken.

Starting from 50 g tissue, 25 mg of pure chymotrypsinogen I and 33 mg of pure

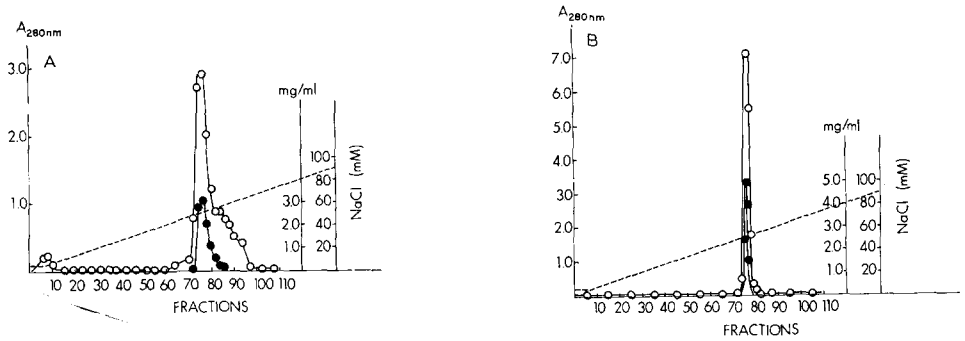


Fig. 2. Rechromatography on CM-cellulose of *P. elegans* chymotrypsinogen I (A) and of chymotrypsinogen II (B), at 4° using a salt and pH gradient (— — —) (see text). Notations are as in Fig. 1.

chymotrypsin II were isolated. The specific activities of these enzymes were measured after activation (Table I). Since the molecular weights of the chymotrypsins from both the turtles and the cow seem to be very similar[6], the differences in specific activities would reflect real differences in the turnover numbers of these enzymes.

*Electrophoresis on cellulose acetate*

Electrophoresis on cellulose acetate membranes at pH 6.2 shows (Fig. 5) *P. elegans* chymotrypsinogen I moving as a single, slightly cationic substance. The activated chymotrypsins I and II have identical mobilities, and the mobility of chymotrypsinogen I is almost the same. Chymotrypsinogen II shows several bands. Since, however, this inhomogeneity disappears completely after activation, so that chymotrypsin II moves in a single band, we conclude that the different components present in the preparation of chymotrypsinogen II represent intermediates of an activation occurring readily during preparation and storage of the material. All these turtle chymotrypsinogens and chymotrypsins are distinctly less cationic at pH 6.2 than is bovine chymotrypsinogen A.

*Analysis of products of activation of zymogens*

The zymogens I and II were activated with bovine trypsin. After subsequent inactivation with TPCK, the protein solutions were gel filtered on Sephadex G-50,

TABLE I

SPECIFIC ACTIVITIES OF CHYMOTRYPSINS

The specific activity is expressed in units of μmoles of BTEE split per min per mg protein under the same standard assay conditions[1] for all, at 25°. The maximum activity found for the purified enzyme is listed; the cow A value was measured on the commercial (thrice recrystallized) material.

| *Species* | *Specific activity* |
|---|---|
| *P. elegans* I | 113 |
| *P. elegans* II | 67 |
| *C. serpentina*[2] | 121 |
| Cow A | 45 |

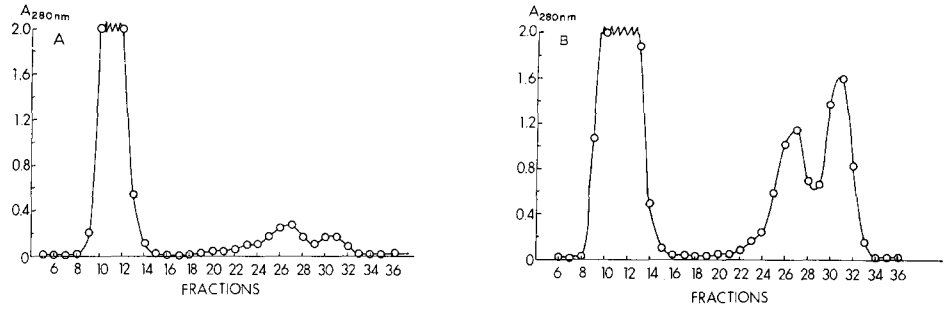


Fig. 3. Gel filtration of the activation products of *P. elegans* chymotrypsin I (A) and of the chymotrypsin II (B), at 25° on a column (30 cm × 1.4 cm) of Sephadex G-50 (fine mesh) in 0.1 M NaCl medium. Fractions of 2 ml were collected at a rate of 15 ml/h. In each case the active enzyme produced was converted to the TPCK-inactivated form prior to the gel filtration.

which separated a high-molecular-weight and a low-molecular-weight fraction (Fig. 3). The latter fraction, derived from 22 mg of zymogen II (Fig. 3B), was proportionately much greater than that from 16 mg of zymogen I (Fig. 3A). The low-molecular-weight fraction of the chymotrypsin II preparation (Fig. 3B) was further resolved into 5 components on Biogel P-2 (Fig. 4). Fraction a (Fig. 4) was purified by a similar refiltration. Each fraction was hydrolyzed in 6 M HCl, and the hydrolysate was analyzed for amino acids. The results are summarized in Table II. Fractions d and e contained no detectable amino acids; they probably consist of breakdown products of the excess inhibitor (TPCK) and salts. Fraction c was analyzed both before and after acid hydrolysis, and only tyrosine was found to be present. The results show that a peptide of 14 amino acids, a tripeptide ($Arg_2$, Phe) (or, just possibly, a dipeptide (Arg, Phe)), and a single amino acid are released from the zymogen II during activation.

Hydrolysis and amino acid analysis were also performed on the combined low-molecular-weight fractions of the chymotrypsin I preparation (Fig. 3A). No amino acid was present at a significant level; the highest was aspartic acid, at about one third of one residue when calculated in proportion to the amount of zymogen that was taken for this experiment. These traces were taken to be contaminants; the slight ultraviolet absorption is attributable to TPCK breakdown products.

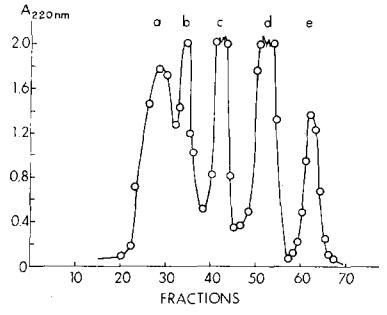


Fig. 4. Gel filtration of the combined low-molecular-weight fractions of *P. elegans* chymotrypsin II (see Fig. 3B) on a column (80 cm × 1 cm) of Biogel P-2 at 25°. The eluant was 0.02 M NaCl. 1-ml fractions were collected at a rate of 10 ml/h. Fractions b, c, d also had considerable absorption at $A_{280\ nm}$ (not shown here).

TABLE II

AMINO ACID COMPOSITION OF FRACTIONS a–c

These are the low-molecular-weight fractions (Fig. 4) produced in the activation of chymotrypsin II (Fig. 3B). The aliquots analyzed correspond to 75 nmoles of the parent zymogen (assuming a molecular weight of 25 000), except for the refiltered Fraction a. Amounts of amino acids found are given as nmoles; the values in parentheses represent the best estimate of the number of residues apparently present. The nmoles values for Fraction b must be too low since, due to the overlap seen in Fig. 4, only the central fractions of the peak were used; however, the purity was sufficient to show that other amino acids are absent in this fraction. The amounts of other amino acids not listed were below 15 nmoles (3 nmoles for refiltered a). Fraction c contained significantly no material other than tyrosine and gave the same analysis when an aliquot was examined without the 6 M HCl hydrolysis. Hydrolyses were 16 h, 110°; no corrections have been applied. Asp and Glu values include their amides.

| *Amino acid* | *a* | *a (refiltered)* | *b* | *c* |
|---|---|---|---|---|
| Arg | 0 | 0 | 94(2) | |
| Asp | 164(2–3) | 66(3) | | |
| Thr | 91(1) | 20(1) | | |
| Ser | 84(1) | 25(1) | | |
| Glu | 147(2) | 47(2) | | |
| Pro | 74(1) | 19(1) | | |
| Gly | 155(2) | 40(2) | | |
| Ala | 120(1–2) | 27(1) | | |
| Val | 79(1) | 20(1) | | |
| Ile | 61(1) | 13(1) | | |
| Leu | 58(1) | 12(1) | | |
| Tyr | 0 | 0 | | 84(1) |
| Phe | 0 | 0 | 54(1) | |
| Total number of residues | 13–15 | 14 | 3 | 1 |

*Reactions with specific inhibitors*

The rechromatographed chymotrypsinogens I and II were used and were activated with bovine trypsin (5 $\mu$g/mg protein). Low concentrations of TPCK completely inactivated both chymotrypsins within 20 h at 25° (Fig. 6A). In a control experiment without inhibitor, the activity decreased during this time only by about 5%. With the same concentration of *N*-tosyl-L-leucine chloromethyl ketone (TLeuCK), the inactivation was incomplete even after 20 h (Fig. 6B). In both cases, the inactivation by TPCK was faster than that by TLeuCK, showing an enzyme specificity toward the inhibitors more like that of bovine chymotrypsin A than that of porcine chymotrypsin C (ref. 4). However, the rates of inactivation with TPCK for the enzymes I and II are very much slower (Table III) than the rate reported[2,3] for bovine chymotrypsin A and are similarly slower than the inactivation rates of the several chymotrypsins from other reptile and fish species that have been tested[2].

In all the TPCK and TLeuCK inactivations conducted on the highly purified *P. elegans* chymotrypsins, the kinetics (as shown in Fig. 6) were found to be first order in semilogarithmic plots. The biphasic behavior that was reported earlier[2] did not appear when the highly purified enzymes were examined. This biphasic form must have been due to the presence of the two chymotrypsins together in the less pure preparations used in the earlier[2] investigations (although the fast initial rates found there are not quantitatively accounted for thus). The spontaneous activity loss found

TABLE III

REACTIONS OF CHYMOTRYPSINS WITH SUBSTRATES AND INHIBITORS

The values of $t_{\frac{1}{2}}$, the half-time for inactivation with 0.3 mM TPCK or TLeuCK (at pH 7.3, 25°), are derived from Fig. 6.

| *Chymotrypsins* | *Activity on BTEE** | $t_{\frac{1}{2}}$ *(min)* | |
|---|---|---|---|
| | *Activity on BLEE* | *TPCK* | *TLeuCK* |
| *P. elegans* I | 50 ± 10 | 165 | 370 |
| *P. elegans* II | 38 ± 10 | 70 | 190 |
| *C. serpentina*[2] | 57 ± 10 | 9 | 25 |
| Cow A (ref. 2) | 80 ± 10 | 8 | 110 |
| Pig A | 75** | | |
| Pig C | 1** | 720*** | approx. 50*** |

* The ratio of these activities measured under standard[2] conditions. Due to imprecision in the BLEE assay, the maximum and minimum limits in each determination were estimated, giving an approximate range of ± 10 in this ratio in these cases, as shown.

** Calculated[2] from the data of ref. 4.

*** Estimated (for 0.3 mM reagent) from the inactivation curves given by TOBITA AND FOLK[4].

in the less pure preparations[2] was greatly reduced here and was presumably due to proteolysis.

*Activity on specific substrates*

The activity of the chymotrypsins, as described so far in this report, was always that in hydrolyzing BTEE. We have previously shown[2] that the rate of hydrolysis of the leucine substrate, *N*-benzoyl-L-leucine ethyl ester (BLEE), when compared with the BTEE rate under standard conditions, provides a method for probing specificity differences between various chymotrypsins. The BTEE to BLEE reaction ratio under standard conditions was, therefore, determined for the two purified *P. elegans* chymotrypsins (Table III). The somewhat greater relative activity at the leucine ester bond, by a factor of about 2-fold, of the *P. elegans* enzymes as compared to the cow enzyme, was noted in a previous approximate determination on a less pure preparation of the former[2] and is confirmed on the pure enzymes.

## DISCUSSION

*P. elegans* pancreas contains two chymotrypsinogens, I and II, which appear to differ slightly in their isoelectric points. These were not separated in our initial investigation[2] but were isolated in pure form here. They each produce, on activation with bovine trypsin, a chymotrypsin. These two enzymes show general molecular similarities: they have the same electrophoretic mobility in our conditions and about the same molecular weight[6] (close to 25 000, as for the bovine A and B chymotrypsins). The specific esterase activity on BTEE (under arbitrary conditions) of chymotrypsin I is almost 3 times that of bovine chymotrypsin A; the corresponding activity of chymotrypsin II is distinctly lower than that of I but still significantly higher than that of bovine A. Both enzymes (I and II) are inactivated by TPCK, by alkylation at the N-3 position of an essential histidine[3] as is the bovine A enzyme; the reaction in both I and II, however, is very much slower (Table III) than in bovine A. These active centers

are not, on the other hand, very similar to that of the C type of chymotrypsin shown in the pig by TOBITA AND FOLK[4], where the reaction with TPCK is also very slow; in that case a relatively fast reaction was observed with TLeuCK, but this is not found in the *P. elegans* enzymes (Table III).

In summary, it appears that some tendency to the C type is still discernable here, but this is definitely not very pronounced. That tendency is seen from the ratio of activities on the leucine and tyrosine substrates, and from the fact that there is some increase (relative to that found for bovine A) in the ratio of the reactivities on TLeuCK compared to TPCK (Table III). This increase is much less dramatic than that reported for porcine chymotrypsin C (Table III). These results, showing a somewhat intermediate behavior, support the suggestion made previously[2] that it is preferable not to make sharp distinctions between A and C types of specificity but rather to consider the types of chymotrypsins in terms of a specificity range.

It is also noteworthy that the pancreas of another reptile, the snapping turtle *C. serpentina*, contains two chymotrypsinogens. Only one of these has been characterized[2] and forms a chymotrypsin which shows a specific activity and a BTEE to BLEE ratio indistinguishable from those of *P. elegans* chymotrypsin I. However, the rate in the TPCK reaction is as fast as that of the bovine A enzyme, while the TLeuCK reaction is also relatively fast (Table III). Hence, the reptilian chymotrypsins so far examined, all show minor differences from both the A and C types of mammalian enzyme and must have subtle variations in the details of their active centers.

On activation of chymotrypsinogen I, a stable stage is rapidly reached in which, so far as can be readily detected, no amino acids are actually released from the molecule, so that only one or more internal cleavages occur. On activation of chymotrypsinogen II, however, several intermediate stages must occur. This is taken to be the explanation of the multiple bands seen in electrophoresis of the zymogen (Fig. 5),

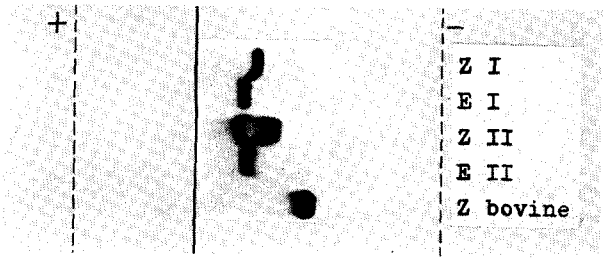


Fig. 5. Cellulose acetate electrophoresis in 0.025 M imidazole–HCl–0.001 M EDTA–0.3 M sucrose (pH 6.2), run for 20 min at 3 mA, 300 V, room temperature. Z, *P. elegans* zymogens; E, *P. elegans* activated chymotrypsins; bovine, bovine chymotrypsinogen A. After staining and drying, strips carrying all the bands were cut out and photographed in transmitted light in a mask of paper, showing the origin (solid line), polarity and path length available (between broken lines).

where a very facile activation apparently commences in the conditions used. After complete activation (initiated by trypsin) only a single electrophoretic and chromatographic species is finally present, and the activity is then stable for many hours at 25° (Fig. 6B), so that one final active enzyme species is formed. The amino acids and peptides released were isolated, but it seems likely that these are the sum of the products released at several stages. That these are true products of activation, and not

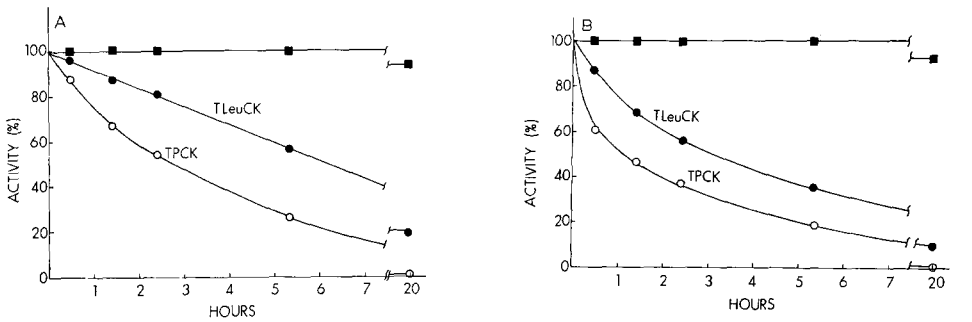


Fig. 6. Inactivations of chymotrypsin I (A) and chymotrypsin II (B). The medium was 0.1 M Tris–HCl–0.05 M $CaCl_2$–0.05 M NaCl (pH 7.3) at 25°. In each case, the enzyme was incubated alone as a control (■—■) or with 0.3 mM TPCK (○—○) or with 0.3 mM TLeuCK (●—●). The inhibitor to enzyme ratio was between 20 and 30. All solutions, including the control, contained 3% (v/v) methanol. Activity was always measured on BTEE.

of further self-degradation, is shown by several features, including their stoichiometric amounts, the relative purity of the various fractions, the stability (at pH 7, 25°) of the enzyme thus formed (Fig. 6B), and the fact that, while the enzyme product was isolated in these experiments (Fig. 3B) in the TPCK-inactivated form, the full specific enzyme activity of chymotrypsin II was found to be present immediately prior to the addition of TPCK, so that the inactivated protein separated here must have the primary structure of the active enzyme.

It is a matter of interest that a relatively large fragment is released from this reptilian chymotrypsin. FOLK AND SCHIRMER[8] found for porcine chymotrypsin C that several stages appeared to occur in tryptic activation, and a large reduction (about 8000) in the molecular weight of the protein was inferred. In the formation of *P. elegans* chymotrypsin II from its zymogen a reduction in molecular weight of about 2000 is indicated by the examination of the activation products.

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