LOCATION OF REACTIVE TYROSINE RESIDUES IN TRYPSIN

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1. Introduction

The results of our preliminary studies on the nitration of bovine trypsin and trypsinogen [1,2] by tetranitromethane have shown that – similarly to other proteins [3,4] – only certain tyrosine residues are nitrated in these two proteins. When analyzing the number of nitrated tyrosines we found that the sum of nitrated plus unmodified residues was always lower than the total number of tyrosines in the molecule of trypsin and that the tyrosine residue obviously had undergone destruction during the reaction. The esterase activity of nitrotrypsin, however, was essentially the same. We decided therefore to isolate from trypsin in parallel experiments all peptides containing tyrosine, both nitrated and unmodified, in order to obtain information on all the ten residues present in the molecule. Our results are discussed from the aspect of similarities existing between trypsin and chymotrypsin [5,6] as suggested by the results of analogous experiments performed on chymotrypsin in this laboratory [7].

While this study was in its final stage, a report on similar experiments of American authors appeared [8]. These authors were able to pick up 5 nitrated tyrosine residues whose positions in the molecule are identical to those determined by us. The authors do not present data on unmodified tyrosine residues.

2. Materials and methods

2.1. *Trypsinogen* was prepared from "precipitate A" described by Northrop [9] after the removal of chymotrypsinogen (Léčiva, Prague) by crystallization from dilute ethanol according to Balls [10]. The procedure for recrystallization was slightly modified and performed in the presence of 0.5% of soy-bean trypsin inhibitor (the weight of the inhibitor added was calculated with respect to the assumed content of dry trypsinogen). Recrystallized trypsin was dissolved in 0.001 N-HCl, the solution was adjusted to pH 3.0 and lyophilized.

2.2. *The solution* of trypsin (1%) was prepared by autoactivation of lyophilized trypsinogen at pH 8.0 (0.02 M Tris-HCl + 0.05 M $CaCl_2$). The final activity of trypsin (toward benzoyl-arginine ethyl ester, determined by titration with 0.1N NaOH at pH 8.0) was 47.4 μeq./mg/min.

2.3. *Tetranitromethane*, a pure reagent grade product of Lachema was redistilled under reduced pressure in a stream of nitrogen and used as a 10% solution in ethanol.

2.4. *The nitration* of 100 ml of 1% trypsin solution was performed according to Riordan and co-workers [3,4] at room temperature by the addition of 2 ml of the tetranitromethane solution, i.e. of an approximately 4-fold molar excess with respect to the number of tyrosine residues. The mixture was stirred 90 minutes in a stoppered flask and then acidified with acetic acid to pH approximately 4. Nitrated trypsin was isolated by gel filtration of the reaction mixture through a Sephadex G-25 column (4.5 × 70 cm) equilibrated with 0.25% acetic acid. The fractions containing nitrotrypsin were pooled and concentrated to a total volume of 40 ml.

2.5. *The number of nitrated tyrosine residues* in trypsin was determined in the solution after gel filtration. The concentration of the nitrated protein was deter-

mined by gravimetric analysis of an aliquot of the solution, the content of tyrosine and nitrotyrosine by measuring the difference absorption at 295 and 430 nm of solutions in 0.1N NaOH and in a buffer at pH 3.0 (0.1M citrate + 0.05M NaCl). The difference extinction coefficient found for tyrosine was ΔE = 2,305 M^{-1} cm^{-1}, for nitrotyrosine ΔE_{430} = 4,700M^{-1} cm^{-1}. Since one tyrosine residue remains masked during spectrophotometric titration (cf. [11]) we measured the time dependence of the spectrophotometric determination of tyrosine in native trypsin and found that 8.7 tyrosine residues out of the possible ten can be picked up when the measurement is made 15 minutes after the addition of 0.1N NaOH to the solution. Therefore the difference absorption was measured always 15 minutes after the addition of NaOH.

2.6. *Amino acid analysis* was performed by the method of Spackman and co-workers [12] in an analyzer of Czechoslovak make *. For the calculation of the nitrotyrosine content, the ninhydrin color value determined with an authentic sample of 3-nitrotyrosine was used. This color value was essentially identical with the value for phenylalanine.

2.7. *S-sulfonation* of the concentrated solution of nitrotrypsin was effected according to Pechère and coworkers [13] after diluting the solution to 65 ml. S-sulfonitrotrypsin was isolated by two-fold passage of the reaction mixture through a Sephadex G-25 column equilibrated with 0.2M$(NH_4)_2CO_3$ (1st run) and with water adjusted to pH 9 with ammonia (2nd run). The protein-containing solution was rotary evaporated to 40 ml at 40°C.

2.8. *Combined enzymatic hydrolysis* of the trypsin derivative was performed as follows. To the solution of the derivative (50 ml) kept at 37°C and at pH 8.5 in the autotitrator were added (in amounts corresponding to a molar enzyme to substrate ratio of 1 : 100) trypsin, 30 minutes later chymotrypsin and 60 minutes thereafter again trypsin. The reaction mixture was allowed to stand 20 hours at room temperature. After this period the mixture was acidified to pH 2.0 and pepsin was added. After 3 hours the addition of pepsin was repeated and the mixture was set aside for 65 hours at room temperature. The precipitate which had formed during the acidification of the mixture (before the addition of pepsin) did not dissolve and was centrifuged off.

2.9. *Fractionation of peptides*

2.9.1. Ion-exchange chromatography. The solution of the hydrolysate was adjusted to pH 2.9 and chromatographed on a Dowex 50 X 2 column (200–400 mesh, 4.5 X 70 cm) at room temperature using elution gradients developed with a series of pyridine acetate buffers at pH 3.0–8.5 and molarity 0.1–2.0. The volume of the mixing chamber was 500 ml. A flow rate of 120 ml/h was maintained and 20-ml fractions were collected. The absorbance of the effluent fractions (undiluted) was measured at 381 nm against water as a blank and evaluated colorimetrically according to Lowry [14] using an 0.1–ml aliquot.

2.9.2. Paper techniques. The peptides were fractionated by descending electrophoresis at pH 5.6 [15] by high-voltage electrophoresis at pH 1.9 [16], or by descending chromatography in the system butanol-acetic acid-pyridine-water (15 : 3 : 10 : 12) [17]. The peptides were detected by ninhydrin, ultraviolet absorption, and by the solution of diazo-sulfanilic acid.

2.10. *Identification* of peptides was achieved by the determination of their quantitative amino acid composition and N-terminal end group by the Dansyl technique [18].

3. Results and discussion

Isolated peptides are summarized in table 1.

The values for the number of nitrated tyrosine residues in trypsin determined both spectrophotometrically and by amino acid analysis (cf. [1]) are lower than the number formed in peptides. These residues obviously undergo destruction during nitration since the sum of analytical values for tyrosine and for nitrotyrosine was always lower than 10. This phenomenon has been observed also in another laboratory [19]. The analytical values for unmodified tyrosine were in good agreement with the results obtained with peptides. The determined number of reactive tyro-

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Table 1
Tyrosine-containing peptides isolated from nitrotrypsin.

Working designation	Composition [a]	Quantity [b] in μmoles		Relative content (%)	Tyrosine	
		Isolated	Original		Form	Serial number [a]
	7 11					
19E	Ile-Val-Gly-Gly-NO_2Tyr	0.288	0.57	100	NO_2	11
21D	Ile-Val-Gly-Gly-NO_2Tyr	1.47	2.94			
	12 20					
13C1	Thr-Cys-Gly-Ala-Asn-Thr-Val-Pro-NO_2Tyr Thr-Cys-Gly-Ala-Asn-Thr-Val-Pro-Tyr	0.057	0.68	60 [c] 40 [c]	NO_2 Tyr	20
	25 28					
18C1	Asn-Ser-Gly-NO_2Tyr	0.75	3.00			
18E3	Asp-Ser-Gly-NO_2Tyr	0.63	2.52	100	NO_2	28
	27 30					
31A2	Gly-NO_2Tyr-His-Phe	0.06	0.24			
	44 48					
19B2	Ala-Ala-His-Cys-NO_2Tyr	0.17	0.68			
	49					
32A2	Ala-Ala-His-Cys-NO_2Tyr-Lys	0.156	0.62	89.3	NO_2	
	41 48					48
5aC2	Val-Val-Ser-Ala-Ala-His-Cys-NO_2Tyr (85%) [c] Val-Val-Ser-Ala-Ala-His-Cys-Tyr (15%) [c]	0.82	2.78 0.49	10.7	Tyr	
	76 83					
31A2	Ser-Ile-Val-His-Pro-Ser-Tyr-Asn	0.177	0.70			
31B3	Ser-Ile-Val-His-Pro-Ser-Tyr	0.77	3.08	75.2	Tyr	
32B1	Ser-Ile-Val-His-Pro-Ser-Tyr	0.50	2.0			
	87					
21C2	Ser-Ile-Val-His-Pro-Ser-Tyr-Asn-Ser-Asn-Thr-Leu	0.07	0.28			82
29B1	Ser-Ile-Val-His-Pro-Ser-NO_2Tyr	0.087	0.35	24.8	NO_2	
30C	Ser-Ile-Val-His-Pro-Ser-NO_2Tyr	0.825	1.65			
	132 137					
15B	Ser-Ser-Gly-Thr-Ser-NO_2Tyr	0.93	5.58	100	NO_2	137
15C2	Ser-Ser-Gly-Thr-Ser-NO_2Tyr	0.52	6.24			
	156 165					
12A4A	Ser-Ala-Tyr-Pro-Gly-Gln-Ile-Thr-Ser-Asn	0.52	4.1			
12A5A	Ser-Ala-Tyr-Pro-Gly-Gln-Ile-Thr-Ser-Asn	0.186	1.5	100	Tyr	158
12A6A	Ser-Ala-Tyr-Pro-Gly-Gln-Ile-Thr-Ser	0.43	3.45			
20D4	Ser-Ala-Tyr	0.08	0.32			
	168 171					
7C2	Cys-Ala-Gly-NO_2Tyr	0.384	1.53			
7D1	Cys-Ala-Gly-NO_2Tyr	0.50	2.0	61.7	NO_2	
	169 171					
5aC3	Ala-Gly-NO_2Tyr	0.10	0.40			
	168 172					171
12D3	Cys-Ala-Gly-NO_2Tyr-Leu (52%) [c] Cys-Ala-Gly-Tyr-Leu (48%) [c]	0.15	0.62 0.58			
8A3	Cys-Ala-Gly-Tyr	0.395	1.58	38.3	Tyr	
	169 171					
8A4	Ala-Gly-Tyr	0.168	0.67			
	211 212					
9B2	Val-Tyr	0.05	0.33	100	Tyr	212
	213 218					
20C	Thr-Lys-Val-Cys-Asn-Tyr	2.65	15.9	100	Tyr	218

a) The numbering system is that used for trypsinogen [23].
b) The quantity of the peptide isolated was calculated from its amino acid analysis, the quantity of the peptide originally present in the digest was assessed assuming 50% losses of material during the fractionation by paper techniques.
c) Determined by amino acid analysis of the peptide which contained both tyrosine and nitrotyrosine.

sines corresponds to the six readily ionizable tyrosines found by other authors [11, 20, 21] in experiments with spectrophotometric titration of trypsin, and also to the six tyrosine residues reactive toward cyanuric fluoride [22].

The esterase activity of trypsin in the reaction mixture decreased during nitration to 70%, nitrotrypsin isolated by gel filtration, however, showed the same full activity as a control sample. It is likely that nitroform formed during nitration partly inhibits trypsin. The potential activity of nitrotrypsinogen, however, was considerably lower (53%).

The disulfide bonds had been cleaved by S-sulfonation, the ultraviolet spectrum of S-sulfo-nitrotrypsin was found to be the same as the spectrum of nitrotrypsin. Therefore the nitro groups had not been reduced.

For the enzymatic hydrolysis of S-sulfo-nitrotrypsin three different enzymes were used in order that possibly smallest peptides be obtained. We did not observe any difference in the susceptibility to cleavage of bonds formed by nitrotyrosine and bonds involving unmodified tyrosine residues. It should be noted that the

$$\overset{158}{\text{Tyr}} - \overset{159}{\text{Pro}}$$

bond was not cleaved while the bond

$$\overset{137}{NO_2\text{Tyr}} - \overset{138}{\text{Pro}}$$

was; this phenomenon, however, has been observed earlier with unmodified peptides [23].

We were able to isolate from the hydrolysate all peptides containing tyrosine, either nitrated or unmodified, and from the yields of these peptides their assumed content in the parent hydrolysate was calculated (cf. table 1). From these values we deduced the relative amount of nitrated and unmodified tyrosine for all the 10 residues in trypsin. This comparison shows that after full nitration of trypsin tyrosine residues [23] Nos. 11, 28, 48 and 137 were nitrated completely, residues Nos. 20 and 171 to approximately 50%, tyrosine No. 82 to a low degree while residues Nos. 158, 212 and 218 remained intact. These results are in agreement with the data reported by American authors [8], in spite of the fact that these authors investigated trypsin nitrated to a lower degree. Nevertheless, their data on the esterase activity of nitrotrypsin and nitrotrypsinogen agree with our findings.

In our opinion, one is justified to assume that the tyrosine residues which react with tetranitromethane are identical with these which are reactive toward cyanuric fluoride [22] and also with the residues which undergo rapid ionization in alkaline media [11, 20, 21]. The fact that the enzymatic activity is retained after the reaction leads us to postulate that these reactive residues are not involved in the function of the enzyme. One of these residues is tyrosine No. 48 which has been hypothesized by Japanese authors [21, 22] to form a hydrogen bond with the aspartic acid residue No. 182 and thus to participate directly on the mechanism of the cleavage. This postulate is in disagreement with the conclusions drawn by us in this study and this discrepancy will no doubt require more detailed examination.

The results of our preliminary experiments with the nitration of trypsinogen [1] show that in this protein is nitrated the tyrosine residue No. 82 which in trypsin is nitrated only to a low degree (to 25%). This situation shows a striking analogy to chymotrypsin in which the analogous tyrosine residue, i.e. No. 94, is not nitrated while the same residue is completely nitrated in chymotrypsinogen [7]. This seems to indicate that during the activation of nitrotrypsinogen nitrotrypsin is formed and that its tyrosine residue No. 82 is considerably more nitrated than the same residue in trypsin nitrated in native state. The activity of such an enzyme should then logically decrease, as we in fact observed. On the other hand, from the aspect of nitration there is a significant difference between chymotrypsin and trypsin in residue No. 158 which in trypsin is completely resistant to nitration while the analogous residue (No. 171) in both chymotrypsinogen and chymotrypsin is nitrated [7]. In our concept of the three-dimensional structure of the molecule of trypsin [6] this difference can be accounted for exclusively by a change in the orientation of the tyrosine residue, i.e. inward the molecule, which is not paralleled by a considerable change in the conformation of the peptide chain. Analogically, the same may hold true for residue No. 82. The complete elucidation of this problem will require also a complete analysis of nitrotrypsinogen and the characterization of all ten tyrosine residues. We intend to perform such a study in the near future even though we are well aware of the fact that it will be somewhat time-consuming.

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