# SPECIFICITY OF NITRATED α- AND δ-CHYMOTRYPSIN

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## 1. Introduction

The nitration of two tyrosyl residues in α-chymotrypsin affects the binding of low molecular ligands to the enzyme [1]. To explore whether this modification alters also the specificity towards polypeptides, our present study was aimed at the comparison of the specificity of cleavage of the chain B of insulin by α- and δ-chymotrypsins and their nitrated forms.

The results obtained with chymotrypsins were compared with those obtained with its structural homologue, trypsin. In contrast with previous papers dealing with the nitrated heterogeneous trypsin [3,4], a homogeneous monomeric nitrated β-trypsin was isolated. Its properties differ partly from those observed previously.

## 2. Materials

Chymotrypsinogen A (5 × crystallized, code CGC), α-chymotrypsin (3 × crystallized, code CDI) and trypsin (2 × crystallized, code TRL) were obtained from Worthington; β-trypsin was purified, from crystallized trypsin, as described by Schroeder and Shaw [5] on a SE–Sephadex C-50 column. B-chain of insulin (oxidized form) was from Boehringer, Mannheim; α-casein and ovomucoïd were purchased from Sigma.

Tetranitromethane and 3-nitrotyrosine were Sigma products. *N*-Acetyl–L-tyrosine ethyl ester, *p*-tosyl–L-arginine methyl ester, *N*-benzoyl–L-arginine ethyl ester, *N*-benzoyl–L-tyrosine ethyl ester were from Sigma, *N*-α-acetyl–L-tryptophan methyl ester from the Cyclo Chemical Corporation, *p*-nitrophenyl-guanidino-benzoate from Merck. Sepharose 4B and Sephadex products were from Pharmacia.

δ-Chymotrypsin was prepared by the activation of chymotrypsinogen A by trypsin (molar enzyme : substrate ratio, 1 : 50). Trypsin was then removed by adsorption on a column of agarose–ovomucoïd [6]. δ-Chymotrypsin and its nitrated form contained 0.43% trypsin activity towards *p*-tosyl–L-arginine methyl ester in contrast with 0.03% for α-chymotrypsin. This residual tryptic activity was inhibited by *N*(*p*-nitrobenzyl-oxycarboxyl) arginine chloromethane [7]. This treatment allowed the residual tryptic activity to decrease to 0.03% whereas the chymotryptic activity towards *N*-benzoyl tyrosine ethyl ester was still 96% of the initial value.

## 3. Methods

### 3.1. *Nitration of proteins and identification of nitrated tyrosyl residues of β-trypsin*

Commercial preparations of chymotrypsinogen A and α-chymotrypsin were nitrated by tetranitromethane as described by Riordan et al. and Shlyapnikov [2,8]. Homogeneity of the purified proteins was tested by chromatography on Sephadex G-100 and gel electrophoresis as reported by Mitchell [9].

Nitration of β-trypsin was performed with a tetranitromethane/enzyme ratio of 40 (four-fold excess on tyrosine). The monomeric form of the nitrated enzyme was obtained by filtration on a Sephadex G-75 column (2.6 × 30 cm) in HCl $10^{-3}$ N at 4°C. Its homogeneity was tested as for chymotrypsin.

For the nitrated proteins, the molar absorbances

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$A_M$ at 280 nm were 5.7 × $10^4$ $M^{-1}$ . $cm^{-1}$ for nitrated α- and δ-chymotrypsins and 5.4 × $10^4$ $M^{-1}$ . $cm^{-1}$ for nitrated β-trypsin on the basis of the protein concentration determined by amino acid analysis. The number of 3-nitrotyrosines was obtained by spectrophotometry at 428 nm ($A_M$ 4.1 × $10^3$ $M^{-1}$ . $cm^{-1}$) [8].

Amino acid analyses were performed according to Spackmann et al. [12] with a Beckman Multichrom B amino acid analyser using a single column procedure [13]. For specific analysis of tyrosine and 3-nitrotyrosine, hydrolysis was carried out for 15 h in presence of 0.2% phenol; 3-nitrotyrosine emerged next to phenylalanine as reported by Sokolovsky et al. [14].

Identification of nitrated tyrosyl residues of β-trypsin was obtained by peptide maps from hydrolysate of nitrated sulfitolyzed β-trypsin essentially as by Holeysovsky et al. [4].

### 3.2. *Enzymic activities*

The esterase activities of α- and δ-chymotrypsins and their nitrated forms were determined with *N*-acetyl–L-tyrosine ethyl ester as substrates as previously reported [1]. The esterase activities of β-trypsin and its monomeric nitrated form towards *p*-tosyl–L-arginine methyl ester were measured spectrophotometrically [15] and towards *N*-benzoyl–L-arginine ethyl ester by titration of liberated protons with 0.05 N NaOH using a pH-stat, at pH 8.0 and 25°C.

The proteolytic activities were measured, at pH 7.6 and 37°C, using α-casein as substrate (0.3% and 1% in NaCl, 0.1 M, $CaCl_2$ 0.02 M) on a pH-stat.

Comparison of specificity between nitrated and native forms of α- and δ-chymotrypsins on the one hand, and of β-trypsin on the other, was made by determinations of the cleaved bonds of oxidized B-chain of insulin.

## 4. Results

### 4.1. *Characterization of nitrated proteins*

Nitrated α-chymotrypsin and nitrated δ-chymotrypsin (obtained by activation of substituted chymotrypsinogen A) contained two 3-nitrotyrosyl residues (Tyr 146 and Tyr 171) and two unmodified tyrosines

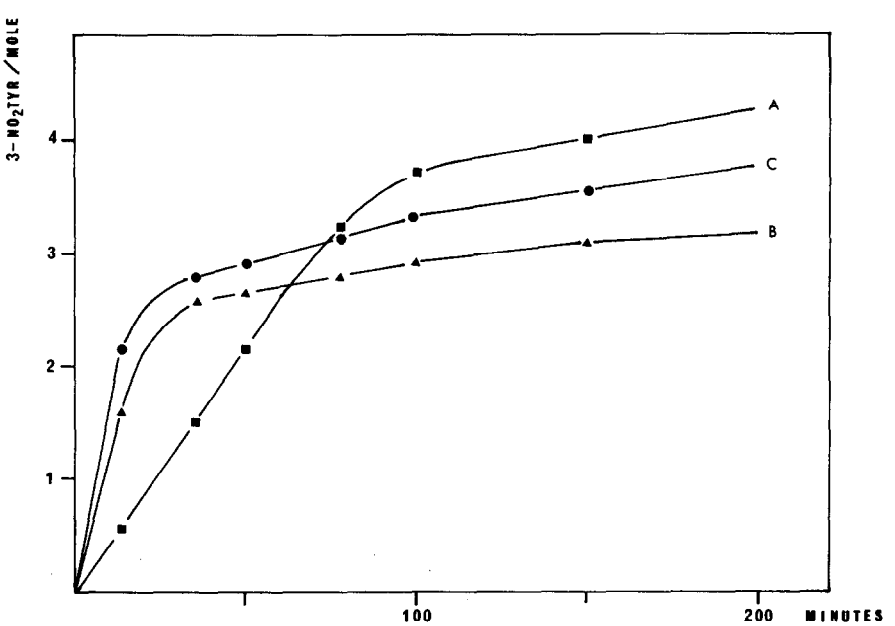


Fig.1. Kinetics of nitration of β-trypsin with (B) and without (C) benzamidine and of crystalline trypsin (A) with benzamidine ($10^{-3}$ M).

per molecule in accordance with previous data [1].

In the time course of nitration of β-trypsin for the same time of reaction (90 min), the same number of nitrated tyrosines was obtained for β-trypsin and crystalline trypsin with and without benzamidine as inhibitor (fig.1).

After purification of the monomerous form of nitrated β-trypsin no loss of tyrosyl residues was observed: the sum of tyrosines and 3-nitrotyrosines was ten (table 1). Peptide maps showed that tyrosine residues 137, 28, 48 and 11 were nitrated, with the residues 137 and 28 the most reactive (table 2).

Table 1
3-Nitrotyrosine content of nitrated β-trypsin

| | Benzamidine | 3-Nitrotyrosines/mol | | | Tyrosine/mol | Phenylalanine/mol |
|---|---|---|---|---|---|---|
| | | a | b | c | c | c |
| Crystalline trypsin | (+) | 4.8 | 4.8 | 4.4 | 5.5 | 3.2 |
| β-Trypsin | (+) | 3.2 | 3.1 | 2.8 | 7.0 | 3.0 |
| | (−) | 3.8 | 3.2 | d | d | d |

[a] Spectrophotometry at the end of the reaction
[b] Spectrophotometry after purification
[c] Amino acid analysis
[d] Not determined

Table 2
3-Nitrotyrosyl peptides from nitro β-trypsin

| Composition | Quantity (nmol) a | Relative content (%) | Total content of 3 $NO_2$Tyr-peptides (%) | Tyrosine Form | Tyrosine Sequence No. b |
|---|---|---|---|---|---|
| $^{7}$Ile–Val–Gly–Gly–$NO_2^{11}$Tyr | 37.0 | 34 | 5.5 | $NO_2$ | 11 |
| Ile–Val–Gly–Gly–Tyr | 73.0 | 66 | | Tyr | 11 |
| $^{21}$Gln–Val–Ser–Leu–Asn–Ser–Gly–$NO_2^{28}$Tyr | 34.2 | 69.5 | 28.3 | $NO_2$ | 28 |
| $^{25}$Asn–Ser–Gly–$NO_2^{28}$Tyr | 155.4 | | | | |
| Gln–Val–Ser–Leu–Asn–Ser–Gly–Tyr | 16.8 | 30.5 | | Tyr | 28 |
| Asn–Ser–Gly–Tyr | 66.6 | | | | |
| $^{41}$Val–Val–Ser–Ala–His–Cys–$NO_2^{48}$Tyr | 80.7 | 74 | 11.0 | $NO_2$ | 48 |
| Val–Val–Ser–Ala–Ala–His–Cys–Tyr | 28.3 | 26 | | Tyr | 48 |
| $^{132}$Ser–Ser–Gly–Thr–Ser–$NO_2^{137}$Tyr | 362.6 | 98 | 54.1 | $NO_2$ | 137 |
| Ser–Ser–Gly–Thr–Ser–Thr | 7.4 | 2 | | Tyr | 137 |

[a] Isolated peptide quantity was estimated from amino acid analyses. Loss due to purification by paper techniques were of 75%
[b] Sequence numbers were from trypsinogen sequence

### 4.2. *Enzymic activities*

The esterasic activities of the nitrated forms of both chymotrypsin and trypsin on synthetic substrate were essentially identical to that of the native proteins. Only a 25–30% decrease of the proteolysis of β-casein could be observed between nitrated and native forms of α- and δ-chymotrypsins [1]. The same two bonds of β-chain of insulin (Arg 22 and Lys 30) were hydrolyzed by both β-trypsin and its nitrated form (table 3). An observed decrease of 25% activity on this substrate in the case of nitrated trypsin can be explained by fluctuation in the active site titration of nitrated β-trypsin by the *p*-nitrophenyl guanidino benzoate method [16]. On the other hand, a quantitative alteration could be observed between the number of cleaved bonds by nitrated and native α-chymotrypsin: one additional bond Phe 24–Phe 25 was hydrolyzed by the nitrated form. The same bond is cleaved by nitrated or native δ-chymotrypsin (fig.2 and table 4).

## 5. Discussion

The previously described change of $K_m$ observed in the case of nitrated α-chymotrypsin seems to indicate a modification in the region of the binding site of the enzyme [1]. The present study shows that the nitration of α-chymotrypsin modifies its tertiary structure in such a way that it cleaves an additional peptidic bond in B-chain of insulin. This same bond is split by nitrated and native δ-chymotrypsin but not by the native form of α-chymotrypsin. The comparison of the residues which correspond to subsites $S_1$, $S_2$ and $S_3$ postulated by Segal [17]

Table 3
Yields of peptides recovered from the hydrolysate of the B-chain of insulin by β-trypsin and nitrated β-trypsin

| Enzymes | $Gly_{23}$–$Lys_{30}$ | | $Ala_{31}$ | |
|---|---|---|---|---|
| | Quantity | Rate | Quantity | Rate |
| β-Trypsin | 41 nmol | 43.6% | 43.6 nmol | 46.3% |
| Nitrated β-trypsin | 31 nmol | 33% | 32 nmol | 34% |

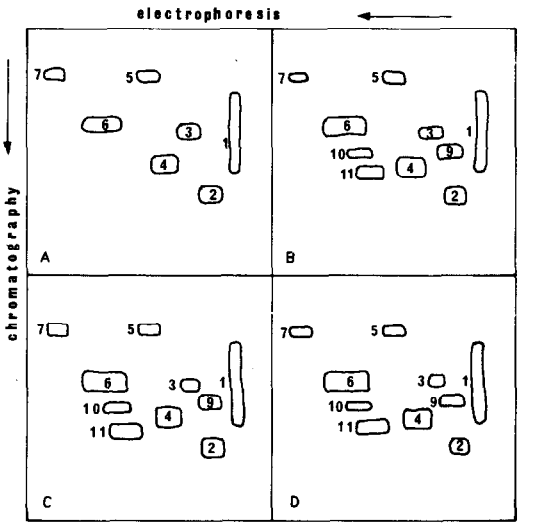


Fig.2. Peptide maps of hydrolysates of B-chain of insulin. (A) α-Chymotrypsin. (B) Nitrated α-chymotrypsin. (C) δ-Chymotrypsin. (D) Nitrated δ-chymotrypsin. Enzyme/substrate ratio 1/6000 in ammonium bicarbonate 0.01 M, 8 h, 37°C. Electrophoresis, pH 3.7 (pyridine/acetic acid/water, 1:10:289, by vol; 80 V/cm, 60 min). Chromatography (butanol/acetic acid/pyridine/water, 15:3:10:12, by vol.).

Table 4
Rate of hydrolysis of peptide bonds in the B-chain of insulin (%)

| | α-chymotrypsin | Nitrated α-chymotrypsin | δ-chymotrypsin | Nitrated α-chymotrypsin |
|---|---|---|---|---|
| $Phe_1$ – $Val_2$ | 5 | 3 | 3 | 8 |
| $Tyr_{16}$ – $Leu_{17}$ | 62 | 44 | 89 | 83 |
| $Phe_{24}$ – $Phe_{25}$ | – | 11 | 21 | 25 |
| $Phe_{25}$ – $Tyr_{26}$ | 95 | 86 | 75 | 64 |
| $Tyr_{26}$ – $Thr_{27}$ | 5 | 3 | 3 | 3 |

Table 5
Subsites of the binding site of chymotrypsin and corresponding sequences of the B-chain of insulin

| $S_3$ | $S_2$ | $S_1$ | Hydrolyzed bond |
|---|---|---|---|
| $P_3$ | $P_2$ | $P_1$ | $-X$ |
| $^{14}$Ala ——— | $^{15}$Leu ——— | $^{16}$Tyr | $-$ $^{17}$Leu |
| $^{23}$Gly ——— | $^{24}$Phe ——— | $^{25}$Phe | $-$ $^{26}$Thr |
| $^{22}$Arg ——— | $^{23}$Gly ——— | $^{24}$Phe | $-$ $^{25}$Phe |

around the bonds split by nitrated α-chymotrypsin (table 5) indicates that in the additional cleavage site a polar residue (Arg) occupies the subsite $S_3$. It can be therefore advanced that nitration of α-chymotrypsin affected the structure of α-chymotrypsin in such a way that subsite $S_3$ is modified. The only difference between α- and δ-chymotrypsin is an opening of the peptide bond adjacent to Tyr 146. The limited change of specificity which approaches nitrated α-chymotrypsin to δ-chymotrypsin can be attributed to the nitration of this residue.

The nitration of β-trypsin did not change its esterasic or proteolytic activity. As regards the tyrosyl residues nitrated, our results confirmed those published by Holeysovsky et al. [4]. On the other hand, after purification of the monomerous form of nitrated β-trypsin we observed neither the drastic decrease of proteolytic activity (4% initial activity) found by Vincent and Ladzunski [18] for nitrated crystalline trypsin, nor the protective effect of benzamidine on the nitration of one tyrosyl residue. Our results seem to fit with the X-ray studies of Bode and Schwager [19] who did not observe any change in the three-dimensional structure of trypsin caused by the interaction with benzamidine: the only observed change was not that of the active site of the enzyme, but a deformation of the inhibitor to fit the enzyme.

The unambiguity of our results was also enhanced by the use of the homogeneous β-trypsin and by the isolation of the pure monomeric nitrated form.

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