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Isolation of Nitrotyrosine-Containing Peptides by Using an Insoluble-Antibody Column

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Antibodies to nitrotyrosine were prepared in goats or rabbits by injecting a nitrotyrosine–protein conjugate. The antibodies were purified by using a protein that had been nitrated with tetranitromethane. These antibodies were used to isolate nitrotyrosine-containing peptides from nitrated lysozyme. The nitrotyrosine-containing peptides were thus purified (55% yield) in one step and the positions of nitration in lysozyme were found to be at tyrosine-20 and tyrosine-23. This method is of general applicability for the determination of the position of nitrotyrosine in proteins.

Nitration of proteins with tetranitromethane, introduced by Sokolovsky, Riordan & Vallee (1966), has become a widespread technique for the study of structure–function relationships in proteins (for review, see Vallee & Riordan, 1969). In such studies it is essential to determine the position of the modified tyrosine residues in the amino acid sequence of the protein. This determination of location requires the isolation of peptides containing nitrotyrosine from the enzymic digest of the nitrated protein. In many cases this requires many steps and presents some difficulties.

Antibodies to a modified amino acid residue may be used as a specific means for the one-step isolation of peptides containing such a modification (Wilchek, Bocchini, Becker & Givol, 1971). Wilchek *et al.* (1971) isolated modified peptides containing either a 2,4-dinitrophenyl group or an azobenzene arsonate group. In the present paper we report the use of antibodies for the isolation of peptides containing 3-nitrotyrosine. The example of nitrolysozyme (Atassi & Habeeb, 1969) was chosen to illustrate this case.

MATERIALS AND METHODS

Antigens and antibodies. A conjugate of bovine serum albumin and nitrotyrosine was prepared by coupling 3-nitrotyrosine (325mg) to the albumin (200mg) with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (300mg) in 16ml of 0.15M-NaCl–0.01M-sodium phosphate, pH7.4, as described by Halloram & Parker (1966). The solution was dialysed against 0.1M-NH_4HCO_3 and freeze-dried. Amino acid analysis showed that the nitrotyrosyl-albumin contained 29mol of nitrotyrosine/mol of albumin. Nitro-(serum albumin) was prepared by nitration of bovine serum albumin with tetranitromethane by the method of Sokolovsky *et al.* (1966) by using a 100-fold molar excess of tetranitromethane over protein. The product contained 6.9mol of nitrotyrosine and no tyrosine/mol of albumin. Rabbit γ-globulin was similarly nitrated, and the resulting nitro-γ-globulin contained (by amino acid analysis) 13mol of nitrotyrosine and 30mol of tyrosine/mol of γ-globulin. Antisera were raised by injecting rabbits or a goat with 1mg of antigen (serum albumin derivatives emulsified with complete Freund's adjuvant) in multiple intradermal sites. The serum was tested for antibodies with the nitro-γ-globulin. In our experience very small amounts of anti-nitrotyrosyl antibodies were produced on injection of the nitro-(serum albumin). On the other hand good response was obtained with nitrotyrosyl-(serum albumin). These antisera contained between 0.5–0.1mg of anti-nitrotyrosyl antibodies as determined by precipitin reaction with nitro-γ-globulin. Antibodies were isolated by adsorption of pooled antisera on a column of nitro-γ-globulin–Sepharose conjugate (Wilchek *et al.* 1971). The column was washed with 0.15M-NaCl until the E_{280} of the effluent was less than 0.1. The adsorbed antibodies were eluted from the column with 0.1M-acetic acid. The average yield of eluted antibodies was 0.8mg/ml of serum. The purified antibodies were dialysed against 0.15M-NaCl–0.01M-phosphate buffer, pH7.4, and stored at −20°C. Precipitin analysis of these antibodies showed that they were 70% precipitable by nitro-γ-globulin.

Nitration of lysozyme. Lysozyme was nitrated with tetranitromethane by using a 10-fold molar excess of tetranitromethane over lysozyme, as described by Atassi & Habeeb (1969). After reaction for 2h the nitrolysozyme was isolated on a Sephadex G-25 column (2cm × 60cm) that was equilibrated and run with 1M-NaCl–0.05M-tris–HCl buffer, pH8.2. The protein-containing fractions were combined, dialysed against 0.05M-NH_4HCO_3 and freeze-dried. The nitrolysozyme

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was reduced in 8M-urea–0.2M-tris–HCl buffer (pH 8.2)–0.1M-2-mercaptoethanol for 1h at 37°C, and alkylated with 0.15M-iodoacetate for 30min. The reduced and alkylated protein was dialysed against 0.1M-NH_4HCO_3 and digested with trypsin (1:50, w/w, ratio of enzyme to protein) for 3h at 37°C. The resulting peptide mixture was used in the experiment for isolation of nitrotyrosine-containing peptides.

Other methods. Coupling of antigens or antibodies to Sepharose was performed as described by Wilchek *et al.* (1971). Amino acid analyses were performed essentially as described by Moore & Stein (1963) by using a one-column system. Nitrotyrosine emerged after phenylalanine at 171min and the colour value of tyrosine was used to calculate its recovery. No corrections were made for destruction during acid hydrolysis (done in 6M-HCl for 24h at 110°C). *N*-Terminal residues of peptides were determined by the dansyl chloride method (Gray, 1968) and the DNS-amino acids were identified by t.l.c. on polyamide sheets (Woods & Wang, 1967). High-voltage paper electrophoresis on Whatman 3MM paper was done at pH 3.5 in pyridine acetate buffer. Peptides were located by dipping in 0.5% (w/v) ninhydrin in acetone. Peptides containing nitrotyrosine were detected as yellow peptides after exposure to NH_3 vapour. The content of nitrotyrosine was measured by its extinction at 381nm ($\epsilon = 2200$ litre·mol^{-1}·cm^{-1}) by using a Zeiss PMQII spectrophotometer. The E_{280} of lysozyme was taken as 2.4 for a 0.1% solution.

RESULTS

Table 1 gives the amino acid composition of the nitrolysozyme derivative. Of the three tyrosine residues of lysozyme only 1.7 were recovered as tyrosine and only 0.9 residue as nitrotyrosine/mol of lysozyme. On the other hand, spectral measurement indicated 1.6mol of nitrotyrosine/mol of nitrolysozyme. This discrepancy may be due either to incomplete recovery of nitrotyrosine on hydrolysis, or to some side-reaction of tetranitromethane with lysozyme that yields coloured derivatives other than nitrotyrosine.

The isolation of nitrotyrosine-containing peptides on the anti-nitrotyrosine–Sepharose column is depicted in Fig. 1. Most of the E_{280} (81%) emerged unretarded from the antibody–Sepharose column. The yellow nitrotyrosyl peptides were adsorbed by the column. After the column had been washed with 0.1M-ammonium bicarbonate the adsorbed yellow peptides were eluted with 1M-ammonia. The overall yield of elute peptides was 55%, as judged from their E_{381}. The column was immediately washed with 0.1M-ammonium bicarbonate and used again. We have used such antibody–Sepharose columns ten times without significant loss of their activity.

A comparative electrophoretogram of the different fractions eluted from the column and of the original tryptic digest is given in Fig. 2, which shows that (*a*) all the peptides present in the original

Table 1. *Amino acid composition of nitrolysozyme*

For experimental details see the text.

Amino acid	Content (mol/mol of lysozyme)	
	Nitrolysozyme	Theoretical*
Lys	6.6	6
His	1.2	1
Arg	11.0	11
Asp	20.6	21
Thr	7.3	7
Ser	10.3	10
Glu	5.4	5
Pro	1.9	2
Gly	12	12
Ala	12	12
Cys	2.9	8
Val	5.9	6
Met	1.7	2
Ile	5.6	6
Leu	8.6	8
Tyr	1.7	3
Phe	3.4	3
NO_2-Tyr	0.9	—

*According to Canfield (1963): tryptophan was not determined.

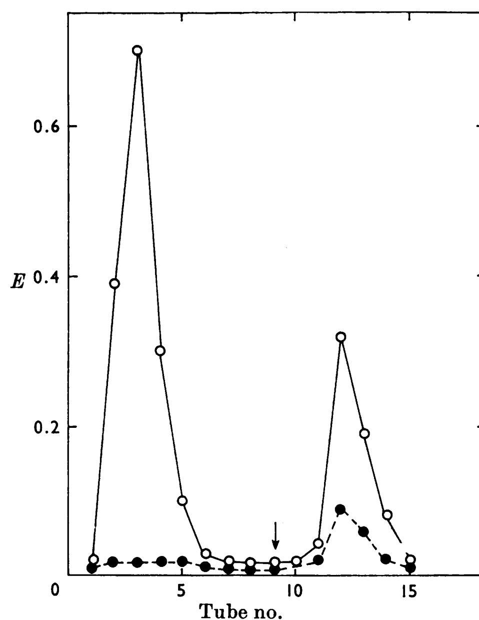

Fig. 1. Isolation of nitrotyrosyl peptides from nitrotyrosyl-lysozyme. A tryptic digest of 2mg of reduced and alkylated nitrolysozyme was applied to a column (1cm×6cm) of anti-nitrotyrosyl antibody–Sepharose conjugate that contained 30mg of antibodies. The column was washed with 0.1M-NH_4HCO_3 and the yellow nitrotyrosyl peptides were eluted with 1M-NH_3 (arrow). ○, E_{280}; ●, E_{381}.

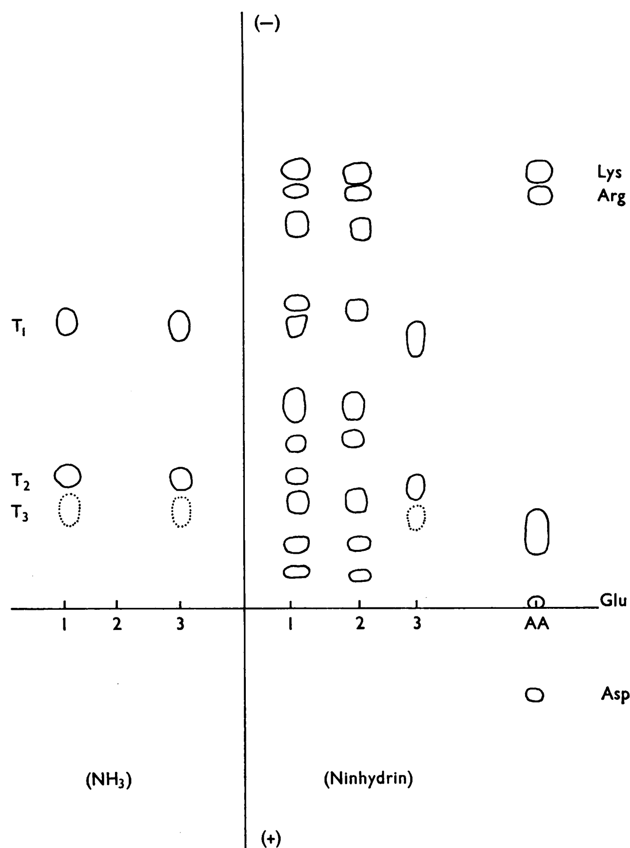

Fig. 2. Comparative electrophoresis of peptides derived from nitrotyrosyl-lysozyme. 1, Tryptic digest of reduced-alkylated nitrotyrosyl-lysozyme; 2, digest after passage through anti-nitrotyrosyl–Sepharose column; 3, peptides eluted from anti-nitrotyrosyl–Sepharose column by 1M-NH_3 (see Fig. 1). AA, mixture of amino acids. Electrophoresis was at 3000V at pH 3.5 for 25 min. The right-hand side of the paper was stained with ninhydrin and the left-hand side was exposed to NH_3 vapour and the yellow spots were marked.

digest, except the nitrotyrosyl peptides, are present in the fraction unadsorbed by the antibody column; (*b*) the same (by mobility) nitrotyrosyl peptides present in the tryptic digest are present in the fraction eluted from the antibody column with 1M-ammonia; (*c*) the fraction eluted with 1M-ammonia contained only nitrotyrosyl peptides and no other ninhydrin-positive material. Thus the nitrotyrosyl peptides were isolated in one step by the antibody–Sepharose column procedure.

Table 2 gives the amino acid analysis and *N*-terminal residues of the three peptides (T_1–T_3, Fig. 2) eluted from paper by 0.05M-ammonia. A comparison of these compositions with the sequence of lysozyme (Canfield, 1963) indicates that peptide T_1 corresponds to the tryptic peptide His-Gly-Leu-Asp-Asn-Tyr-Arg that occupies residues 15–21. Peptides T_2 and T_3 are very similar in their composition and identical in their *N*-terminal residue (Gly). They must be derived from the region corresponding to residues 22–33 (Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys). The recovery of two peptides, differing in their electrophoretic mobility, from this region may be due to oxidation of carboxymethyl-cysteine. These results thus indicate that tyrosine-20 and tyrosine-23 are nitrated under the conditions employed, in agreement with the results of Atassi & Habeeb (1969).

DISCUSSION

The method described in the present paper is another example of the use of an antibody column for the isolation of modified peptides (see Wilchek

Table 2. *Amino acid composition of nitrotyrosyl-containing peptides eluted from paper* (*see Fig.* 2)

For experimental details see the text. Abbreviations: CMCys, carboxymethylcysteine; n.d., not determined.

	Content (mol/mol of peptide)				
Amino acid	T_1	Theoretical*	T_2	T_3	Theoretical†
Lys			1.0	0.8	1
His	1.0	1			
Arg	1.1	1			
Asp	1.9	2	1.0	1.1	1
Ser			0.8	0.8	1
Gly	1.1	1	1.7	1.9	2
Ala			2.0	1.3	2
CMCys			0.6	0.1	1
Val			1.0	0.7	1
Leu	1.3	1	1.1	1.0	1
Tyr		1			1
NO_2-Tyr	1.0		0.6	0.6	
Trp			n.d.	n.d.	1
Remarks					
N-Terminus	His	His	Gly	Gly	Gly
Yield‡	21%		15%	13%	

* Composition of the tryptic peptide corresponding to residues 15–21 in lysozyme.

† Composition of the tryptic peptide corresponding to residues 22–33 in lysozyme.

‡ Yield per mol of lysozyme after elution from paper.

et al. 1971). In previous cases antibodies to a group (dinitrophenyl or azobenzenearsonate) that was coupled to a residue of the peptide chain were used (Wilchek *et al.* 1971). Nitrotyrosine, however, is a part of the peptide chain itself and it seems that antibody production to such a moiety is more difficult. However, if antibodies produced against nitrotyrosyl–protein conjugates are purified on a nitroprotein, they are suitable as a reagent for the isolation of nitrotyrosyl peptides. The yield of the nitrotyrosyl peptides recovered from the column (55%) is somewhat lower than yields of peptides isolated by this method in other systems (Wilchek *et al.* 1971). This may be correlated with the discrepancy between the nitrotyrosine content of lysozyme estimated by amino acid analysis and spectral measurement, and suggests that part of the E_{381} was not due to nitrotyrosine.

The conditions used here for elution of the peptides from the antibody column (1M-ammonia) did not decrease the capacity of the antibody column and it can be re-used many times. In addition, under these conditions no antibodies are released from the column (Wilchek *et al.* 1971) and the modified peptides can be isolated in one step. This procedure is rapid and convenient and can be applied to any kind of chemical modification of proteins, provided that antibodies to the modified residue are available.

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