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# THE DIGESTION OF THE OXIDIZED B CHAIN OF INSULIN BY HUMAN NEUTROPHILE PROTEASES: ELASTASE AND CHYMOTRYPSIN-LIKE PROTEASE

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## Summary

The specificities of human neutrophile elastase and chymotrypsin-like protease towards oxidized insulin B chain were studied. The neutrophile elastase was found to differ from porcine pancreatic elastase in its specificity towards insulin B chain. The neutrophile elastase preferred mostly valine near the cleaved bond in contrast to pancreatic elastase which preferred alanine as well as valine near the cleaved bond. Human neutrophile chymotrypsin-like protease was found to cleave mostly bonds involving leucine and phenylalanine.

## Introduction

Human neutrophile elastase has been shown to have similar substrate specificity to porcine pancreatic elastase. Both enzymes can hydrolyze the natural substrate, elastin [1]. Several synthetic substrates, such as Boc-Ala-ONp and Boc-$(Ala)_3$-OMe, were easily hydrolized by both enzymes [2]. On the other hand Boc-$(Ala)_3$-Nan, which is accepted as a 'good' substrate for porcine elastase, was found to be a poor one for the neutrophile elastase [3].

Zimmernam and Ashe [4] have reported that the synthetic substrates specificity of neutrophile elastase was different from that of the pancreatic elastase.

Abbreviations: Boc-Ala-ONp, *t*-butyloxycarbonyl-L-alanine-4-nitrophenyl ester; Boc-$(Ala)_3$-OMe, *t*-butyloxycarbonyl-L-alanyl-L-alanyl-L-alanine-methyl ester; Boc-$(Ala)_3$-Nan, *t*-butyloxycarbonyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; Bz-Leu-OEt, *n*-benzoyl-L-tyrosine-ethyl ester; Ac-Tyr-Nan, *N*-acetyl-L-tyrosine-*p*-nitroanilide.

They showed that the human enzyme preferred valine at the cleaved bond.

Porcine pancreatic elastase has been reported to have specificity for cleaving peptide bonds involving carboxyl groups of aliphatic amino acids, mainly alanine [5,6]. Studies with highly purified pancreatic elastase suggested that its greatest activity was directed towards the peptide bonds of Ala-14, Val-18 and Gly-23 in the B chain and Ser-18 in the A chain of oxidized insulin [7]. While the present work was under progress Blow [8] had reported his results on the specificity of human lysosomal elastase. Blow and Barrett [9] had reported on the action of human cathepsin G on the oxidized insulin B chain.

In the present study we investigated the specificity of the human neutrophile elastase using the B chain of oxidized insulin as a substrate. We also tested the specificity of another human neutrophile enzyme, the chymotrypsin-like protease [10] on the same substrate. This enzyme was found to have about $\frac{1}{3}$ the specific activity of bovine pancreatic α-chymotrypsin when the enzymes were tested on Bz-Ty-OEt. It was also found that, like bovine pancreatic chymotrypsin, it hydrolyzed Bz-Tyr-OEt faster than Bz-Leu-OEt, but unlike pancreatic chymotrypsin, it hydrolyzed Ac-Tyr-Nan [11].

## Experimental

*Materials*

Fresh buffy coat was obtained from the blood bank at the Bellinson Hospital, Petah Tikva, Israel. Human neutrophile elastase and chymotrypsin-like protease were prepared according to the method previously described [3,11] using buffy coat as a source of neutrophiles. Oxidized insulin B chain was obtained from Schwarz/Mann Orangeburg, NY, U.S.A. Bz-Tyr-OEt and Boc-Ala-ONp were from Sigma Chemical Co., St. Louis, MO. Pyridine was obtained from Matheson Coleman and Bell, Norwood, OH, and phenylisothiocyanate from Y.T. Baker Chemicals, Philadelphia, NJ. These last two reagents were redistilled before use. 4-Phenylbutylamine was from Aldrich Chemical Co., Milwaukee, WI. Affrigel-10 from Bio-Rad Lab., Richmond, CA. Trifluoroacetic acid and *n*-butylacetate were purchased from E. Merck, Darmstadt, F.R.G. Porcine pancreatic elastase was a gift of Dr. A. Gertler from the Hebrew University.

*Methods*

*Enzymic assays* were carried out at 30°C in 0.1 M Tris buffer, pH 7.0, as described previously [3,11]. A Varian Techtron Spectrophotometer (Model 635) was used.

*Digestion of oxidized insulin B chain.* The enzymes were added to the insulin, dissolved in 0.05 M ammonium acetate (pH 8.0—9.0) in ratios of 1 : 100, 1 : 50 or 1 : 25. Aliquots were taken out at different time intervals. The reaction was stopped by lowering the pH with glacial acetic acid. The samples were lyophilized. Each aliquot contained 120 nmol of insulin so that 30 nmol were added to each one of the tubes for the Edman degradation. In the first tube no degradation took place, in the second, third and fourth, one, two or three degradations were carried out.

*Determination of the cleaved peptide bond.* A modification of the substruc-

tive Edman degradation [12] was used for the determination of the cleaved peptide bond. Samples containing 30 nmol oxidized insulin B chain, previously treated with pancreatic elastase, neutrophile elastase or chymotrypsin-like protease, were put into Edman degradation test tubes and dried under vacuum. Two drops of equal volumes of 5% phenylisothiocyanate in pyridine and 50% pyridine in water, were added. The tubes were flushed with nitrogen and sealed. The stoppered tubes were then incubated at 40°C for 1 h. After the incubation time they were opened and the phenylisothiocyanate was evaporated under vacuum in a heated desiccator (Electrothermal, U.K.), for 2 h at 60°C over $H_2SO_4$.

The next step was the addition of 2 drops redistilled trifluoroacetic acid, again under $N_2$ atmosphere. The stoppered tubes were incubated at 40°C for 45 min and thereafter dried in a desiccator under vacuum for 4 h over NaOH. At this stage, one Edman cycle was completed. The phenylthiohydantoin amino acid was extracted by butylacetate. The aqueous solution containing the remained digested insulin was dried in a desiccator over NaOH and $H_2SO_4$ in vacuum. The samples were now ready for amino acid analysis. A Beckman-Unichrom amino acid analyzer was used utilizing the chromatographic procedures of Hamilton [13]. Since three Edman cycles were carried out we could determine the sequence of the three degraded amino acids knowing the sequence of the B chain [14].

TABLE I

RESULTS OF EDMAN DEGRADATION OF OXIDIZED INSULIN B CHAIN AFTER INCUBATION WITH PORCINE PANCREATIC ELASTASE

Arrows indicate the cleaved bond. Details of the experiments are given in the text.

| Digestion time | Degraded amino acid | | | Sequence |
|---|---|---|---|---|
| | Edman cycle 1 | 2 | 3 | |
| Zero | Phe | Val | Asp | $Phe_1$ -$Val_2$-$Asp_3$ |
| 20 min | Phe | Gly | Asp | $Phe_1$ ↓ -$Val_2$-$Asp_3$ |
| | Leu | Val | Glu | $Ala_{14}$ — $Leu_{15}$ ↓ -$Tyr_{16}$-$Leu_{17}$ |
| | $Cys(O_3H)$ | Tyr | Leu | $Val_{18}$ — $Cys(O_3H)_{19}$ -$Gly_{20}$-$Glu_{21}$ |
| 1 h | Phe | Val | Asp | $Phe_1$ ↓ -$Val_2$-$Asp_3$ |
| | Leu | Tyr | Glu | $Ala_{14}$ — $Leu_{15}$ ↓ -$Tyr_{16}$-$Leu_{17}$ |
| | $Cys(O_3H)$ | Phe | Leu | $Val_{18}$ — $Cys(O_3H)_{19}$ ↓ -$Gly_{20}$-$Glu_{21}$ |
| | Phe | Gly | Tyr | $Gly_{23}$ — $Phe_{24}$ -$Phe_{25}$-$Tyr_{26}$ |
| 24 h | As after 2 h of incubation plus: | | | As after 1 h of incubation plus: ↓ |
| | His | Leu | Val | $Ser_9$ — $His_{10}$ -$Leu_{11}$-$Val_{12}$ |

## Results and Discussion

The specificities of neutrophile elastase and chymotrypsin-like protease, using oxidized insulin B chain as substrate were investigated. The modified substructive Edman degradation described by Konigsberg [12] was used. Porcine pancreatic elastase served as reference to test the reliability of the method. The data of Table I shows that the specificities of the pancreatic

TABLE II

RESULTS OF EDMAN DEGRADATION OF OXIDIZED INSULIN B CHAIN AFTER INCUBATION WITH NEUTROPHILE

Arrows indicate the cleaved bond. Details of the epxeriments are given in the text.

| Digestion time | Degraded amino acid | | | Sequence | |
|---|---|---|---|---|---|
| | Edman cycle 1 | 2 | 3 | | |
| Zero | Phe | Val | Asp | $Phe_1$ | $-Val_2-Asp_3$ |
| 1 h | Glu | Gly | Asp | $Phe_1$ | $-Val_2-Asp_3$ |
| | Gly | Ala | Glu | $Val_{12} - Glu_{13}$ | $-Ala_{14}-Leu_{15}$(?) |
| | | | | $Val_{18} - Cys(O_3H)_{19}$ | $-Gly_{20}-Glu_{21}$ |
| | Phe | Val | Val | $Arg_{22} - Gly_{23}$ | $-Phe_{24}-Phe_{25}$ |
| | $Cys(O_3H)$ | | Phe | ? — ? | - ? -Val |
| 4 h | Glu | Gly | Asp | $Phe_1$ | $-Val_2-Asp_3$ |
| | Phe | Ala | Glu | $Val_{12} - Glu_{13}$ | $-Ala_{14}-Leu_{15}$ |
| | $Cys(O_3H)$ | | Val | $Val_{18} - Cys(O_3H)_{19}$ | $-Gly_{20}-Glu_{21}$ |
| | | Leu | Leu | $Arg_{22} - Gly_{23}$(?) | $-Phe_{24}-Phe_{25}$ |
| | | Phe | Phe | ? — ? | -Leu-Val |
| 10 h | Glu | Gly | Asp | $Phe_1$ | $-Val_2-Asp_3$ |
| | Gly | Ala | Glu | $Val_{12} - Glu_{13}$ | $-Ala_{14}-Leu_{15}$ |
| | Phe | Val | Val | $Val_{18} - Cys(O_3H)_{19}$ | $-Gly_{20}-Glu_{21}$ |
| | $Cys(O_3H)$ | Phe | Leu | $Arg_{22} - Gly_{23}$ | $-Phe_{24}-Phe_{25}$ |
| | | | Phe | ? — ? | - ? -Val |
| 24 h | Glu | Gly | Asp | $Phe_1$ | $-Val_2-Asp_3$ |
| | Gly | Ala | Glu | $Val_{12} - Glu_{13}$ | $-Ala_{14}-Leu_{15}$ |
| | Tyr | Val | Val | $Leu_{15} - Tyr_{16}$ | $-Leu_{17}-Val_{18}$ |
| | Phe | Leu | Leu | $Val_{18} - Cys(O_3H)_{19}$ | $-Gly_{20}-Glu_{21}$ |
| | $Cys(O_3H)$ | Phe | Phe | $Arg_{22} - Gly_{23}$ | $-Phe_{24}-Phe_{25}$ |

enzyme are in good agreement with those previously reported using other methods [5,7]. Table II summarizes results of Edman degradation of insulin B chain following incubation with neutrophile elastase for different time intervals. After 1 h incubation, two main splits-$Val_{12}$-$Glu_{13}$ and $Val_{18}$-Cys $(O_3H)_{19}$ were observed. A sedoncary cleavage between Arg-22 and Gly-23 could be detected after 10 h incubation. Supporting evidence for these splits was obtained from experiments with carboxypeptidases Y and B, as well as by the identification of the corresponding phenylthiohydantoin-amino acids. A fourth split, $Leu_{15}$-$Tyr_{16}$, was detected after 24 h incubation.

The data suggest that although the pancreatic and neutrophile elastases have similar main specificities towards nonpolar aliphatic amino acids, the two enzymes seem to differ in their preference to the amino acid located at the $P_1$ position, as well as in their secondary specificities. Thus, the neutrophile enzyme cleaved mainly valyl peptide bonds ($Val_{12}$-$Glu_{13}$, $Val_{18}Cys(O_3H)_{19}$,

TABLE III

RESULTS OF EDMAN DEGRADATION OF OXIDIZED INSULIN B CHAIN AFTER INCUBATION WITH NEUTROPHILE CHYMOTRYPSIN-LIKE PROTEASE

Arrows indicate the cleaved bond. Details of the experiments are given in the text.

| Digestion time | Degraded amino acid | | | Sequence |
|---|---|---|---|---|
| | Edman cycle | | | |
| | 1 | 2 | 3 | |
| Zero | Phe | Val | Asp | $Phe_1$-$Val_2$-$Asp_3$ |
| 1 h | Tyr | Val | Asp | $Phe_1$-$Val_2$-$Asp_3$ |
| | | | | ↓ |
| | Phe | Leu | Val | $Leu_{15}$ — $Tyr_{16}$-$Leu_{17}$-$Val_{18}$ |
| 4 h | Tyr | Val | Asp | $Phe_1$-$Val_2$-$Asp_3$ |
| | | | | ↓ |
| | | Leu | Thr | $Leu_{15}$ — $Tyr_{16}$-$Leu_{17}$-$Val_{18}$ |
| | | | | ↓ |
| | Phex2 | Tyr | Val | $Phe_{24}$ — $Phe_{25}$-$Tyr_{26}$-$Thr_{27}$ |
| 10 h | Phex2 | Val | Asp | $Phe_1$-$Val_2$-$Asp_3$ |
| | Tyr | Tyr | Thr | ↓ |
| | Val | Leu | Val | $Leu_{15}$ — $Tyr_{16}$-$Leu_{17}$-$Val_{18}$ |
| | | | $Cys(O_3H)$ | ↓ |
| | | | Ala | $Phe_{24}$ — $Phe_{25}$-$Tyr_{26}$-$Thr_{27}$ |
| | | | | ↓ |
| | | | Glu | ? — Val-?-Ala |
| 24 h | Val | Glu | Asp | $Phe_1$-$Val_2$-$Asp_3$ |
| | | Val | Thr | ↓ |
| | Tyr | Leu | Gly | $Leu_{15}$ — $Tyr_{16}$-$Leu_{17}$-$Val_{18}$ |
| | | | | ↓ |
| | Phex2 | Tyr | Ala | $Phe_{24}$ — $Phe_{25}$-$Tyr_{26}$-$Thr_{28}$ |
| | | | Val | |
| | | | | ↓ ↓ $Cys(O_3H)_{19}$-$Gly_{20}$ |
| | | | Leu | $Leu_{(11\ or/and\ 17)}$ — $Val_{(12\ or\ 18)}$ |
| | | | | $Glu_{13}$-$Ala_{14}$ |

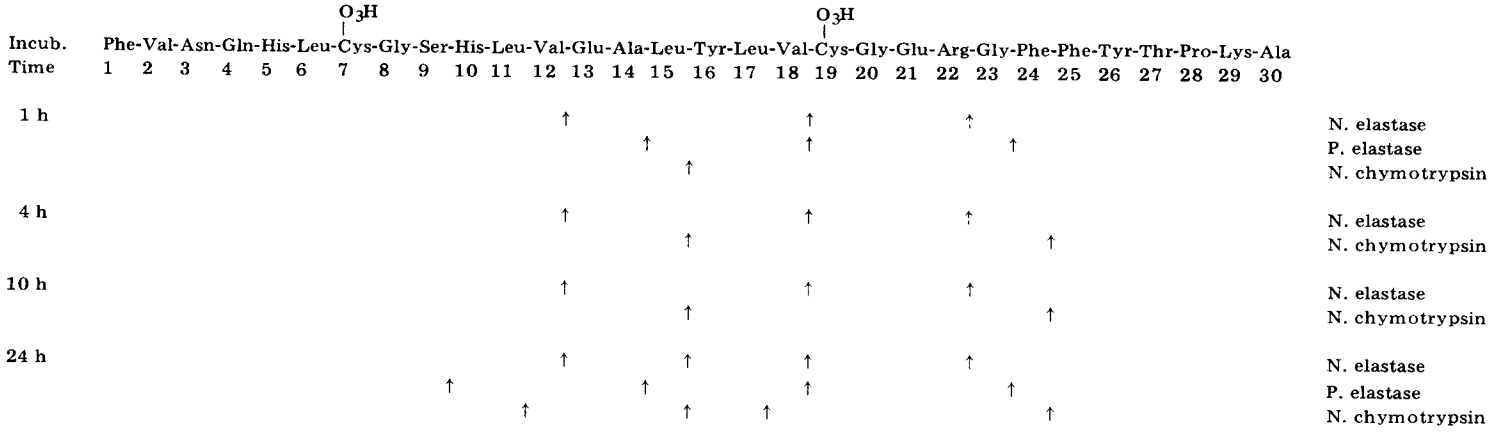


Fig. 1. The peptide bond specificities of porcine pancreatic, neutrophile elastase and neutrophile chymotrypsin-like protease. Full line arrows indicate major cleavage, while the dashed line arrows indicate a slow cleaved peptide bond.

see Table II) as compared with $Ala_{14}$-$Leu_{15}$ and $Val_{18}$-Cys $(O_3H)_{19}$ in the case of pancreatic elastase (see Table I).

We did not detect the split $Ala_{14}$-$Leu_{15}$ reported by Blow [8]. The discrepancies between the results could possibly be attributed to the fact that the enzymes preparations were obtained from different sources. (neutrophiles or spleen cells). Such a view is in accord with the data of Powers et al. [15] who reported that the neutrophile elastase reacted more slowly with the tetrapeptide chloromethylketone Ac-Ala-Ala-Pro-Ala$CH_2Cl$ than pancreatic elastase.

The secondary cleavage at the $Leu_{15}$-$Tyr_{16}$ bond observed after 24 h incubation could be possibly due to contamination of the elastase with chymotrypsin-like protease; this does not seem very likely however, since no chymotrypsin-like protease contaminations were detected by acrylamide gel electrophoresis using both protein and activity staining [3,11]. Furthermore, the neutrophile elastase has been shown to cleave peptide bonds near leucine in synthetic substates [4].

The digestion of the insulin B chain by neutrophile chymotrypsin-like protease is summarized in Table III. After 1 h incubation only one bond. $Leu_{15}$-$Tyr_{15}$, was cleaved. A secondary split, $phe_{24}$-$phe_{25}$, was observed after 4 h. On prolonged incubation (24 h), additional leucyl peptide bonds (Leu-11, Leu-17) were cleaved. These results suggest that the bonds most susceptible to hydrolysis by chymotrypsin-like protease are $Leu_{15}$-$Tyr_{16}$ and $Phe_{24}$-$Phe_{25}$. These conclusions are in agreement with the model peptide studies of Zimmerman and Ashe [4] who reported the hydrolysis of leucyl and phenylanyl peptide bonds by neutrophile chymotrypsin-like protease. In this context it is of interest to note that the specificities of chymotrypsin-like protease reported in this communication are in several respects similar to those of Cathepsin-G [9], claimed by Starkey and Barrett to be immunologically identical to the neutrophile chymotrypsin-like protease [16].

In conclusion, the specificity of human neutrophile elastase, using oxidized insulin B chain as substrate, was found to differ from that of the porcine pancreatic enzyme. Human neutrophile chymotrypsin-like protease was found to cleave mostly bonds involving leucine and phenylanine. Fig. 1 summarizes these findings.

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