# AMINO ACID SEQUENCE STUDIES OF HORSERADISH PEROXIDASE†. TRYPTIC GLYCOPEPTIDE CONTAINING TWO HISTIDINE RESIDUES AND A DISULFIDE BRIDGE††

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## 1. Introduction

In the elucidation of the primary structure of horseradish peroxidase, reports have previously appeared on the amino acid sequences of 24 out of 29 possible tryptic peptides [1] and on 120 peptides obtained on thermolysin digestion [2]. Together, these peptides account for all the amino acid residues of the horseradish peroxidase peptide chain. Furthermore, 8 sites of carbohydrate attachment were assigned [2]. The 24 tryptic peptides were isolated exclusively by high voltage paper electrophoresis. However, it was experienced that the successive electrophoretic steps resulted in complete loss of the larger disulfide bridged peptides. Column chromatography procedures have now been adapted to the isolation of these tryptic peptides.

Lack of information as to the nature of the amino acid residues located proximally and distally to the ferric protoporphyrin moiety of horseradish peroxidase, has long been an obstacle to the detailed interpretation of physical, kinetic and chemical modification studies on the enzyme. The primary structure of the tryptic peptide reported in the present communication indicates that this sequence is positioned distally to the heme [3].

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*Abbreviations:*
dansyl, 1-dimethylaminonaphtalene-5-sulfonyl; GlcN, glucosamine.

## 2. Methods

Materials (horseradish peroxidase, Mann Research Laboratories, Inc., lot U1929) and methods were essentially the same as previously [1, 2]. Characterization of dansyl amino acids was carried out on 5 cm × 5 cm polyamide layer sheets [4]. Performic acid oxidation was accomplished in 3 hr at 0° with a mixture of 30% hydrogen peroxide and formic acid (1:19) which had been heated to 50° for 3 min and subsequently chilled to 0°. Amino acid composition was determined on a Bio-Cal automatic amino acid analyzer, model BC 201 using a one column program. The designation of peptides previously characterized or sequenced has been preserved. Glycopeptides are indexed by an asterisk as is the amino acid residue identified as a site of carbohydrate attachment. Glycopeptides were not analyzed for content of neutral sugars. The recovery of glucosamine on automatic amino acid analysis has not been corrected for destruction during hydrolysis.

## 3. Results

A tryptic digest of apo-horseradish peroxidase was fractionated on a Sephadex G-50 column in 0.05 acetic acid. The first histidine positive peak was rechromatographed on Bio-Gel P4 in the same eluant. The paper electrophoretic mobility and staining characteristics of this fraction indicated that it contained the histidine positive disulfide bridged peptide, previously designated T9* [1]. The fraction was lyophilized and oxidized by

Table I
The amino acid sequence of T9*.

| Peptide | Mobility pH 6.5 | Yield (%) | Amino acid composition and sequence |
|---|---|---|---|
| Known thermolytic peptides: | | | Leu –His \| Phe –His –Asp –Cys \| Phe –Val –Asn –Gly –Cys –Asp –Ala –Ser \| Ile –Leu –Leu –Asp –Asn* –Thr –Thr –Ser \| Phe –Arg– |
| | | | H132 \| H33-ox1 \| Bridge B \| H33-ox2 \| I3* \| H43 or H44 |
| Performic acid oxidation: | | | |
| T9-ox* | –0.25 | | [GlcN] Leu –His –Phe –His –Asx –$O_3$H Cys –Phe –Val –Asx –Gly ($O_3$H Cys ,Asx ,Ala ,Ser ,Ile ,Leu ,Leu ,Asx ,Asx ,Thr ,Thr ,Ser ,Phe ) Arg |
| | | | 1.51 0.97 0.89 1.06 0.89 1.03 0.94 1.06 1.12 1.03 1.00 0.94 1.03 1.16 1.00 0.90 0.97 0.97 1.03 1.03 0.98 0.98 1.00 1.06 1.06 |
| Partial acid hydrolysis: | | | |
| T9-oxH1 | +0.48 | 20 | Leu (His ,Phe ,His ) |
| | | | 0.96 1.02 0.99 1.02 |
| T9-oxH5 | –0.67 | 30 | $O_3$H Cys (Phe ,Val ) |
| | | | 0.97 0.99 1.03 |
| T9-oxH6 | –0.85 | 40 | $O_3$H Gly –Cys |
| | | | 0.91 1.09 |
| T9-oxHn | 0.00 | 10 | Ala (Ser ,Ile ,Leu ,Leu ) |
| | | | 0.99 1.19 0.91 0.96 0.96 |
| T9-0xH2 | +0.36 | 10 | Thr (Thr ,Ser ,Phe ) Arg |
| | | | 0.90 0.90 1.15 1.00 1.04 |
| T9-oxH3* | +0.20 | 10 | [GlcN] Asn (Thr ,Thr ,Ser ,Phe ) Arg |
| | | | 0.91 1.00 0.92 0.92 1.17 1.08 0.91 |
| T9-oxH4* | +0.15 | 10 | [GlcN] Asn (Thr ,Thr ,Ser ,Phe ) Arg |
| | | | 1.32 1.05 0.90 0.90 1.30 0.89 0.95 |
| T9-oxH7 | –1.00 | | Asp |
| T9*, final sequence | | | Leu –His –Phe –His –Asp –Cys –Phe –Val –Asn –Gly –Cys –Asp –Ala –Ser –Ile –Leu –Leu –Asp –Asn* –Thr –Thr –Ser –Phe –Arg |

performic acid and subsequent refractionation on Bio-Gel P4 yielded two large peptides which were eluted together, and a small peptide. The histidine positive peptide, T9-ox*, was isolated by high voltage paper electrophoresis at pH 1.8. The almost complete loss (95%) of the accompanying histidine-negative peptide, during this electrophoretic step, is discussed below.

The amino acid sequence of T9* and the supporting evidence for this sequence are summarized in table 1. The amino acid composition of T9-ox* shows it contains two cysteic acid residues and two histidine residues per arginine residue. Dansyl-Edman analysis places the thermolytic histidine peptides III32 and III33-ox1, and also shows that II33-ox1 is followed by the thermolytic peptide II33-ox2. II33-ox1 and II33-ox2 were previously shown to be disulfide linked [2]. Consequently an internal disulfide loop is present in T9*. Subtracting the amino acid composition of these known thermolytic peptides from that of T9-ox*, a glycopeptide composition was left which could only be accomodated by thermolytic glycopeptide I3*. This leaves only a phenylalanine and an arginine residue. Therefore the sequence of the thermolytic peptides is (III32)–(II33-ox1)–(II33-ox2), (I3*), (Phe)–Arg. The peptides obtained on partial acid hydrolysis of T9-ox* were separated by paper electrophoresis and were found to fully support this sequence (lower half of table 1).

## 4. Discussion

The amino acid analysis of T9-ox* shows that the carbohydrate side chain of this glycopeptide contains more than one glucosamine residue. Two glycopeptide varieties of identical amino acid compositions were obtained on partial acid hydrolysis, the slower T9-oxH4*, and the faster T9-oxH3* which judged from its lower glucosamine content and higher mobility presumably has lost part of its carbohydrate side chain. It is also noteworthy that the peptide bond involving the carboxyl group of carbohydrate linked asparagine is hydrolyzed only slowly in 0.03 N HCl at 105°.

Welinder et al., [1] previously experienced that successive steps of paper electrophoresis gave complete loss to some of the larger tryptic peptides of HRP. But at that time the responsible electrophoretic step or factor could not be distinguished. As mentioned above the peptide T9-ox* was eluted together with another large tryptic peptide. A preliminary electrophoretic experiment showed that these peptides were easily separated at pH 1.8, and normally recovered when spotted on a 10 cm strip of Whatman No. 1 paper. Preparative electrophoresis on a 38 cm strip of Whatman No. 3 MM paper under identical conditions resulted in an almost complete loss of the histidine-negative peptide. The temperature of the Varsol coolant was less than 25° after each of the electrophoretic experiments. It is tentatively concluded that the greater local heat generation in the preparative run, caused the extraction of the lost peptide.

A succeeding paper [3] discusses the homology of the presented sequence to that of the distal histidine sequence of the globin family and the probable catalytic capacities which this sequence might be responsible for as a part of the active site of horseradish peroxidase.

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