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3-chlorotyrosine formation versus other molecular changes induced by hypochlorous acid in proteins: A study using dairy proteins as a model

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ABSTRACT

General markers of HOCl induced changes in food proteins were evaluated and compared with the specific indicator 3-chlorotyrosine using dairy proteins at various oxidant/protein ratios and at different pHs. Protein aggregation was more pronounced at alkaline pH and already observed at a ratio of 0.3 mmol HOCl/g protein. Tryptophan, methionine, tyrosine and lysine in whey proteins showed more degradation at pH 8.0, whereas methionine and histidine in caseins were more vulnerable for degradation at pH 5.8. Total thiol content was strongly decreased, up to 75% at 4.8 mmol HOCl/g whey protein with more degradation at acidic pH while in caseins it remained constant. The available lysine content notably decreased upon HOCl treatment and was more pronounced at pH 8.0. The levels of 3-chlorotyrosine increased as function of the oxidant/protein ratio and reached a maximum at 2.8 mmol HOCl/g whey and casein proteins. The 3-chlorotyrosine concentration was observed the least at pH 8.0, while the increase in protein carbonyls depended only on the HOCl/protein ratio, but not on the pH. It is concluded that 3-chlorotyrosine provides a more accurate assessment of the impact of HOCl damage on proteins in foods.

1. Introduction

Hypochlorous acid (HOCl) is a strong oxidizing agent, destroying target organisms by oxidation of their cellular material. HOCl has been shown to be effective in eliminating large populations of microorganisms and to extend the shelf life of many foods, including meat, poultry, fish and fish products, fruits and vegetables (Report of a Joint FAO/WHO Expert Meeting, 2008; Reuter, 1998; Stober, Dinnel, Hurlburt, & DiJulio, 1980; Vandekinderen et al., 2009). The reaction of HOCl is however not limited to microorganisms but also organic compounds found in food matrices. Several studies in the 80s and 90s have reported incorporation of chlorine into beef, pork, chicken and shrimp (Johnston, Ghanbari, Wheeler, & Kirk, 1983; Cunningham & Lawrence, 1977). Chlorine (1.1%) was shown to oxidize cysteine and methionine, destroy a proportion of tyrosine and histidine and cause some deamidation

Abbreviations used: HOCl, hypochlorous acid; SDS-PAGE, sodium dodecyl sulphate – polyacrylamide gel electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; TCA, trichloroacetic acid.

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of wheat proteins (Ewart, 1968). In addition, chlorine that was applied to cake flour at levels of 0–8.93 g/kg caused some changes in the sulfhydryl content, degradation of the aromatic amino acids, and the chlorination of tyrosine have also been reported (Tsen, Kulp, & Daly, 1971).

Currently, the use of chlorine as a sanitizer to decontaminate animal products is prohibited in the EU. It is however used as a hygienic processing aid in for instance water used for irrigation or for washing fresh cut fruits and vegetables (Alegria et al., 2009; Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009; Rico, Martin-Diana, Barat, & Barry-Ryan, 2007). Apart from potential abuse of hypochlorite as a decontamination agent, applied concentrations used in sanitizing applications can be too high because of insufficient care or ignorance. Moreover consumer's acceptance to the use of bleach in contact with foods is not general, turning it into a quality issue (MacRitchie, Hunter, & Strachan, 2014).

Recently we reported potential use of 3-chlorotyrosine as an indicator for the treatment of fish fillet with hypochlorite (Bao Loan, Devlieghere, Van Hoeke, & De Meulenaer, 2015). Reaction of free tyrosine with HOCl resulted in the formation of 4-hydroxyphenylacetaldehyde, 3-chlorotyrosine, and 3,5-dichlorotyrosine. In protein or peptide-bound tyrosine however, only the formation of 3-chlorotyrosine and 3,5-dichlorotyrosine was reported (Fu, Wang, Davies, & Dean, 2000). Obviously, hypochlorite induces considerably more changes in proteins than only the conversion of tyrosine to 3-chlorotyrosine and further to 3,5-dichlorotyrosine. Indeed, various authors described the formation of protein carbonyls and the reaction products of various amino acids with HOCl. Protein carbonyls are readily formed when proteins are treated with hypochlorite due to the decomposition of the formed chloramines (Chapman, Senthilmohan, Winterbourn, & Kettle, 2000). However, protein carbonyls can be formed via many other routes, and thus cannot be considered as a specific marker (Winterbourn & Kettle, 2000). Similarly, tryptophan, lysine and histidine are readily reacting with hypochlorite, but the reactions products formed can also be produced via other oxidation routes (Ferrer, Alegria, Farre, Abellan, & Romero, 2003; Hawkins, Pattison, & Davies, 2003; Pattison & Davies, 2001). In addition, other changes with the sulphur-containing side chains of methionine, cystine and cysteine residues, are difficult to access because of the instability of the reaction products, thus limiting their applicability to be used as a biomarker for hypochlorite exposure (Monnier, Nemet, Sell, & Weiss, 2011; Pitt & Spickett, 2008).

Previously, we reported on the oxidation of dairy proteins with hypochlorite at one particular pH, but 3-chlorotyrosine was not one of the markers monitored (Kerkaert et al., 2011). As an elaboration of this earlier work, the impact of hypochlorite on these model food proteins was currently studied at various pH levels. Various molecular changes on protein level were considered and additionally with the formation of 3-chlorotyrosine. In consequence, the potency of 3-chlorotyrosine was evaluated as an indicator for the use of hypochlorite in contact with foods in comparison with the other molecular changes in proteins.

2. Materials and methods

2.1. Chemicals

Whey protein isolate (Lacprodan® DI-9224) and caseinate (Miprodan® 30) both containing 88% protein, 1.5% fat, 0.3% sugar, 4% ash and 6% moisture were delivered by Acatris Food Belgium (Londerzeel, Belgium). Gel and standards for SDS-PAGE were from Bio-Rad (Nazareth, Belgium). Pyridine and trifluoroacetic acid were of analytical grade and obtained from Merck (Darmstadt, Germany). Acetonitrile HPLC grade was obtained from VWR (Leuven, Belgium). All other chemicals and reagents were of analytical or HPLC grade and obtained from Sigma Aldrich (Bornem, Belgium) and Chemlab (Zedelgem, Belgium).

2.2. Oxidation of milk proteins with HOCl

Proteins and HOCl were dissolved in a 0.1 M potassium phosphate buffer at pH 8.0, 7.4 and 5.8 and in an acetate buffer containing 0.1 M acetic acid and 0.013 M sodium acetate at pH 3.8. For caseins, the experiments were only performed at pH 8.0, 7.4 and 5.8 since they precipitate at pH 3.8 (below their isoelectric point of 4.6). The HOCl stock standardization, protein oxidation and protein content determination were done as described earlier (Kerkaert et al., 2011).

2.3. SDS-PAGE

Proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described earlier (Kerkaert et al., 2011). Diluted samples were 1:1 mixed with Laemmli buffer, heated (5 min at 90 °C) and centrifuged. 10 μg of protein was brought onto a 12% or 15% polyacrylamide Tris–HCl gel. Gels were developed Biosafe Coomassie and read using a Bio-Rad Gel Doc™ Imager (Nazareth, Belgium).

2.4. Carbonyl assay

Protein carbonyls were determined as described earlier (Kerkaert et al., 2011). Proteins were reacted with 2,4-dinitrophenylhydrazine (DNPH) and unreacted DNPH was removed by precipitating the proteins using trichloroacetic acid (TCA) and washing the protein pellet with ethanol:ethylacetate (1:1, v/v). Absorbance of the dissolved pellet (in 0.5 mL of 6 M urea in 20 mM phosphate buffer) was read at 370 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer (Nazareth, Belgium). The carbonyl content was expressed in μmol carbonyls/g protein using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.5. Amino acid analysis

Proteins were hydrolysed to their constituent amino acids which were then derivatized with ortho-phthaldialdehyde and 9-fluorenylmethyl chloroformate and separated on HPLC as described earlier (Kerkaert et al., 2011). Before hydrolysis, the oxidized proteins were precipitated with TCA and then incubated on ice during 10 min followed by a centrifugation at 4053 ×*g* for 10 min at 4 °C. The obtained pellet was redissolved in 5 mL of 0.1 M phosphate buffer. For the acid hydrolysis, 1.3 mL of the redissolved pellet was added to 3.7 mL of 8 M HCl. For basic hydrolysis, 2 mL of redissolved pellet was added to 2 mL of 8 M NaOH. Both mixtures were incubated for 24 h at 110 °C, and further neutralised before analysis. Amino acids were derivatized in the injector of an Agilent 1100 system (Agilent Technologies, Switzerland) and separated on a Zorbax Eclipse AAA Rapid Resolution column (4.6 × 150 mm, Agilent Technologies).

2.6. Total thiol groups

The oxidation of the sulphur containing amino acids was monitored by the Ellman method as described earlier (Kerkaert et al., 2011). One mL of 10 M urea (in Tris–HCl) and 20 μL of mercaptoethanol was added to 0.2 ml of protein solution. After incubation (1 h), proteins were precipitated with TCA (20%). The pellet was washed with TCA (20%) and redissolved in 3 mL of 50 mM Tris–HCl buffer (pH 8.0) containing 8 M urea. Subsequently 20 μl of