# SELECTIVE CLEAVAGE OF THE SINGLE TRYPTOPHANYL PEPTIDE BOND IN HORSE HEART CYTOCHROME *c*

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## 1. Introduction

Several reagents capable of chemical cleavage of peptide bonds have been studied, but in the great majority of cases they are not applicable to proteins because of numerous and various side reactions [1]. Cleavage at the methionine peptide bonds [2] remains the only really satisfactory chemical method of cleaving proteins.

Tryptophan is another potential point of attack on the polypeptide chain. *N*-Bromosuccinimide [3] is not a selective reagent for cleaving the tryptophan peptide bond, since both tyrosine and histidine residues are attacked. Moreover, the yields of cleavage are rather poor, so that the reagent has proved of no practical utility for the fragmentation of proteins [1].

This report describes the selective chemical cleavage of the single tryptophanyl peptide bond in horse heart cytochrome *c* by BNPS-skatole (2-(2-nitrophenylsulfenyl)-3-methyl-3-bromo-indolenine) [4, 5].

## 2. Experimental

Horse heart cytochrome *c* (type III, Sigma Chem. Company, St. Louis, Mo., USA) (200 mg; 16 μmoles) was dissolved in 8 ml water and 8 ml acetic acid with 20 mg of phenol. To the solution were added under stirring 60 mg (160 μmoles; 10 equivalents) of BNPS-skatole (Pierce Chem. Company, Rockford, Ill., USA) dissolved in 8 ml of glacial acetic acid. After 48 hr in the dark, 100 μl of β-mercaptoethanol were added and the solution let stand at 37° for 5 hr. The reaction mixture was then diluted with 70 ml water and several times extracted with ethyl acetate in order to remove excess reagent and by-products. The solution was concentrated in vacuo at 37° and directly applied to a Sephadex G-50-SF (Pharmacia, Uppsala, Sweden) column. The peptide fragments were further purified by ion exchange chromatography on a CM-cellulose (Serva, Heidelberg, Germany) column.

## 3. Results

The cleavage of horse heart cytochrome *c* by BNPS-skatole was carried out in 65% acetic acid with 10 equivalents of reagent for 48 hr at room temp. It was found that the yields of cleavage were not improved by using larger excess of reagent up to 100 equivalents. The reaction was performed in presence of phenol as a scavenger for tyrosine destruction. Since the reagent oxidizes the methionine residue to its sulfoxide excess of β-mercaptoethanol was added at the end of the reaction in order to reverse the methionine modification [4].

The BNPS-skatole reagent also quantitatively removes the heme group from cytochrome *c*. Kinetic analysis by thin-layer chromatography on cellulose plates in butanol:acetic acid:pyridine:water (42:24:4:30) (the heme moves with the solvent front) showed that under the experimental conditions used for tryptophan cleavage the heme removal is complete in about 2 hr. Since BNPS-skatole is a brominating agent [4, 5], the mechanism by which the thioether bridges linking the heme to the protein are cleaved is

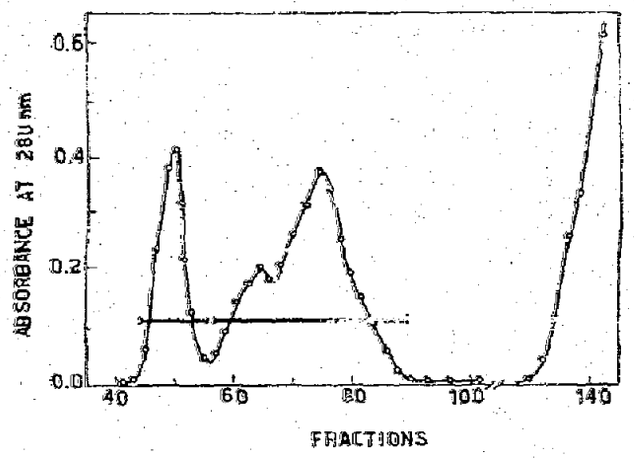


Fig. 1. Fractionation of BNPS-skatole treated cytochrome *c* on a Sephadex G-50-SF column (3.8 × 132 cm) equilibrated and eluted with 10% acetic acid. The flow rate was 20 ml/hr and fractions of 10 ml were collected. Horizontal bars indicate fractions pooled.

probably similar to the iodine cleavage recently described by Lederer and Tarin [7].

The elution profile on a Sephadex G-50-SF column of the reaction mixture of cytochrome *c* treated with BNPS-skatole is shown in fig. 1. From the amino acid composition (table 1), the first material eluted corresponded to modified but uncleaved cytochrome *c*, the second to the mixture of the two fragments residues 1–59 and residues 60–104, and the third peak low molecular weight material (excess reagent, heme, phenol). The two peptide fragments were then separated by ion exchange chromatography on a CM-cellulose column, eluted by a gradient of ammonium acetate buffer, pH 6.5. The sequences 60–104 and 1–59 were eluted, respectively, at about 0.1 and 0.4 ammonium acetate molarity (fig. 2). The yield of cleavage, in three different experiments, was 55–65%, as judged from the dry weights of the lyophilized fragments.

From the amino acid composition of oxidized apocytochrome and of the fragments shown in table 1 it appears that the isolated peptides are pure and that no loss of any amino acid occurred. This fact is in agreement with the high specificity of the reagent [4].

The good recovery of cystine on the amino acid analyzer suggests that the emi-cystine residues are under the form of a disulfide bridge in the isolated

Table 1

Amino acid composition of oxidized apocytochrome *c* and of the peptide fragments obtained by BNPS-skatole cleavage of cytochrome *c*. (Theoretical values are in parentheses).*

| | Cytochrome *c* | Apocytochrome *c* oxidized | Peptide fragments 1–59 | Peptide fragments 60–104 |
|---|---|---|---|---|
| Lysine | 18.1 (19) | 18.6 (19) | 10.1 (10) | 8.3 (9) |
| Histidine | 3.1 ( 3) | 3.0 ( 3) | 3.1 ( 3) | 0 (0) |
| Arginine | 1.9 ( 2) | 2.2 ( 2) | 0.9 ( 1) | 0.8 (1) |
| Aspartic acid | 7.8 ( 8) | 9.3 ( 8) | 5.0 ( 5) | 3.0 (3) |
| Threonine | 9.8 (10) | 10.2 (10) | 5.8 ( 6) | 3.7 (4) |
| Glutamic acid | 11.5 (12) | 12.6 (12) | 5.2 ( 5) | 7.6 (7) |
| Proline | 4.0 ( 4) | 4.3 ( 4) | 1.9 ( 2) | 2.2 (2) |
| Glycine | 11.6 (12) | 12.8 (12) | 9.7 (10) | 2.1 (2) |
| Alanine | 6.7 ( 6) | 7.2 ( 6) | 3.2 ( 3) | 2.9 (3) |
| Emicystine | 1.4 ( 2) | 1.7 ( 2) | 1.9 ( 2) | 0 (0) |
| Valine | 2.7 ( 3) | 2.9 ( 3) | 2.8 ( 3) | 0 (0) |
| Methionine | 1.6 ( 2) | 1.5 ( 2) | 0 ( 0) | 1.6 (2) |
| Isoleucine | 4.6 ( 6) | 4.8 ( 6) | 2.0 ( 2) | 4.1 (4) |
| Leucine | 6.0 ( 6) | 6.5 ( 6) | 2.2 ( 2) | 4.0 (4) |
| Tyrosine | 3.8 ( 4) | 3.7 ( 4) | 1.1 ( 1) | 2.9 (3) |
| Phenylalanine | 4.0 ( 4) | 3.7 ( 4) | 3.0 ( 3) | 1.1 (1) |

* Composition was determined after 22 hr hydrolysis in sealed tubes under vacuum in 6 N HCl at 110°. Amino acid analyses were carried out with a C. Erba analyzator, Model 3A27. No correction for destruction or low recovery of amino acid were made. The results are expressed in terms of residues per mole of protein or peptide.

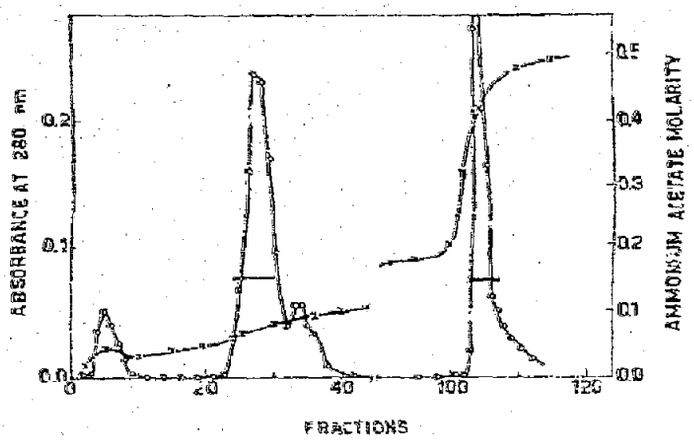


Fig. 2. Ion exchange chromatography of the second peak of fig. 1 on a CM-cellulose column (1.8 × 12 cm) equilibrated with 0.01 M ammonium acetate buffer, pH 6.5. The column was eluted with a gradient (×—×—×) from 0.01 to 0.5 M of the same buffer, at a flow rate of 36 ml/hr. Fractions of 5.5 ml were collected. Horizontal bars indicate fractions pooled.

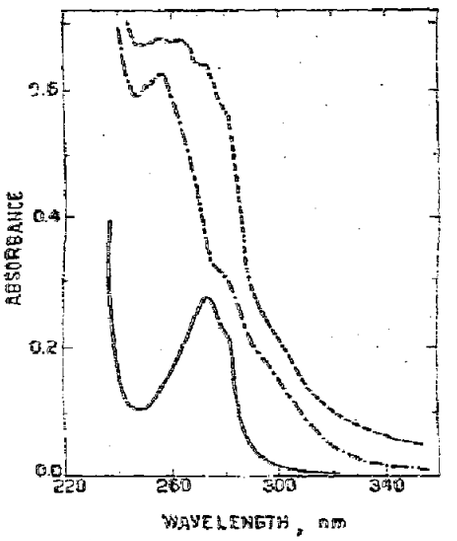


Fig. 3. Absorption spectra in 0.1 N acetic acid of oxidized apocytochrome *c* (---) and of the fragments 1–59 (·---·) and 60–104 (——).

apocytochrome and in the peptide fragment 1–59. No free sulfhydryl groups were detected by the Ellman reagent 5,5′-dithiobis-(2-nitrobenzoic acid) [8]. Furthermore, the elution position of the peptide fragments from Sephadex G-50 (fig. 1) indicates that the disulfide bonds cannot be intermolecular, and hence must connect cysteine residues 14 and 17.

The absorption spectra of the peptide fragments as well as of the modified apocytochrome are shown in fig. 3. The spectrum of the fragment 60–104 is typical of a tyrosine containing peptide. The modified apocytochrome and the fragment 1–59 show an absorption spectrum which could result from the contribution of tyrosine and 2-oxy-tryptophan absorption. The 2-oxy-tryptophan chromophore ($\lambda_{max}$ near 250 nm and shoulder at 280 nm) has been shown to be produced by *N*-bromosuccinimide, oxidizing reagent analogous to BNPS-skatole [9]. It may be inferred therefore that the tryptophan residue is modified by BNPS-skatole to 2-oxy-tryptophan.

Attempts to improve the yields of cleavage by changing the solvent composition, reaction time and equivalents of the reagent were so far unsuccessful.

## 4. Discussion

The results reported here point out that BNPS-skatole is a useful reagent for the selective chemical fragmentation of proteins, approaching in its utility the cyanogen bromide reaction.

The molecule of cytochrome *c* from a large variety of eukaryotic organisms contains a single tryptophanyl residue at position 59 in the amino acid sequence and this residue has been classified as invariant [10]. In view of this fact, the method here described will be a general route to cleave the cytochrome *c* molecule into only two fragments of similar length. These peptide fragments, if obtained from different organisms, may be compared for common and variable regions and facilitate the structural work of sequence determination.

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