# Neutrophils convert tyrosyl residues in albumin to chlorotyrosine

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**Abstract Hypochlorous acid chlorinates tyrosyl residues in small peptides to produce chlorotyrosine. Detection of chlorotyrosine has the potential to unequivocally identify the contribution hypochlorous acid makes to inflammation. I have developed a selective and sensitive HPLC assay for measuring chlorotyrosine. When albumin was exposed to reagent hypochlorous acid, or that produced by myeloperoxidase and stimulated neutrophils, tyrosyl residues in the protein were converted to chlorotyrosine. About 2% of the hypochlorous acid generated by neutrophils was accounted for by the formation of chlorotyrosine. These results demonstrate that chlorotyrosine will be a useful marker for establishing a role for hypochlorous acid in host defence and inflammation.**

*Key words:* Myeloperoxidase; Neutrophil; Hypochlorous acid; Chlorotyrosine; Inflammation

## 1. Introduction

Neutrophils are considered to cause much of the tissue damage that occurs in inflammation [1]. To date, the contribution their oxidants make to tissue injury remains equivocal. This is primarily due to the difficulty of detecting short-lived oxidants, and the lack of availability of unique markers for these reactive intermediates. Hypochlorous acid, generated by myeloperoxidase, hydrogen peroxide and chloride, is the strongest oxidant produced by neutrophils and monocytes in appreciable amounts [2,3]. Identification of a specific marker for hypochlorous acid is essential to understanding the extent to which this oxidant is involved in inflammation and microbial killing.

Hypochlorous acid converts tyrosyl residues in small peptides to chlorotyrosine [4]. If chlorotyrosine is formed in proteins at sites of inflammation, it has the potential to be a marker for the production of hypochlorous acid. However, it has yet to be shown that chlorination of tyrosyl residues in proteins occurs. In this investigation I have developed a sensitive and selective assay to measure chlorotyrosine in hydrolysed proteins. Using this assay, I determined the ability of hypochlorous acid to chlorinate tyrosyl residues in human serum albumin.

## 2. Materials and methods

Myeloperoxidase was purified from human leukocytes as described previously [5]. Its purity index ($A_{430}/A_{280}$) was greater than 0.72 and its concentration was determined using $\varepsilon_{430}$ 91,000 $M^{-1} \cdot cm^{-1}$ per haem [6]. Albumin was purified from human blood by ion exchange chromatography [7] and was a gift from Dr. Stephen Brennan, of the Molecular Pathology Laboratory at the Christchurch School of Medicine. Phorbol myristate acetate, substituted tyrosines, pronase E, bovine liver catalase, bovine erythrocyte superoxide dismutase, 5,5′-dithiobis(2-nitrobenzoic acid), and 1-nitroso-2-naphthol were purchased from the Sigma Chemical Co. (St. Louis MO, USA). Chlorotyrosine was also synthesized by adding HOCl to Gly-Gly-Tyr-Arg and then digesting the peptide with pronase [4]. 5-Thio-2-nitrobenzoic acid was prepared from 5,5-dithiobis(2-nitrobenzoic acid) as described previously [8]. Hydrogen peroxide solutions were prepared by diluting a 30% stock and calculating its concentration using $\varepsilon_{240}$ 43.6 $M^{-1} \cdot cm^{-1}$ [9]. Hypochlorous acid was purchased from Reckitt and Colman (NZ) Ltd, Auckland, New Zealand. Its concentration was determined by measuring the loss in absorbance at 290 nm after reacting it with monochlorodimedon ($\varepsilon_{290}$ 19,000 $M^{-1} \cdot cm^{-1}$) [10].

### 2.1. Isolation of human neutrophils

Neutrophils were isolated from blood of healthy donors by Ficoll-Hypaque centrifugation, dextran sedimentation and hypotonic lysis of red cells [11]. Cell preparations contained 95–97% neutrophils. After isolation, neutrophils were resuspended in phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, pH 7.4, with 140 mM sodium chloride) containing 1 mM calcium chloride, 0.5 mM magnesium chloride and 1 mg/ml of glucose.

### 2.2. Chlorination of albumin in cell free systems

Albumin was dissolved in PBS to give a final concentration of 500 $\mu g \cdot ml^{-1}$, and hypochlorous acid was added while vortexing the solution. To ensure all the HOCl had reacted, the solutions were left for an hour at room temperature. Pronase (200 $\mu g \cdot ml^{-1}$) was then added to digest the albumin, and the solution was incubated for 24 h at 37°C. In this time, at least 85% of the tyrosyl residues in albumin were hydrolysed to free tyrosine. When pronase was incubated alone over this period there was negligible release of tyrosine.

Albumin was also chlorinated with myeloperoxidase, hydrogen peroxide and chloride. Reactions were at 20°C and started by adding hydrogen peroxide to PBS containing 50 nM myeloperoxidase and 500 $\mu g \cdot ml^{-1}$ of albumin. To prevent inactivation of myeloperoxidase, hydrogen peroxide was added at 10 min intervals in aliquots of 50 $\mu$M or less. After an hour, samples were digested with pronase as described above.

### 2.3. Chlorination by stimulated neutrophils

Neutrophils ($2 \times 10^6$/ml) were suspended in PBS containing 1.0 mM calcium chloride, 0.5 mM magnesium chloride, 1 $mg \cdot ml^{-1}$ of glucose, and 500 $\mu g \cdot ml^{-1}$ of albumin. After 5 min at 37°C, the cells were stimulated with 100 ng/ml of phorbol myristate acetate and after a set time they were pelleted by centrifugation. The supernatant was removed and pronase was added to digest the albumin as described above.

### 2.4. Hypochlorous acid production by neutrophils

To determine hypochlorous acid production, cells were stimulated in the presence of 15 mM taurine and 500 $\mu$g/ml of albumin. The formation of taurine chloramine was measured by its ability to oxidise 2-nitro-5-thiobenzoate [12].

### 2.5. Derivatization of substituted tyrosines

Tyrosine was derivatized with 1-nitroso-2-naphthol essentially as described by Waalkes and Udenfriend [13]. Pronase digests were diluted 1:1 with 0.6 M trichloroacetic acid and after 10 min were centrifuged at 10,000 rpm to pellet any undigested protein. To 100 $\mu$l of the supernatant, or 100 $\mu$l of substituted tyrosines in 0.3 M trichloroacetic acid, were added 167 $\mu$l each of 1.5 M nitric acid, 0.1 M sodium nitrite, and

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7.5 mM 1-nitroso-2-naphthol in 95% ethanol. Solutions were then mixed and heated at 60°C for 20 min. After allowing the solutions to cool, the derivatized tyrosines were extracted by adding 0.4 ml of deionised water and 4 ml of methylene chloride. The mixtures were then shaken vigorously and centrifuged for 5 min at $1000 \times g$. The upper aqueous phase was removed for analysis. 3-Chlorotyrosine and 3-bromotyrosine also reacted with 1-nitroso-2-naphthol to give fluorescent products with excitation and emission spectra similar to those of derivatized tyrosine (not shown). The fluorescence of these products was about 25% and 10% of that of derivatized tyrosine, respectively. Other potential products of tyrosyl residues in proteins, including 3-iodotyrosine, *o*-tyrosine, *m*-tyrosine, 3-nitrosotyrosine, and DOPA did not give compounds with appreciable fluorescence after reaction with 1-nitroso-2-naphthol. Fluorescence of the derivatized tyrosines was measured in an Aminco Bowman spectrophotofluorimeter with excitation and emission wavelengths of 460 nm and 570 nm, respectively.

*2.6. Separation and analysis of derivatized tyrosines*

Derivatized tyrosine and chlorotyrosine were separated by reversed phase high performance liquid chromatography (HPLC) using a 250 × 4.6 mm Nucleosil 5 μm C18 AB column (Macherey-Nagel, Düren, Germany). The eluant was water/acetonitrile/acetic acid (77:23:0.1) and the flow rate was 0.8 ml/min. After 15 min of elution, the column was washed for 5 min with 100% acetonitrile, then re-equilibrated with the eluant for 10 min. Typically 50 to 100 μl of derivatized tyrosines were injected on to the HPLC column and they were detected using a Jasco FP-920 fluorescence detector with excitation and emission wavelengths of 375 and 530 nm, respectively. Concentrations of tyrosine and chlorotyrosine in digested peptides were calculated by comparing their peak areas in HPLC chromatograms to those obtained for a series of standards of known concentration.

## 3. Results

*3.1. HPLC separation of derivatized tyrosine and 3-chlorotyrosine*

When tyrosine and 3-chlorotyrosine were derivatized with 1-nitroso-2-naphthol, they were readily separated by reversed phase chromatography (Fig. 1, trace a). The peak area of fluorescence for chlorotyrosine increased proportionally as 0.2 to 100 pmoles of derivatized chlorotyrosine was loaded onto the HPLC column. The fluorescence of the tyrosine derivative was linear between 1 and 200 pmoles. Due to tailing of the tyrosine peak, a maximum of 1 chlorotyrosine per 1000 tyrosines could be detected in this assay. Bromotyrosine could also be detected and it eluted immediately after chlorotyrosine (results not shown).

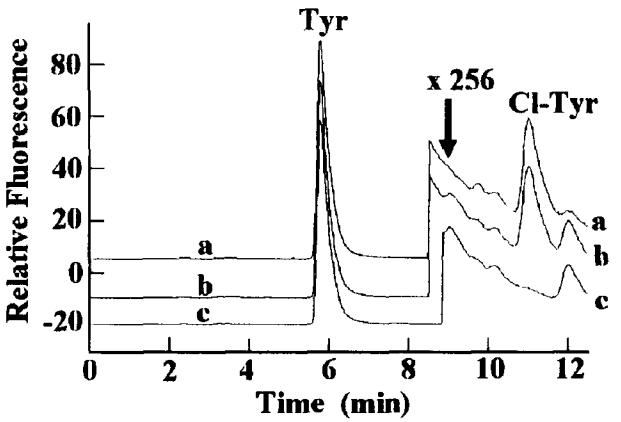


Fig. 1. Detection of chlorotyrosine by reversed phase HPLC. (a) A mixture of tyrosine (250 pmoles) and chlorotyrosine (2.5 pmoles) was derivatized with 1-nitroso-2-naphthol and separated by HPLC with fluorescence detection. (b) Albumin from the supernatants of stimulated or (c) unstimulated neutrophils was hydrolysed and derivatized with 1-nitroso-2-naphthol. Reaction and HPLC conditions plus the derivatization procedure are described in section 2. The arrow indicates where the sensitivity of the fluorimeter was increased 256 times. Tyr and Cl-Tyr indicate positions of the tyrosine and chlorotyrosine peaks, respectively.

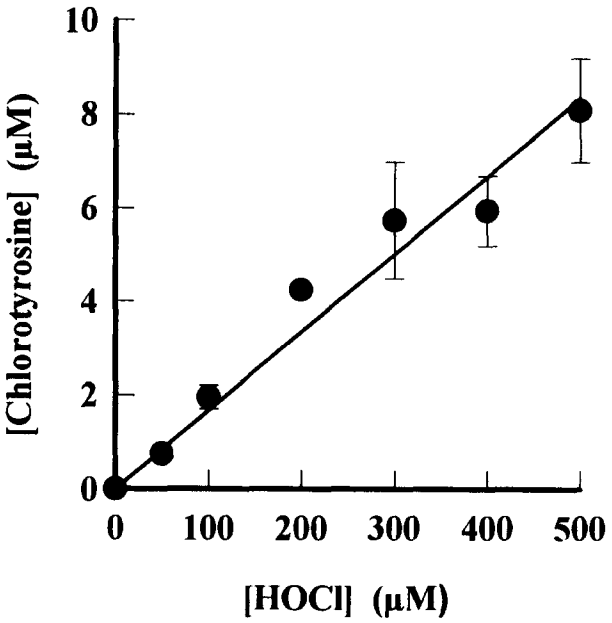


Fig. 2. Chlorination of albumin by hypochlorous acid. Hypochlorous acid was added to 500 μg/ml of albumin in PBS. Conversion of tyrosyl residues to chlorotyrosine was determined as described in section 2. Results are means and ranges of duplicate experiments.

*3.2. Chlorination of albumin by hypochlorous acid and myeloperoxidase*

Addition of increasing concentrations of hypochlorous acid to albumin produced increasing amounts of chlorotyrosine (Fig. 2). About 2% of the hypochlorous acid was accounted for by the production of chlorotyrosine. Myeloperoxidase, hydrogen peroxide and chloride also chlorinated albumin to form chlorotyrosine. As shown in Fig. 3, chlorination of tyrosyl residues became apparent at concentrations of hydrogen peroxide above 10 μM. Approximately 3% of the hydrogen peroxide was accounted for by the production of chlorotyrosine. No chlorination occurred in the absence of enzyme, hydrogen peroxide or chloride.

*3.3. Chlorination of albumin by stimulated human neutrophils*

Neutrophils were incubated with human serum albumin and stimulated with phorbol myristate acetate for 40 min. When the proteins from the supernatant were hydrolysed, derivatized with 1-nitroso-2-naphthol, and subjected to HPLC, a peak eluted at 11 min which corresponds to that for authentic chlorotyrosine (Fig. 1, compare traces a and b). Chlorotyrosine mixed with hydrolysed supernatant gave only one distinct peak which eluted at 11 min (not shown). No peak corresponding to chlorotyrosine eluted when cells were not stimulated (Fig. 1, trace c), or when albumin was omitted from the incubation (result not shown). Chlorotyrosine production was detected after a lag of 5 min following stimulation and continued for at least 60 min (Fig. 4). Formation of chlorotyrosine was inhibited

by the haem poison azide, by catalase, which scavenges hydrogen peroxide, and by methionine and taurine which scavenge hypochlorous acid (Fig. 5). The neutrophils produced 43 ± 14 μM ($n = 4$; S.D.) of hypochlorous acid and 0.66 ± 0.22 μM ($n = 4$; S.D.) of chlorotyrosine in 30 min. This indicates that 1.5% of the hypochlorous acid generated was accounted for by the formation of chlorotyrosine. It represents chlorination of about 1 in 200 tyrosyl residues of albumin in 30 min.

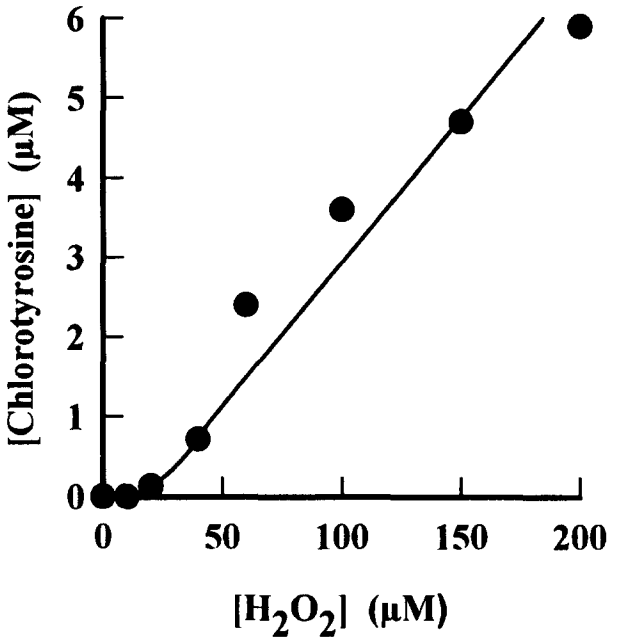


Fig. 3. Chlorination of albumin by myeloperoxidase, hydrogen peroxide and chloride. Increasing concentrations of hydrogen peroxide were added to 50 nM myeloperoxidase in PBS containing 500 μg/ml of albumin. Conversion of tyrosyl residues to chlorotyrosine was determined as described in section 2. Results are representative of three experiments.

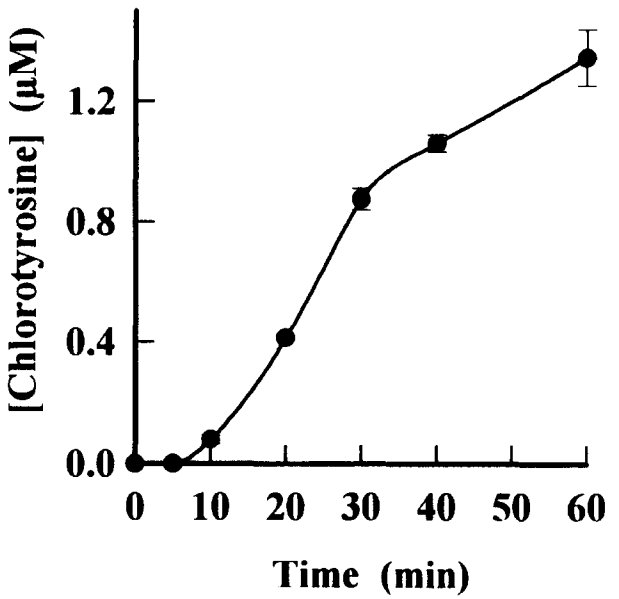


Fig. 4. The time course for production of chlorotyrosine in albumin by stimulated neutrophils. Neutrophils were incubated with 500 μg/ml of albumin and stimulated with phorbol myristate acetate. Reaction conditions and determination of chlorotyrosine are described in section 2. Results are expressed as means and ranges of duplicate experiments.

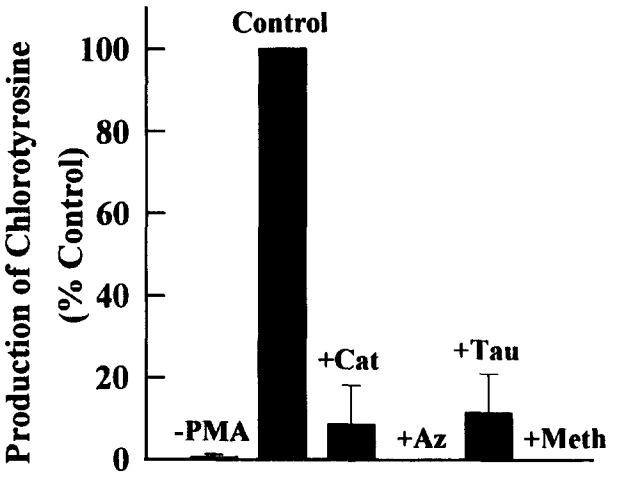


Fig. 5. Effect of inhibitors on the chlorination of albumin by neutrophils. Neutrophils were incubated with 500 μg/ml of albumin and stimulated with phorbol myristate acetate (PMA) for 30 min in the absence (control) or presence of 50 μg/ml of catalase (Cat), 1 mM azide (Az), 10 mM taurine (Tau), or 1 mM methionine (Meth). Reaction conditions and determination of chlorotyrosine are described in section 2. Results are expressed as means and standard deviations of four separate experiments.

## 4. Discussion

In this investigation I have demonstrated that hypochlorous acid reacts with human serum albumin to chlorinate its tyrosyl residues. Chlorination also occurred with purified myeloperoxidase and with stimulated neutrophils. Chlorotyrosine production by neutrophils was dependent on myeloperoxidase and hypochlorous acid, since the reaction was inhibited by the haem poison azide, and was blocked by catalase, taurine and methionine. The time course for the formation of chlorotyrosine was similar to that for hydrogen peroxide production by neutrophils [14], and is consistent with chlorotyrosine being a product of the respiratory burst.

Production of chlorotyrosine accounted for less than 3% of the hypochlorous acid that reacted with albumin. Others have shown that hypochlorous acid reacts with proteins by preferentially oxidising cystyl, methionyl, and tryptophanyl residues, and chlorinating amine groups [15–17]. The latter breakdown to form aldehydes and nitriles [18]. Although chlorotyrosine is a minor reaction product, it is the only modification of proteins identified to date that is unique to hypochlorous acid. Halogenation of aromatic rings is an enzymatic activity that is specific to peroxidases and myeloperoxidase is the only human enzyme capable of producing hypochlorous acid, and of chlorinating tyrosyl residues [19]. Therefore, detection of chlorotyrosine has the potential to unequivocally identify the contribution myeloperoxidase makes to inflammation.

The HPLC assay developed to measure chlorotyrosine in hydrolysed proteins is selective for a limited number of tyrosine derivatives, without interference from any other naturally occurring amino acids [13]. It is extremely sensitive and can be used to detect as little as 200 femtomoles of chlorotyrosine. The assay will be appropriate to use for analysing protein samples

taken from sites of inflammation, or from target cells exposed to neutrophils. Thus, analysis of chlorotyrosine can now be used to probe the as yet uncertain role of hypochlorous acid in inflammation and host defense.

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