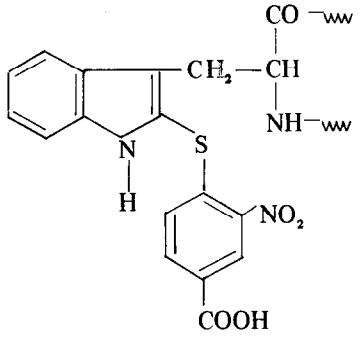
MODIFICATION OF TRYPTOPHAN 108 IN LYSOZYME BY 2-NITRO-4-CARBOXYPHENYLSULFENYL CHLORIDE

Francesco M. VERONESE, Enrico BOCCU, and Angelo FONTANA
Institute of Pharmaceutical Chemistry
(Centro di Chimica del Farmaco e dei Prodotti Biologicamente Attivi del C.N.R.)
and of Organic Chemistry of the University, Padova, Italy

Received 7 February 1972

1. Introduction

Sulfenyl halides have been found to be highly specific reagents for modification of tryptophan and cysteine residues in proteins [1–6]. The valuable feature of this technique lies in the possibility of a wide choice of reagents for protein modification studies. In this communication we report the modification of hen egg-white lysozyme with 2-nitro-4-carboxyphenylsulfenyl chloride (NCPS-Cl) (see footnote*). A single molecular specie of the modified lysozyme was isolated by ion exchange chromatography and the modified tryptophan residue, 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan, Trp(NCPS) (I), located in the polypeptide sequence by enzymatic degradation.



* Abbreviations: NCPS-Cl, 2-nitro-4-carboxyphenylsulfenyl chloride; Trp(NCPS), 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan; NCPS–lysozyme, lysozyme modified with NCPS-Cl at the tryptophan residue 108.

North-Holland Publishing Company – Amsterdam

2. Materials and methods

Hen egg-white lysozyme (Worthington, LYSF 8 KA) was modified by dissolving the protein (30 mg/ml) in 5% acetic acid and by adding under vigorous stirring the desired amount of NCPS-Cl dissolved in glacial acetic acid to give a final conc. of 25% acetic acid. After 5 min cold acetone was added to precipitate the modified protein and to separate the material from excess reagent and its decomposition products. The protein was separated by centrifugation and dried. A sample of lysozyme was run in the same conditions without the addition of the reagent.

The extent of modification was determined by spectrophotometric reading at 354 nm, using an extinction coefficient for the Trp(NCPS) residue of 4700 [4]. Protein concentrations were determined by amino acid analysis of an aliquot of the solution hydrolyzed in 6 N HCl at 110° for 24 hr in sealed tubes under vacuum. Amino acid analyses were performed with a Spinco Model 120B instrument.

The activity of the modified lysozyme was determined by following spectrophotometrically the extent of lysis of a suspension of dead cells of *Micrococcus lysodeikticus* (Difco) [7].

The modified lysozyme was purified by column chromatography on a Bio-Rex 70, –400 mesh cation exchange resin (Bio-Rad), at 30° using 0.2 M sodium phosphate buffer (pH 7.18) as eluent.

Trypsin treated with L-(1-tosilamido-2-phenyl)-ethyl chloromethyl ketone and carboxypeptidase A and B were obtained from Worthington.

NCPS-Cl was prepared according to [8] and recrystallized from anhydrous ethylene dichloride, mp 185°.

3. Results

The tryptophan residues of lysozyme were modified by using increasing amounts of NCPS-Cl in 25% acetic acid. The extent of modification was determined by spectrophotometric reading of the Trp (NCPS)-chromophore at 354 nm [4]. With about 15–20 equivalents of reagent all 6 tryptophan residues were modified. Conversely, the lytic activity towards *Micrococcus lysodeikticus* of the enzyme decreased gradually upon modification, and was completely abolished when about 3–4 residues were modified (fig. 1).

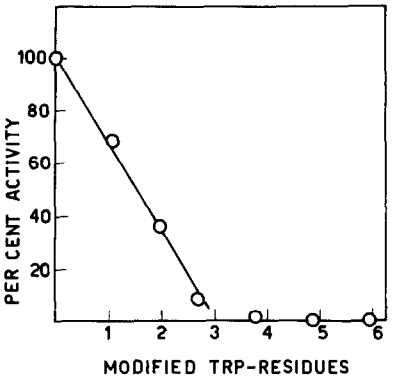


Fig. 1. Effect of progressive modification of tryptophan residues on lysozyme activity. The activity test was performed as described by Jollès [16].

The sample of lysozyme modified with one equivalent of NCPS-Cl, which retained about 70% activity, was resolved on a column of carboxylic ion exchange resin into its components, as shown in fig. 2. Along with unmodified lysozyme (peak R), different peaks of modified protein were eluted from the column.

The *major component* (peak P, in the chromatogram of fig. 2) contained one tryptophan residue modified per mole of protein, and retained about 10% of the lytic activity. The amino acid analysis of the modified protein showed no change in any other amino acid residue.

In order to localize the modified residue, the sample of the NCPS–lysozyme (3 μmoles) was reduced with β-mercaptoethanol in 6 M guanidinium hydrochloride and carboxymethylated with iodoacetic acid [9]. After dialysis against 0.1 M ammonium bicarbonate, the sample was digested with trypsin. The tryptic hydrolyzate was separated on a Sephadex G-50 column (1.9 × 150 cm) with 30% acetic acid as eluent. The unique peak of yellow material (absorption at 354 nm) eluted from the column was lyophilized and further purified by preparative paper electrophoresis (pH 1.9, 2500 V. 45 min). Only one yellow band was detected on the chromatogram. The Trp(NCPS)-containing peptide, eluted with 30% acetic acid, was judged to be pure by paper chromatography and amino acid analysis and corresponded to the sequence 98–112 of lysozyme [10]: Arg 1.00 (1); Asp 3.02(3); Ser 1.05(1); Gly 2.20(2); Ala 1.80 (2); Val 1.90(2); Met 0.85(1); Ile 0.95(1) (the values of Val and Ile obtained after 72 hr of hydrolysis). The yield of the peptide was 1.4 μmoles.

Samples of the peptide were digested with carboxypeptidase A plus B at different times at 40°. After 10 min digestion only arginine and traces of tryptophan were released, after 30 min also alanine was present, and after 4 hr the following analysis was obtained: Arg 1.00: Trp 0.9; Ala 1.0; Val 0.3. After 6 hr of hydrolysis the value of valine was slightly increased, but no Trp(NCPS) was released. This derivative is eluted before tryptophan from the basic column of the analyzer.

The data are in agreement with the sequence of peptide T-19 of Canfield [10] and consistent with the modification of tryptophan 108: –Trp(NCPS) –Val–Ala–Trp–Arg. The limited release of valine by carboxypeptidase may be related to the bulky moiety of the sulfenyl group attached to the indole ring of the preceding tryptophan.

4. Discussion

The use of the carboxy-substituted sulfenyl chloride, NCPS-Cl, for tryptophan modification of hen egg-white lysozyme allowed isolation of a single molecular specie of the modified protein by ion exchange chromatography. The reagent is more suitable of the parent compound 2-nitrophenylsulfenyl chloride (NPS-Cl) (i.e. without the carboxyl group) [6] for protein modification studies. In fact, fractionation of the reaction product of lysozyme with one equivalent of NPS-Cl by ion exchange chromatography was not successful. In addition, the

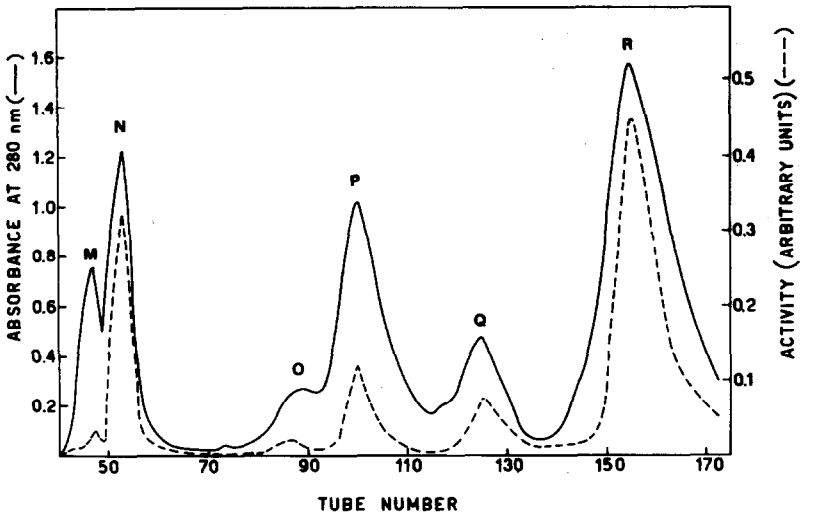


Fig. 2. Elution pattern of lysozyme modified with one equivalent of NCPS-Cl. Chromatography was performed on a column (2 × 90 cm) of Bio-Rex 70, −400 mesh with 0.2 M sodium phosphate buffer, pH 7.18. Fractions of 3 ml were collected at a flow rate of 18 ml/hr. (——): Absorbance at 280 nm; (– – –): activity (arbitrary units).

carboxyl group enhances solubility in aqueous solutions of the modified protein.

The 108-NCPS-derivative of lysozyme, which retains about 10% activity, could be useful for a better understanding of the role of the tryptophan 108 in the lytic activity of the enzyme. Recent X-ray studies demonstrated the importance of some of the tryptophan residues for the biological activity [11,12]. The tryptophan 108 appears to be involved in inhibitor binding [13] and also to interact with glutamic acid 35, a group which has been suggested to play an intimate role in catalysis [14, 15]. The results obtained so far by chemical modification are not conclusive, since the 108-oxindole–lysozyme obtained by N-bromosuccinimide treatment [16] is inactive, whereas the 108-N′-formyl-kynurenine–lysozyme prepared by ozone oxidation is fully active [17]. This has prompted a crystallographic study of the 108-oxindole lysozyme; any clear reason for the loss of catalytic activity could not be found [18, 19].

Studies are going on on the enzymological and conformational characteristics of the NCPS–lysozyme in order to differentiate between the effects of the modification on the catalysis, affinity or conformation of the enzyme.

Acknowledgements

The authors wish to thank Professor E. Scoffone and Professor C.A. Benassi for their interest in this work. The excellent technical assistance of Mrs. S. Agostinetti is also acknowledged.

References

[1] E. Scoffone, A. Fontana and R. Rocchi, Biochemistry 7 (1968) 971.
[2] A. Fontana, E. Scoffone and C.A. Benassi, Biochemistry 7 (1968) 980.
[3] A. Fontana, F.M. Veronese and E. Scoffone, Biochemistry 7 (1968) 3901.
[4] F.M. Veronese, E. Boccú and A. Fontana, Ann. Chim. (Rome) 58 (1968) 1309.
[5] F.M. Veronese, E. Boccú and A. Fontana, Int. J. Protein Res. 2 (1970) 67.
[6] A. Fontana and E. Scoffone, in: Methods in Enzymology, Vol. 25, eds. C.H.W. Hirs and S.N. Timasheff (Academic Press, New York, 1972) 482.
[7] P. Jollés, in: Methods in Enzymology, Vol. 5, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1961) p. 137.
[8] A.J. Havlik and N. Kharasch, J. Am. Chem. Soc. 77 (1965) 1150.

[9] J. Jollès, J. Jauregui-Adell, I. Bernier and P. Jollès, Biochim. Biophys. Acta 78 (1963) 668.
[10] R.E. Canfield, J. Biol. Chem. 238 (1963) 2691, 2698.
[11] C.C.F. Blake, D.F. Koenig, G.A. Mair, A.C.T. North, D.C. Phillips and V.R. Sarma. Nature 206 (1965) 757.
[12] L.N. Johnson and D.C. Phillips, Nature 206 (1965) 761.
[13] D.C. Phillips, Sci. Am. 215 (5) (1966) 78; Proc. Natl. Acad. Sci. U.S. 57 (1967) 484.
[14] C.C.F. Blake, L.N. Johnson. G.A. Mair, A.C.T. North, D.C. Phillips and V.R. Sarma, Proc. Roy. Soc. (London) B167 (1967) 378.
[15] C.C.F. Blake, D.F. Koenig, G.A. Mair, A.C.T. North, D.C. Phillips and V.R. Sarma. Proc. Roy. Soc. (London) B167 (1967) 365.
[16] F.J. Hartdegen and J.A. Rupley, J. Am. Chem Soc. 89 (1967) 1743.
[17] A. Previero, M.A. Coletti-Previero and P. Jolles, J. Mol. Biol. 24 (1967) 261.
[18] C.C.F. Blake, Proc. Roy. Soc. (London) B167 (1967) 435.
[19] J.A. Rupley, 162nd National Meeting, Amer. Chem. Soc., Washington D.C., 12 – 17 September 1971, Abstr. Commun., Biol. Section, 140.