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Hypochlorous and Peracetic Acid Induced Oxidation of Dairy Proteins

Barbara Kerkaert, Frédéric Mestdagh, Tatiana Cucu, Philip Roger Aedo, Shen Yan Ling, and Bruno De Meulenaer*

NutriFOODchem unit, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

Hypochlorous and peracetic acids, both known disinfectants in the food industry, were compared for their oxidative capacity toward dairy proteins. Whey proteins and caseins were oxidized under well controlled conditions at pH 8 as a function of the sanitizing concentration. Different markers for protein oxidation were monitored. The results established that the protein carbonyl content was a rather unspecific marker for protein oxidation, which did not allow one to differentiate the oxidant used especially at the lower concentrations. Cysteine, tryptophan, and methionine were proven to be the most vulnerable amino acids for degradation upon hypochlorous and peracetic acid treatment, while tyrosine was only prone to degradation in the presence of hypochlorous acid. Hypochlorous acid induced oxidation gave rise to protein aggregation, while during peracetic acid induced oxidation, no high molecular weight aggregates were observed. Protein aggregation upon hypochlorous acid oxidation could primarily be linked to tryptophan and tyrosine degradation.

KEYWORDS: Protein oxidation; whey proteins; caseins; hypochlorous acid; peracetic acid

INTRODUCTION

Hypochlorous acid is a strong antimicrobial agent, commonly used in the food industry as a disinfection agent. It is used to sanitize food products such as vegetables (*1*), fruits (*2*), and meat (*3*, *4*) besides the disinfection of processing equipment (*5*) and waste water (*6*). Hypochlorous acid is a potent oxidant which induces a cascade of oxidation reactions resulting in the loss of valuable nutrients and the potential production of toxic chlorinated compounds in foods (*2*, *5*, *7*). The formation of these harmful byproducts resulted recently in the evaluation of new sanitizers such as peracetic acid, chlorine dioxide, and hydrogen peroxide (*2*). These products seemed effective in enhancing the shelf life of different food products (*1*−*3*) without affecting the sensorial quality of the product. Nevertheless, the use of these sanitizers is still under discussion since little is known about the nutritional losses and the formation of potential toxic byproducts. While the use of decontaminating treatments on meat carcasses is approved by the United States Food and Drug Administration (FDA), it is still not authorized in Europe. Recently, the European Food Safety Authority (EFSA) reported that poultry carcass decontamination with trisodium phosphate, acidified sodium chlorite, chlorine dioxide, or peracetic acid poses no toxicological risk to human health (*8*). Despite the fact that the reactivity of hypochlorous acid toward specific amino acids and plasma proteins has been studied in detail (*7*, *9*, *10*), studies on food proteins are scarce. Moreover, for peracetic acid no major studies on its interaction with proteins were published. Therefore, it was the aim of this study to investigate and compare the chemical changes in two major food proteins, caseins and whey proteins, due to their interactions with these two oxidizing agents.

The reaction mechanism of hypochlorous acid toward amino acids and proteins was thoroughly studied and elucidated by Hawkins et al. (*7*) and Pattison and Davies (*10*), who both described that hypochlorous acid readily attacks proteins and forms unstable chloramines on the α-amino group and lysine side chain, decomposing to reactive carbonyls (**Figure 1**). As illustrated in **Figure 1**, a Schiff base reaction between these carbonyls and the free amino groups results in protein aggregation (*7*, *11*). Besides α- and γ-amino groups, sulfur side chains and aromatic amino acids are vulnerable to hypochlorous acid (*7*). An unstable sulfenylchloride will be formed upon hypochlorous acid induced oxidation of cysteine, which will further react resulting in protein cross-linkage (**Figure 1**). The reaction between the different aromatic amino acids and hypochlorous acid is described by Hawkins et al. (*7*), who also reported that hypochlorous acid has the highest activity toward methionine and cysteine, followed by histidine, α-amino groups, tryptophan, lysine, and tyrosine at pH 7.4. As mentioned before, in contrast to hypochlorous acid, the reactivity of peracetic acid toward proteins is not thoroughly investigated. It is, however, known that peracetic acid in solution is consumed by spontaneous decomposition and hydrolysis resulting in acetic acid (AA), hydrogen peroxide (H_2O_2), and oxygen (O_2), thus representing a loss of oxidative power (*12*). Besides decomposition and hydrolysis of peracetic acid, homolysis of this oxidant will occur, resulting in peroxyl and hydroxyl radicals (**Figure 2**). The hydroxyl radicals formed are known for their high reactivity toward food components and are even supposed to

*Corresponding author. Tel: +3292646166. Fax:+3292646215. E-mail: Bruno.DeMeulenaer@UGent.be.

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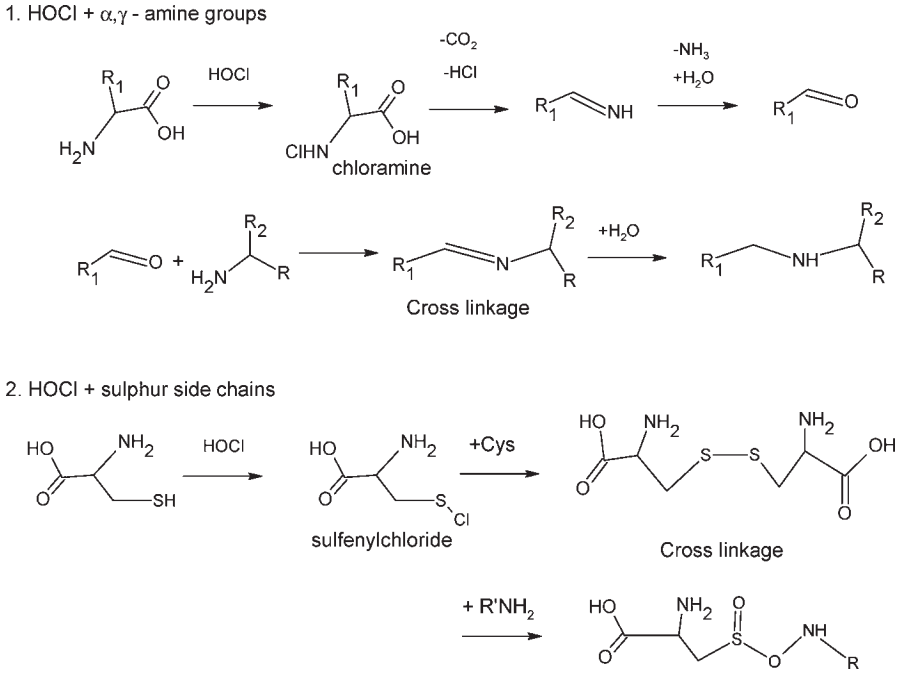

Figure 1. Reaction between HOCl and amino acid side chains (*7*).

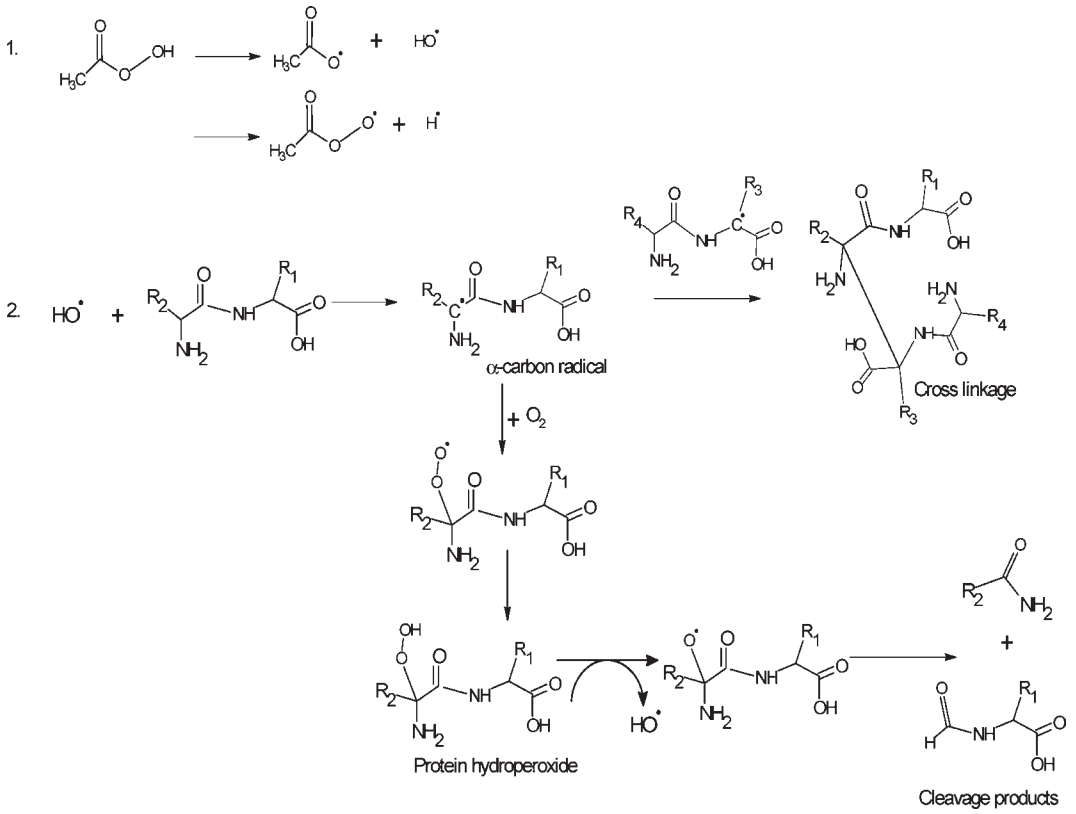

Figure 2. Radical production from peracetic acid and the reaction with proteins (*13*).

have a higher reactivity toward proteins than hypochlorous acid (*7*, *13*). These radicals are expected to primarily attack the protein backbone by hydrogen abstraction from the α-carbon position as illustrated in **Figure 2**. In the presence of oxygen, the α-carbon radical will react to a protein peroxyl radical which decomposes, resulting in different protein fragments, mainly carbonyl compounds (*14*). The same α-carbon radical can give rise to protein aggregates by cross-linkage, probably, however, to a smaller extent than protein fragmentation because of sterical hindrance (**Figure 2**).

In view of the limited information available with respect to the interactions between peracetic acid and proteins in general and between hypochlorous acid and food proteins in particular, this study intended to gain more knowledge in this respect, using two major food proteins, caseins and whey proteins. The modifications in both proteins upon hypochlorous and peracetic acid oxidation

were determined as a function of the oxidant concentration. The concentration varied between 0.02%, which is the highest concentration applied in the food industry, and 0.3% (*1*, *2*, *4*). Notwithstanding the fact that concentrations above 0.02% are less relevant from a practical point of view, we applied them in the present study in order to investigate the effect of both oxidants under extreme conditions.

MATERIALS AND METHODS

Whey protein isolate (WPI), 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). Peracetic acid was obtained from Christeyns (Ghent, Belgium). Acetonitrile and methanol were of HPLC grade and obtained from VWR (Leuven, Belgium). All other chemicals and reagents were all of analytical grade and were obtained from Sigma Aldrich (Bornem, Belgium) and Chemlab (Zedelgem, Belgium).

Oxidation of Milk Proteins with Hypochlorite or Peracetic Acid. The concentration of both hypochlorous acid and peracetic acid were determined by an iodometric titration. The solutions were incubated with potassium iodide, and the liberated iodine was titrated with a sodium thiosulphate solution. Both protein and oxidant were dissolved in a 0.1 M potassium phosphate buffer at pH 8. The protein solution was oxidized, under well controlled standardized conditions, by adding the oxidant solution to the protein solution at a ratio of 1/4 (v/v). The mixture was vortexed and incubated for 10 min at 30 °C. After incubation, the solution was placed on ice. Oxidation was performed in triplicate just as the different analyses to determine the protein modifications. All results are expressed as a function of the oxidant/protein ratio, which varied between 0.2 and 6.5 mmol oxidant/g protein. The protein content was determined by a Kjeldahl analysis, using a nitrogen-to-nitrogen conversion factor of 6.38, to express the results per mg protein (*15*).

Carbonyl Assay. Protein carbonyls were determined following a protocol adapted from Fenaille et al. (*16*). Carbonyls were derivatized with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of a stable dinitrophenyl hydrazone product. Briefly, 0.4 mL of DNPH (10 mM in 2 M HCl) was added to 0.3 mL of oxidized protein (±6−7 mg/mL), the mixture was incubated in the dark for 60 min. After incubation, 0.7 mL of TCA was added (10% final concentration), and samples were incubated on ice during 10 min followed by centrifugation at 10 000 rpm for 3 min. Protein pellets were washed three times with 1 mL of ethanol/ethylacetate (1:1, v/v) in order to remove the excess DNPH. The final pellet was dissolved in 0.5 mL of 6 M urea in 20 mM phosphate buffer (pH 2.3), and the absorbance was measured at 370 nm. Results were expressed in nmol carbonyls per mg protein using a molar absorption coefficient of 22 000 M^{-1} cm^{-1} on blank subtracted data.

Determination of Thiol Groups. The free and total thiol content was determined by the Ellman method adjusted from Beveridge et al. (*17*). For free thiols, 0.5 mL of oxidized protein was dissolved in 2.5 mL of 50 mM Tris-HCl buffer (pH 8) containing 8 M urea. Twenty microliters of 10 mM 5,5-dithiobis nitro-benzoic acid (DTNB) in Tris-HCl was added. After 5 min of incubation, the absorbance of the samples was measured at 412 nm before and after adding DTNB against a blank without protein. The thiol content was calculated using the molar extinction coefficient of 13 600 M^{-1} cm^{-1} on blank subtracted data. To determine the total thiol content, the disulfide bridges were first broken by adding 1 mL of 10 M urea (in Tris-HCl) and 20 μL of mercaptoethanol to 0.2 mL of oxidized protein. This mixture was incubated for 1 h after which the proteins were precipitated with 10 mL of TCA (20%). The obtained pellet was resuspended twice in 5 mL of TCA (20%) to remove the excess of mercaptoethanol. The final pellet was dissolved in 3 mL of the same buffer as samples for free thiols, and the procedure was followed as described above. Results were expressed in μmol thiol groups per g protein.

Determination of Free Lysine. The available lysine was determined by a fluorescence method based on Vigo et al. (*18*). Therefore, 25 μL of protein (±6−7 mg/mL) was added to 25 μL of 12% SDS. After overnight incubation at 4 °C, the samples were sonicated for 15 min at 30 °C after which 25 μL of the sample was mixed with 750 μL of OPA reagent (40 mg *ortho*-phthaldialdehyde (OPA), 1 mL of ethanol, 25 mL of 0.1 M borate buffer at pH 9.3, 2.5 mL of 20% SDS, and 0.1 mL of β-mercaptoethanol made up to 50 mL with distilled water) during 30 s on a vortex. The mixture was incubated for 90 s in the dark and vortexed another 15 s, and finally fluorescence was measured at an excitation of 340 nm and an emission of 450 nm (with a Spectramax Gemini XPS fluorimeter, Molecular Devices, Brussels, Belgium). Results were expressed in mg lysine/mg protein and calculated on the basis of a calibration curve of casein containing 11 residues of lysine.

Amino Acid Analysis. Proteins were hydrolyzed to their constituent amino acids which were then derivatized with OPA and 9-fluorenylmethylchloroformate (FMOC) and separated on HPLC (*19*). Before hydrolysis, the oxidized proteins (100 mg) were precipitated with TCA (15% final) and redissolved in 5 mL of 0.1 M phosphate buffer (pH 8) to which 50 μL of sodium hydroxide (10 M) was added. Acid and basic hydrolysis were performed on the redissolved pellet, together with Kjeldahl analysis (*15*). For the acid hydrolysis, 1.3 mL of the redissolved pellet was added to 3.7 mL of 8 M HCl containing 0.1% phenol and 0.1% Na_2SO_3 in a 5 mL glass tube with a Teflon screw cap. For basic hydrolysis, 2 mL of the redissolved pellet was added to 2 mL of 8 M NaOH. Both mixtures were vortexed, incubated for 24 h at 105 °C, neutralized, and further diluted to 20 mL. All of the amino acids were automatically derivatized with OPA, except proline with FMOC, in the injector of an Agilent 1100 system (Agilent Technologies, Switzerland). The derivatized amino acids were separated on a Zorbax Eclipse AAA Rapid Resolution column (4.6 × 150 mm, Agilent Technologies) and detected fluorometrically. A flow rate of 2 mL/min was applied with a gradient of solvent A (45% methanol, 45% acetonitrile, and 10% water) and solvent B (45 mM $NaH_2PO_4 \cdot H_2O$, 0.02% NaN_3, pH 7.8). Eluting conditions expressed as a portion of solvent B were from 0 to 1.9 min, 95%; at 17.9 min, 43%; at 18.4 min, 0%; until 22.2 min and at 23 min, 95%.

Determination of Amino Groups. Primary amino groups were determined after the reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS), following Fields (*20*). The protein solution was diluted in phosphate buffer to a concentration of 0.5 mg protein/mL. Then, 0.5 mL of this diluted solution was added to 0.5 mL of 0.1 M borate buffer dissolved in 0.1 M sodium hydroxide. After mixing the sample, 20 μL of TNBS was added to the mixture, which was vortexed and incubated for exactly 10 min. Finally, 2 mL of stopping solution (1.5 mL of 0.1 M sodium thiosulphate with 98.5 mL of 0.1 mL sodium phosphate) was added. The absorbance of the samples was measured at 420 nm, and the results were calculated by applying a molar extinction coefficient of the TNB complex of 22 000 M^{-1} cm^{-1} on blank subtracted data. The results were expressed as percentage of free amino groups of the initial concentration at 0 mmol oxidant/g protein.

Succinylation. Solid succinic anhydride (34 mg) was added to 5 mL of the 2 mg/mL oxidized protein solution to give a 50-fold excess of anhydride over protein amine groups (*21*). The anhydride was added slowly, and the pH was monitored. To maintain the pH at 8, 200 μL of 2 M NaOH was added. After the addition of anhydride, the solution was incubated for 30 min and subsequently diluted for SDS−PAGE analysis.

SDS−PAGE. Proteins were subjected to SDS−PAGE under reducing conditions. Therefore, 10 μL of diluted sample (1 mg protein/mL) was mixed with 10 μL of Laemmli buffer (Bio-Rad, Nazareth, Belgium) containing 0.05% mercaptoethanol. Samples were heated for 5 min at 90 °C and centrifuged for 3 min at 10 000 rpm. Subsequently, 10 μg of protein was brought onto a 12% or 15% polyacrylamide Tris-HCl gel (Bio-Rad), and electrophoresis was performed at a constant voltage of 150 V. The gels were stained with Biosafe Coomassie (Bio-Rad), and a precision plus protein standard (Bio-Rad) was used as a molecular weight marker.

RESULTS AND DISCUSSION

The oxidation of whey and casein proteins by both disinfectants resulted in different modifications depending on the type of oxidant and oxidant concentration. Before comparing both oxidants, a kinetic study was performed in which it was observed that the oxidative modifications were completed within 60 s at 30 °C (data not shown). For practical reasons, however, a standardized reaction time of 10 min was applied. Previously, hypochlorous acid induced protein oxidation was already reported to occur rapidly (*7*). Our findings indicate that peracetic acid also reacts

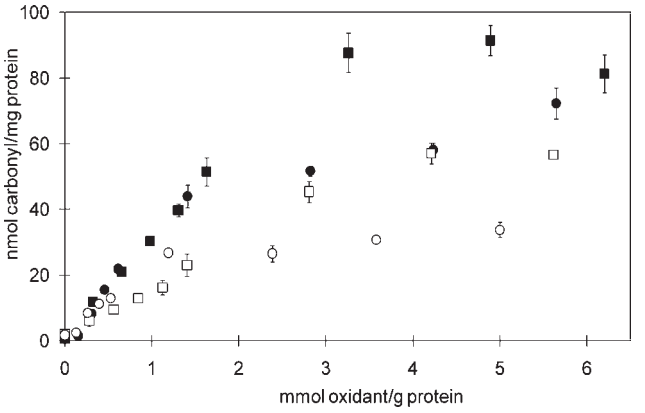

Figure 3. Carbonyl groups as a function of the oxidant concentration: hypochlorous acid−whey proteins (■); hypochlorous acid−caseins (□); peracetic acid−whey proteins (●); peracetic acid−caseins (○) (error bars, $n = 3$).

fast with proteins. As a further consequence, the oxidizing agents themselves were not detectable anymore by iodometric titration, soon after their application.

Amino Acid Modifications upon Hypochlorous and Peracetic Acid Oxidation. As described before, hypochlorous acid primarily reacts with the amino acid side chain, while peracetic acid rather attacks the protein backbone. In both cases, the modifications result in carbonyl compounds as illustrated in **Figures 1** and **2**. Therefore, protein carbonyl groups were determined as a general marker for protein oxidation together with the losses in specific amino acids prone to oxidation, namely, the sulfur amino acids, the aromatic amino acids, and lysine.

Protein Carbonyl Formation. As expected, protein carbonyls increased upon an increasing oxidant/protein ratio and this was the case for both oxidants (**Figure 3**). The concentrations determined after applying 0.5 mmol oxidant/g protein were comparable with the carbonyl content observed in heat treated milk, which was on average 12.4 nmol/mg (*22*). Up to a 1.5 mmol oxidant/g protein ratio, we observed that the protein carbonyl content for both oxidants showed a similar dependency upon the oxidant/protein ratio (**Figure 3**). At higher ratios, more protein carbonyls were formed after oxidation with hypochlorous acid in comparison to that with peracetic acid induced oxidation, and this was the case for both whey proteins and caseins. Protein carbonyls for peracetic acid treated proteins leveled off to, respectively, a 60 and 33 nmol/mg protein level for whey proteins and caseins, while for hypochlorous acid, a maximum plateau of, respectively, 90 and 57 nmol carbonyls/mg protein was reached from a ratio of 4 mmol oxidant/g protein onward. A possible reason for this observation could be the necessity of peracetic acid for transition metal ions to facilitate the radical induced protein oxidation. At higher oxidant/protein ratios, the trace amounts of metal ions inevitably present in the whey protein isolate used became the limiting factor in the peracetic acid induced protein carbonyl generation. The carbonyl formation was moreover stronger in whey proteins compared to that in caseins at all oxidant/protein ratios studied. In the case of hypochlorous acid induced oxidation, an almost 30% higher content of carbonyls (90 nmol/mg) in whey compared to that in caseins (60 nmol/mg) was observed, while for peracetic acid induced oxidation, the carbonyl content in whey proteins was nearly double. Considering the amino acid composition of both proteins, it was observed that the higher carbonyl content in whey proteins corresponded to a higher tryptophan and lysine content compared to that in caseins. These data indicate that tryptophan and/or lysine could play an important role in the generation of protein carbonyls upon hypochlorous and peracetic acid oxidation. In order to investigate this further, the degradation of both amino acids was determined together with the losses of other vulnerable amino acids such as cysteine, methionine, tyrosine, and histidine.

Table 1. Amino Acid Profile of Whey Proteins Oxidized with Hypochlorous or Peracetic Acid[a]

Hypochlorous Acid Oxidation				
mmol hypochlorous acid/g protein	0.5	1	2.5	5
Trp	5.1 a	4.9 a	0.0 b	0.0 b
Met	34.9 a	4.3 b	4.6 b	0.0 c
Tyr	90.6 a	55.3 b	5.2 c	0.0 d
His	96.1 a*	93.3 a*	65.6 b	17.2 c
Lys	95.1 a*	89.4 a	68.5 b	76.7 b
Peracetic Oxidation				
mmol peracetic acid acid/g protein	0.5	1	2.5	5
Trp	96.0 a*	73.4 b	44.9 c	20.4 d
Met	85.9 a	78.6 a	61.5 b	29.5 c
Tyr	97.0 a*	97.7 a*	97.7 a*	73.2 b
His	97.1 a*	96.9 a*	92.8[ab]	88.1 b
Lys	93.8 a*	94.4 a*	94.5 a*	89.2 a

[a] The results are expressed as % AA left of the initial content as a function of the oxidant concentration expressed as mmol oxidant/g protein. The initial amounts of Trp, Met, Tyr, Lys, and His were, respectively, 1.56; 2.43; 3.22; 9.74; and 1.88 g/100g protein. Each result is the mean of four repetitions. *Not significantly different from the initial content

Table 2. Amino Acid Profile of Caseins Oxidized with Hypochlorous or Peracetic Acid[a]

Hypochlorous Acid Oxidation				
mmol hypochlorous acid/g protein	0.5	1	2.5	5
Trp	5.3 a	3.7 a	0.0 b	0.0 b
Met	35.9 a	13.7 b	0.0 c	0.0 c
Tyr	101.7 a*	65.3 b	5.7 c	1.2 c
His	103.7 a*	107.8 a*	77.4 b	26.0 c
Lys	97.3 a*	93.1 a*	61.0 b	56.6 b
Peracetic Acid Oxidation				
mmol peracetic acid/g protein	0.5	1	2.5	5
Trp	79.8 a	50.1 b	17.8 c	4.1 d
Met	87.8 a	69.0 b	33.1 c	8.7 d
Tyr	100.0 a*	98.4 a*	87.4 b	52.1 c
His	100.6 a*	100.5 a*	100.9 a*	98.6 a*
Lys	99.6 a*	99.7 a*	100.7 a*	97.9 a*

[a] The results are expressed as % AA left of the initial content as a function of the oxidant concentration expressed as mmol oxidant/g protein. The initial amounts of Trp, Met, Tyr, Lys, and His were, respectively, 1.11; 2.65; 5.05; 6.85; and 2.51 g/100g protein. Each result is the mean of four repetitions. *Not significantly different from the initial content.

Degradation of Amino Acids. All amino acids were determined by amino acid analysis after acid and basic hydrolysis except for cysteine, which was determined by the Ellman method (*17*) since it is not stable upon hydrolysis. The losses in specific amino acids are given as a function of the oxidant concentration in **Tables 1** and **2**.

For hypochlorous acid induced oxidation, dramatic losses of tryptophan and methionine in both proteins were observed already at the lowest oxidant/protein ratio evaluated. Apart from a total tryptophan and methionine loss at the highest hypochlorous

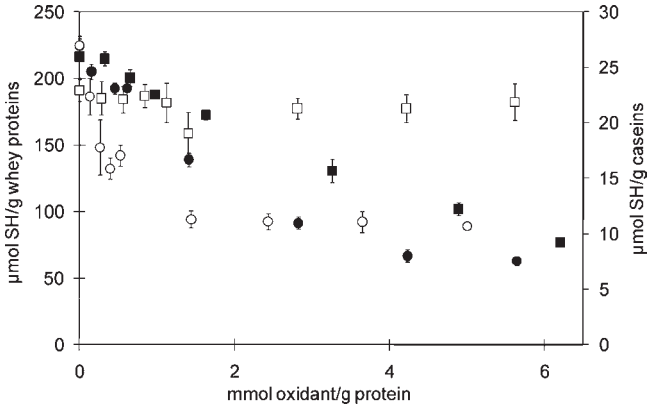

Figure 4. Total thiol content as a function of the oxidant concentration: hypochlorous acid−whey proteins (■); hypochlorous acid−caseins (□); peracetic acid−whey proteins (●); peracetic acid−caseins (○) (error bars, $n = 3$).

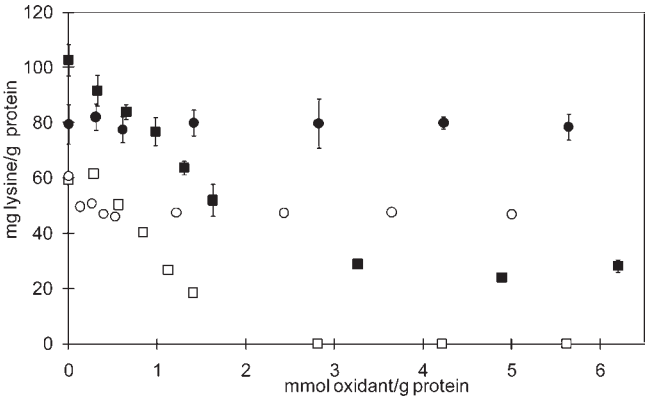

Figure 5. Free lysine as a function of the oxidant concentration: hypochlorous acid−whey proteins (■); hypochlorous acid−caseins (□); peracetic acid−whey proteins (●); peracetic acid−caseins (○) (error bars, $n = 3$).

acid/protein ratio studied, also tyrosine was completely destroyed. Histidine and lysine were also prone to hypochlorous acid degradation, albeit to a considerably lower extent. These two amino acids were only degraded after applying hypochlorous acid concentrations higher than 1 mmol/g protein. While histidine losses were more pronounced in whey proteins, lysine degradation was stronger in caseins upon hypochlorous acid treatment.

For peracetic acid induced oxidation, again tryptophan and methionine proved to be very vulnerable for degradation, although no complete loss was observed, even at the highest oxidant/protein ratio evaluated. Tyrosine and histidine were fairly stable in the presence of peracetic acid, only showing significant degradation at the highest oxidant/protein ratios considered. At the highest peracetic acid/protein ratio, respectively, 73 and 52% of the original tyrosine was still present in whey proteins and caseins. Histidine, however, only significantly decreased in whey proteins, while it remained stable in caseins. Lysine was not affected by peracetic acid, not even at the highest oxidant/protein ratio.

Despite the fact that methionine was shown to be very prone for oxidation, tryptophan was even more vulnerable, and this was irrespective of the oxidant. For hypochlorous acid, this was somewhat unexpected in view of the earlier reported rate constants for the reaction of this oxidant with methionine (10^7 M^{-1} s^{-1} at pH 7) and tryptophan (10^5 M^{-1} s^{-1} at pH 7). Similarly, the observed reactivity of hypochlorous acid toward tyrosine was unexpectedly high compared to that of histidine in view of the earlier reported discrepancy between the rate constant for tyrosine (44 M^{-1} s^{-1} at pH 7) and histidine (10^5 M^{-1} s^{-1} at pH 7) (*7*). Thus, it seems that the incorporation of amino acids in a more complex protein structure can result for some amino acids in a higher sensitivity toward hypochlorous acid induced degradation, while for other amino acids, a protective effect is induced. It remains unclear though to what extent this phenomenon could be protein specific, despite the fact this trend was observed for both caseins and whey proteins.

Since the amino acid analysis was not able to determine the cysteine content, the free and total thiol contents were determined by the Ellman method (**Figure 4**). Only the total thiols are given since the free thiols reacted away readily with the lowest oxidant concentration applied. Pattison et al. (*10*) reported earlier that the reaction of cysteine and methionine with hypochlorous acid was too fast for accurate measurement as a function of time. For the whey proteins, the total thiol content decreased with an increasing oxidant concentration after hypochlorous acid oxidation. This was, however, not observed for the caseins. For both proteins, the degradation was more pronounced upon oxidation with peracetic acid compared to that with hypochlorous acid, which was somewhat surprising because of the stronger carbonyl formation observed during hypochlorous acid oxidation. Remarkable as well was the fact that even at high oxidant/protein ratios a residual amount of disulfide bridges remained. This could potentially be attributed to steric effects preventing the oxidant from reacting with the disulfide groups present in the interior 3-D protein structure, although this seems less likely in the case of caseins.

Notwithstanding the fact that the lysine content was determined after hydrolysis by amino acid analysis, the available lysine content was also determined by the OPA method (**Figure 5**) in order to compare both methods. The available lysine content decreased to, respectively, 0 and 25 mg/g in caseins and whey proteins upon hypochlorous acid oxidation at a 3 mmol/g protein ratio. During peracetic acid oxidation, the available lysine content was not affected as was previously observed from **Tables 1** and **2**. While the results of the lysine content determined by both methods were comparable for peracetic acid treated proteins, strong discrepancies were observed between the lysine content of hypochlorous acid treated proteins determined by the mild OPA method and by amino acid analysis (**Figure 5** and **Tables 1** and **2**). In the latter conditions, eventual lysine−protein carbonyl adducts and chloramines were possibly hydrolyzed, resulting in an apparent overestimation of the available lysine content compared to those obtained by the mild OPA method measured on the intact protein. To which extent lysine−protein carbonyl adducts were formed is, however, not clear. While Hawkins et al. (*7*) and Matoba et al. (*11*) described the Schiff base formation between a protein carbonyl and a free amino group of lysine upon hypochlorous acid treatment, Chapman et al. (*22*) did not find any evidence for Schiff base formation during hypochlorous acid induced protein oxidation. In order to assess to what extent the ε-amino group of lysine is likely to react with protein carbonyls, oxidized whey proteins were incubated with boc-lysine. Since no decrease was observed in the protein carbonyls, even when an excess of 13 mol boc-lysine/mol carbonyls was applied (data not shown), it could be concluded that lysine−protein carbonyl interaction was very limited. These results moreover imply that the overestimation of the lysine content in hypochlorous acid treated proteins determined by amino acid analysis was mainly due to the hydrolysis of lysine chloramines.

The effect of the oxidizing agents on the free amino group content of the proteins was investigated (**Figure 6**). The method applied did not only determine the α-amino groups (*20*), making this method complementary to the mild OPA method, which only

determined the ε-amino group of lysine (*18*, *23*). Application of hypochlorous acid resulted in a total loss of free amino groups from a 3 mmol oxidant/g protein ratio irrespective of the protein considered. For peracetic acid, however, 40% of free amino groups remained intact even at the highest oxidant over protein ratio studied. Similar to the results observed for the lysine content upon hypochlorous acid oxidation, the degradation in free amino groups after hypochlorous acid treatment could be attributed to the formation of chloramines given the high molar excess of hypochlorous acid compared to the amount of free amino groups. In view of the reaction mechanism illustrated in **Figure 2**, it was expected that the peracetic acid treatment would result in fragmentation and as such an increase in the free amino groups. A decrease was, however, observed indicating that there are other more dominant reactions taking place during the peracetic acid treatment of proteins.

Comparing the impact of both oxidants used on the amino acid modifications, it became obvious that hypochlorous acid was a more potent oxidant compared to peracetic acid, even at oxidant/protein ratios lower than 2 mmol/g, which is the level below which no difference in the protein carbonyl content could be observed.

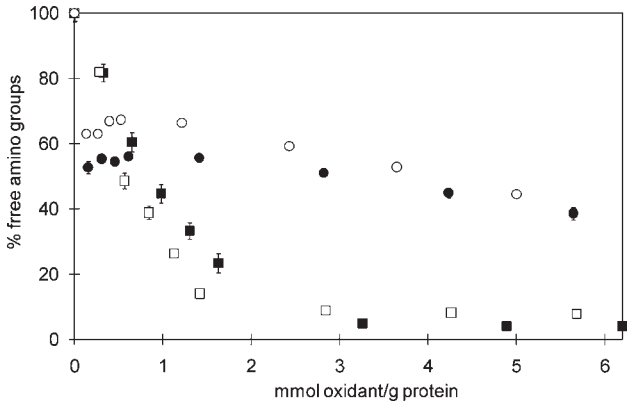

Figure 6. Percentage of loss of free amino groups as a function of the oxidant concentration: hypochlorous acid−whey proteins (■); hypochlorous acid−caseins (□); peracetic acid−whey proteins (●); peracetic acid−caseins (○) (error bars, $n = 3$).

In a similar perspective, it should be mentioned that at the higher oxidant/protein ratios tested, further amino acid degradation took place, despite the protein carbonyl content already reaching a plateau at an oxidant/protein ratio of 3 mmol/g. These observations illustrate that the protein carbonyl content is a less specific protein oxidation indicator which cannot be used to detect the fraudulent use of oxidative disinfectant agents for hygienic reasons. More specific markers, such as the tryptophan or methionine concentrations and the detection of their degradation products seem to be more appropriate. Meltretter et al. (*24*) suggested earlier that MALDI-TOF-MS could be a reliable tool to monitor chemical modifications of nutritional proteins.

SDS−PAGE. As illustrated before, the hypochlorous acid and peracetic acid induced modifications on an amino acid level can result in protein aggregation and less likely in fragmentation (**Figures 1** and **2**). In order to know to what extent these modifications occurred, SDS−PAGE was performed under reducing conditions (**Figure 7**). The bands for β-lactoglobulin (BLG, 18 kDa), α-lactalbumin (ALA, 14 kDa), and bovine serum albumin (BSA, 69 kDa) disappeared at an increasing hypochlorous acid/protein ratio (Gel A). Furthermore, new bands appeared between 25 and 37 kDa which intensified with the oxidant concentration until a hypochlorous acid/protein ratio of 4.9 mmol/g. These bands indicate the formation of BLG and ALA dimers. Besides dimers, high molecular weight aggregates were formed, observed from the protein band at the boundary of the running and the stacking gel (> 250 kDa). These bands, however, disappeared at the highest oxidant/protein ratio due to the formation of precipitates which were not loaded on the gel. The formation of high molecular weight aggregates was also observed upon hypochlorous acid oxidation of caseins (Gel C). Already, at the lowest oxidant/protein ratio evaluated, a smear could be observed in the 75−250 kDa range besides the presence of larger aggregates at the top and the bottom of the stacking gel. As a consequence, α_{s1}-casein (23 kDa), α_{s2}-casein (25 kDa), and β-casein (24 kDa) bands tended to disappear completely at a hypochlorous acid/protein ratio of 3.2. At higher oxidant/protein ratios, aggregates in the 75−250 kDa range and at the end of the stacking gel decreased while the band at the top of the stacking gel became more intense. Moreover, at this highest protein/oxidant ratio, precipitation was observed.

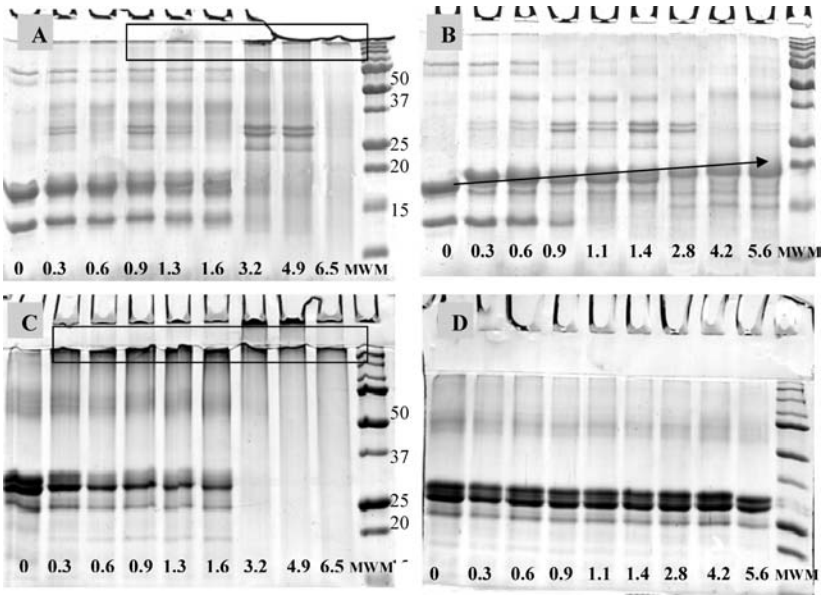

Figure 7. SDS−PAGE profiles of oxidized milk proteins as a function of the oxidant concentration (mmol oxidant/g protein): hypochlorous acid−whey proteins (**A**); peracetic acid−whey proteins(**B**); hypochlorousacid−caseins (**C**); peracetic acid−caseins (**D**). MWM, molecular weight marker.

In contrast to hypochlorous acid assisted oxidation, peracetic acid oxidation did not result in the formation of high molecular weight aggregates (Gel B, D). Nevertheless, low peracetic acid/protein ratios of, respectively, 0.3 and 0.9 mmol peracetic acid/g resulted in the loss of BLG and ALA. The loss of both bands was related to the appearance of a new band at 19 to 20 kDa whose mass further increased with peracetic acid concentration. Besides the formation of a modified BLG adduct, new bands appeared between 25 and 37 kDa and above 37 kDa at a 0.9 mmol peracetic acid/g protein ratio, indicating the formation of diand trimers of ALA and BLG. Upon peracetic acid oxidation of caseins (Gel D), neither aggregation nor fragmentation of the caseins could be observed.

The fact that upon peracetic acid oxidation no higher aggregates were formed could be an indication that the radicals produced in the protein backbone are not able to form oligomers with other protein radicals, most probably due to sterical hindrance. Thus, only minor bands for BLG dimers were observed at the highest peracetic acid concentration. Since the origin of the remarkable band appearing at a MW of 19−20 kDa is not clear, it deserves further investigation. This band could be a BLG adduct whose mass increased with oxidant concentration. However, it is also possible that it is an intramolecularly cross-linked form with a distinct electrophoretic mobility.

The protein aggregation observed in the case of hypochlorous acid oxidation could be attributed to different factors, in view of other changes on a molecular level discussed above. Since tryptophan was proven to be one of the major oxidation targets, it cannot be excluded that the earlier reported reactive intermediate 3-chloroindoline, which is formed upon a reaction between tryptophan and hypochlorous acid, plays a key role in cross-linking tryptophan with nucleophilic side chains of other amino acids via nucleophilic substitution reactions (*25*). Since tyrosine is well-known to induce protein cross-linking via dimerization, the role of this amino acid in the observed cross-linking also cannot be ignored (*7*, *26*, *27*). Dalle-Donne et al. (*27*) earlier assigned the cross-linking of proteins upon hypochlorous acid oxidation to the presence of dityrosine. Interestingly, BLG dimers were also formed at the highest peracetic acid/protein ratios used, ratios at which an appreciable loss of tyrosine was also noticed. Indeed, in the case of peracetic acid induced oxidation, the 3-chloroindoline cannot be produced, which resulted in an absence of high molecular weight aggregates. As mentioned previously, it seems according to our observations less likely that lysine−protein carbonyl adduct formation would have a serious impact on protein cross-linking in the applied reaction conditions, which contrasts with the results of Matoba et al. (*11*). However, Chapman et al. (*21*) did not support covalent linkage during hypochlorous acid induced oxidation at all, including Schiff base interaction, sulphonamide bonds, or the formation of dityrosine. They ascribed the hypochlorous acid induced aggregation of human plasma proteins to noncovalent interactions, supported by the fact that their aggregates were dissociated after succinylation (*21*). In our study, however, no dissociation of aggregates was seen after succinylation (data not shown) implying that the hypochlorous acid induced aggregate formation was a consequence of covalent linkage. In view of the specific effects seen with respect to tyrosine and especially tryptophan degradation, it could be concluded that these amino acids played an important role in the observed aggregation process.

ABBREVIATIONS USED

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BLG, β-lactoglobulin; ALA, α-lactalbumin; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5-dithiobis(2-nitro-benzoic)acid; TNBS, trinitrobenzenesulfonic acid; OPA, *ortho*-phthaldialdehyde; FMOC, 9-fluorenylmethylchloroformate; TCA, trichloroacetic acid; AA, acetic acid; H_2O_2, hydrogen peroxide; O_2, oxygen; TRIS, tri(hydroxymethyl)aminomethane.

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