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# ON SUBUNIT II OF BOVINE PROCARBOXYPEPTIDASE A. ENZYMATIC SPECIFICITY AFTER TRYPTIC ACTIVATION

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## SUMMARY

The similarities shown in the preceding paper existing between the structure of subunit II of bovine procarboxypeptidase A and porcine chymotrypsinogen C were found to extend to the specificities of the corresponding enzymes. Both cleave leucyl bonds in peptides and synthetic esters at a high rate, and are readily inhibited by tosyl-L-leucine chloromethyl ketone.

## INTRODUCTION

The tryptic activation of subunit II of bovine procarboxypeptidase A-S6 is known to give rise to an endopeptidase which hydrolyses the typical chymotrypsin substrate *N*-acetyl-L-tyrosine ethyl ester (ATEE). Its amino acid composition, molecular weight and N-terminal residues[1] resemble more those of porcine chymotrypsinogen C (refs 2 and 3) than those of bovine and porcine chymotrypsinogens A and B. Moreover, the short chain formed during the activation of subunit II contains, like that of chymotrypsin C, 13 residues instead of the 15 known to be present in the corresponding chain of chymotrypsinogens A and B. This latter difference is due to an apparently characteristic deletion at positions 12 and 13 (ref. 4). Bovine subunit II and porcine chymotrypsinogen C, therefore, can be expected to possess a high degree of structural homology.

The purpose of the present work is to show by a series of assays with substrates and inhibitors that this structural homology in the zymogens results in a marked similarity in the specificity of the corresponding enzymes.

## MATERIALS AND METHODS

### *Enzymes, substrates and inhibitors*

Bovine procarboxypeptidase A-S6 and the two related Subfractions IIb and IIc were prepared according to methods described in the preceding paper[1]. Porcine

chymotrypsinogen C and chymotrypsin C were purified from fresh and autolyzed pancreas, respectively[3,5]. Solutions of the zymogen (10 $\mu$M in 50 mM Tris–HCl buffer, pH 7.8) were activated by a 60 min incubation at 0 °C with 1:100 trypsin (EC 3.4.4.4) (weight ratio). For these and other assays, the concentration of the protein solutions was calculated using the following extinction coefficients ($\varepsilon^{1\%}_{1\,cm}$ at 280 nm): 21.5 for bovine $\alpha$-chymotrypsin; 23.0 for porcine chymotrypsinogen C and chymotrypsin C; 19.0 for bovine procarboxypeptidase A-S6 and the two subfractions IIb and IIc. Trypsin and $\alpha$-chymotrypsin (2 times crystallized) were obtained from Worthington and used without further purification.

The S-carboxymethylated B chain of insulin and the synthetic ester benzoyl-L-tyrosine ethyl ester (BTEE) were Schwarz-Mann products. The esters ATEE and acetyl-L-leucine methyl ester (ALME) were obtained from Industrie Biologique Française and Cyclo Chemicals, respectively. Benzoyl-L-leucine ethyl ester (BLEE) was synthetized in this laboratory by Dr F. W. Stevens from pure benzoyl-L-leucine. The inhibitors tosyl-L-phenylalanine chloromethyl ketone (TPCK) and tosyl-L-leucine chloromethyl ketone (TLCK) were a generous gift of Dr M. Mousseron-Canet and Miss Christine Demontès (Ecole Nationale Supérieure de Chimie, University of Montpellier).

*Assays with the S-carboxymethylated B chain of insulin*

To a suspension of the chain (10 mg) in water (870 $\mu$l) were added 30 $\mu$l of a 0.04% phenol red solution in 1 mM NaOH and 100 $\mu$l of a 0.1% solution of the enzyme. The mixture was adjusted to pH 8.2 (red shift of the indicator) with 0.1 M ammonium carbonate and incubated at 37 °C for 1 h with the occasional addition of ammonium carbonate to stabilize the pH at 8.2. The digest was fingerprinted and individual peptides were analyzed in a Spinco-Beckman automatic analyzer Model 120 C after acid hydrolysis.

*Assays with synthetic esters*

The leucine esters ALME and BLEE and the tyrosine ester ATEE were dissolved in a 50 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl and 3% methanol. The methanol concentration was increased to 6% for the more insoluble BTEE.

Activity measurements were carried out at 25 °C with the aid of a Radiometer recording pH-stat.

*Inhibition studies*

0.21% solutions of TPCK and TLCK in methanol were added (25 $\mu$l per ml) to the enzyme dissolved in a 50 mM Tris–HCl buffer at pH 7.0. Final concentrations were 0.01 mM for the enzyme and 0.15 mM for the inhibitor. The mixtures were incubated at 25 °C and controls were run simultaneously without inhibitor but with the same methanol concentration as the assays. After suitable time intervals, aliquots were removed and the remaining activity was measured titrimetically with ATEE as substrate.

## RESULTS AND DISCUSSION

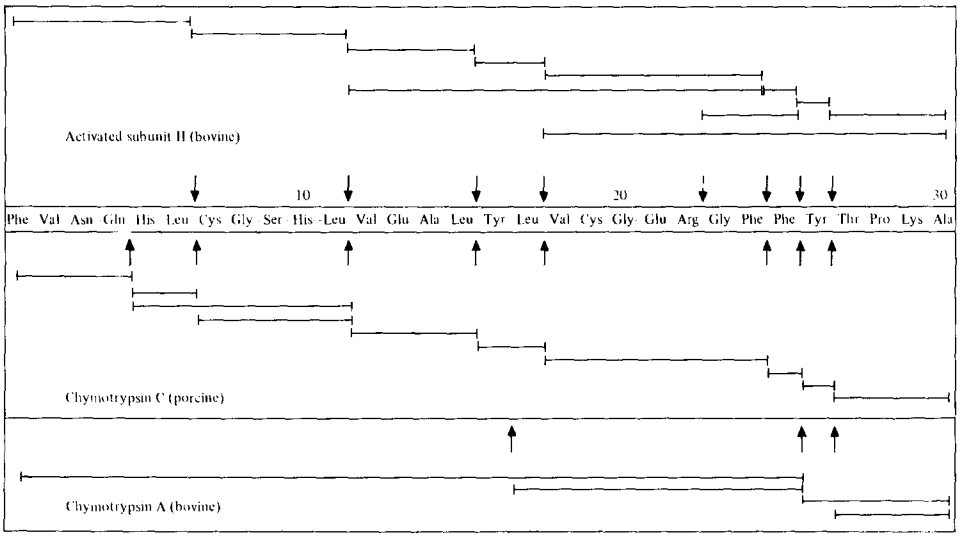
*Digestion of S-carboxymethylated insulin B-chain*

It was reported by Folk and Cole[6] that porcine chymotrypsin C had a broader

TABLE I

HYDROLYSIS OF S-CARBOXYMETHYLATED INSULIN B-CHAIN

The vertical arrows indicate the bonds cleaved by the enzymes and the horizontal bars indicate the identified peptides after enzymatic digestion.




specificity than bovine chymotrypsin A (EC 3.4.4.5). The enzyme was observed to cleave in several peptide substrates (oxidized insulin A- and B-chains, glucagon and oxidized oxytocin), not only bonds involving the carboxyl group of aromatic amino acids, but also leucyl, glutaminyl and methionyl bonds which were not split by chymotrypsin A under the same conditions. Similarly, chymotrypsin C rather than chymotrypsin A was recently used for the preparation of neochymotrypsinogens involving the specific cleavage of two leucyl bonds (Bonds 10 and 13) in bovine chymotrypsinogen A (ref. 7).

Table I shows that chymotrypsin A is also unable to hydrolyze any of the four leucyl bonds of S-carboxymethylated insulin B-chain. All these bonds are cleaved by chymotrypsin C and activated subunit II. This result is a first indication that the latter two enzymes may share the ability to cleave leucyl bonds at an especially high rate in peptide substrates. However, the activated subunit does not appear to cleave the Glu–His bond in position 4–5, which is readily attacked by chymotrypsin C.

TABLE II

KINETIC PARAMETERS OF SYNTHETIC ESTERS HYDROLYSIS

$k_{cat}$ and $K_m$ are expressed in $s^{-1}$ and mM, respectively.

| *Enzyme* | *Tyrosyl esters* | | | | *Leucyl esters* | | | |
|---|---|---|---|---|---|---|---|---|
| | *ATEE* | | *BTEE* | | *ALME* | | *BLEE* | |
| | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ |
| Bovine chymotrypsin A | 175 | 2 | 70 | 0.2 | 14 | 25 | 2 | 0.7 |
| Porcine chymotrypsin C | 170 | 20 | 80 | 2 | 50 | 10 | 28 | 0.4 |
| Bovine subunit II | 210 | 7 | 60 | 0.4 | 100 | 15 | 26 | 0.3 |

*Hydrolysis of tyrosine and leucine esters*

Another aspect of the comparison between the specificity of the two enzymes is given by Table II in which the kinetic parameters ($k_{cat}$ and $K_m$) of synthetic tyrosine and leucine esters hydrolysis are listed. As expected, tyrosine esters are better substrates for chymotrypsin A (10-fold lower $K_m$; same $k_{cat}$) whereas leucyl esters are better substrates for chymotrypsin C (2-fold lower $K_m$; 3–14 fold higher $k_{cat}$). Activated subunit II appears to occupy an intermediary position for tyrosyl esters, but behaves very much like chymotrypsin C for leucyl esters. In other words, results obtained with synthetic esters confirm that bonds involving the carboxyl group of leucine and aromatic amino acids are equally well cleaved by activated subunit II.

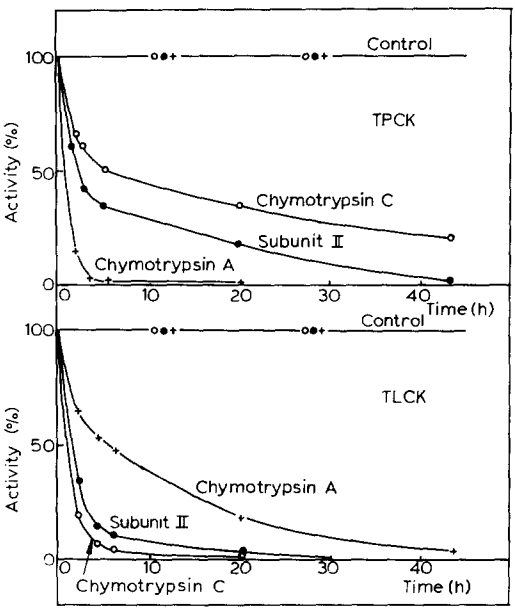



Fig. 1. Inhibition by TPCK and TLCK of bovine chymotrypsin A, activated subunit II and porcine chymotrypsin C.

*Action of active site-directed inhibitors*

It was also of interest to compare the action on the enzymes of chloromethylketones containing phenylalanine or leucine[8] as a substrate-like moiety. The results illustrated in Fig. 1 are in perfect agreement with those obtained with synthetic esters. Inhibition of activated subunit II by the phenylalanyl chloromethylketone is intermediary between that of chymotrypsin A and C, whereas it is nearly as fast as that of chymotrypsin C when the leucine derivative is used.

In conclusion, this work and the preceding one appear to be fully consistent with the already expressed view[2,4] that subunit II of bovine procarboxypeptidase A is a chymotrypsinogen C.

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