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 (325 mg) to the albumin (200 mg) with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (300 mg) in 16 ml of 0.15 M-NaCl-0.01 M-sodium phosphate, pH 7.4, as described by Halloran & Parker (1966). The solution was dialysed against 0.1 M-NH₂HCO₃ and freeze-dried.

Amino acid analysis showed that the nitrotyrosyl-albumin contained 29 mol of nitrotyrosine/mol of albumin. Nitro- (serum albumin) was prepared by nitrination 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 6.9 mol 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) 13 mol of nitrotyrosine and 30 mol of tyrosine/mol of γ-globulin. Antiserum were raised by injecting rabbits or a goat with 1 mg 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 nitroatyrosyl-(serum albumin). These antisera contained between 0.5–0.1 mg of anti-nitrotyrosyl antibodies as determined by precipitin reaction with nitro-γ-globulin. Antibodies were isolated by adsorption of pooled antiserum on a column of nitro-γ-globulin-Sepharose conjugate (Wilchek et al. 1971). The column was washed with 0.15 M-NaCl until the E₂₈₀ of the effluent was less than 0.1. The adsorbed antibodies were eluted from the column with 0.1 M-acetic acid. The average yield of eluted antibodies was 0.8 mg/ml of serum. The purified antibodies were dialysed against 0.15 M-NaCl-0.01 M-phosphate buffer, pH 7.4, and stored at -20°C. Precipitin analysis of these antibodies showed that they were 70% precipitable by nitro-γ-globulin.

Nitrination 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 2 h the nitrolysozyme was isolated on a Sephadex G-25 column (2 cm x 60 cm) that was equilibrated and run with 1 M NaCl-0.05 M-tris-HCl buffer, pH 8.2. The protein containing fractions were combined, dialysed against 0.05 M-NH₂HCO₃ and freeze-dried. The nitrolysozyme...
was reduced in 8M-urea–0.2M-tris–HCl buffer (pH8.2)–
0.1M-2-mercaptoethanol for 1 h at 37°C, and alkylated with 0.15M-iodoacetate for 30 min. The reduced and
alkylated protein was dialysed against 0.1M-NH₄HCO₃
digested with trypsin (1:50, w/w, ratio of enzyme to
protein) for 3 h 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 phenyl-
alanine at 171 min 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 24 h at 110°C). N-Terminat 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 NH₃ vapour. The content of
nitrotyrosine was measured by its extinction at 381 nm
(ε = 2200 litre·mol⁻¹·cm⁻¹) by using a Zeiss PMQII
spectrophotometer. The E₃₈₀ 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 1.6 mol of nitrotyrosine/mol of nitrolysozyme.
This discrepancy may be due either to incomplete recovery of nitrotyrosine on
hydrolysis, or to some side-reaction of tetranitro-
methane with lysozyme that yields coloured
derivatives other than nitrotyrosine.

The isolation of nitrotyrosine-containing pep-
tides on the anti-nitrotyrosine–Sepharose column
is depicted in Fig. 1. Most of the E₃₈₀ (81%) emerged unretarded from the antibody–Sepharose
column. The yellow nitrotyrosyl peptides were
adsorbed from 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 E₃₈₁. 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 differ-
et fractions eluted from the column and of the
original trypsin digest is given in Fig. 2, which shows
that (a) all the peptides present in the original

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

Fig. 1. Isolation of nitrotyrosyl peptides from nitrotyrosyl-lysozyme. A trypic digest of 2 mg of reduced and alkylated nitrolysozyme was applied to a column (1 cm × 6 cm) of anti-nitrotyrosyl antibody–Sepharose conjugate that contained 30 mg of antibodies. The column was washed with 0.1M-NH₄HCO₃ and the yellow nitrotyrosyl peptides were eluted with 1M-NH₃ (arrow). ○, E₃₈₀; •, E₃₈₁.
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 (T₁–T₃, Fig. 2) eluted from paper by 0.05M-ammonia. A comparison of these compositions with the sequence of lysozyme (Canfield, 1963) indicates that peptide T₁ corresponds to the tryptic peptide His-Gly-Leu-Asp-Asn-Tyr-Arg that occupies residues 15–21. Peptides T₂ and T₃ 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 carboxymethylcysteine. 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
and the are released from under these addition, column and peptides from lysozyme estimated 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.

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.

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Remarks

N-Terminus: His
Yield: 21% 15% 33%

* 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 azobenzeneasorionate) 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 (1 M-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.

REFERENCES