not require molecular oxygen. The much higher consumptions measured previously (Cheynier and Van Hulst, 1988) actually resulted from the reduction of the free quinones by the sulfite ions added when sampling to inhibit the enzymatic reactions.

The oxidation sequence established in model solutions is consistent with the reactions of phenolic compounds observed in musts. Under normal processing conditions, caftaric acid is oxidized very fast. Its quinones react primarily with the available glutathione to form 2-*S*-glutathionylcaffeoyltartaric acid (Cheynier et al., 1986; Singleton et al., 1985). Following glutathione depletion, the excess caftaric acid quinones are involved in coupled oxidation mechanisms in which the glutathionyl adduct or catechin can serve as the reductant. Among phenolic compounds, epicatechin gallate, procyanidins, and condensation products, which have lower redox potentials than their monomer precursors (Singleton, 1987), should be the best targets for these coupled oxidations. However, other must components such as ascorbic acid and when sulfur dioxide is used, sulfite ions, should be more readily oxidized and, therefore, protect phenolic compounds from oxidation.

Registry No. Polyphenoloxidase, 9002-10-2.

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Deamination of Lysine as a Marker for Nitrite–Protein Reactions

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The deamination of ϵ-amino groups in poly-L-lysine by nitrite at low pH produces two amino acid isomers, δ- and ϵ-hydroxynorleucines, which elute with acidic amino acids as separate peaks during amino acid analysis. Chlorinated derivatives of the hydroxynorleucines, formed during HCl hydrolysis, elute later with neutral amino acids but are absent in mercaptoethanesulfonic acid hydrolysates. Nitrite treatment of proteins at low pH followed by HCl hydrolysis produces identical deaminated and chlorinated derivatives of lysine residues. The results indicate the potential for lysine deamination and suggest a way to monitor the fate of nitrite as well as to detect reactions that proteins may undergo in nitrite-processed foods. Hydroxynorleucines and their chlorinated derivatives are readily detected by routine amino acid analysis; in contrast, special amino acid analysis techniques are required to observe changes in all other protein amino acid residues that can react with nitrite.

The fate of nitrite used in food processing and the mechanism of nitrosamine production have been difficult to assess partly because of the lack of specific information on the chemistry of nitrite interactions with reactive groups in food components. Reactions of nitrite with nearly all of the protein amino acids having reactive side chains have been extensively characterized (Bonnet and Nicolaidou, 1977), but deamination reactions at the lysine ϵ-amino group have not been rigorously examined. Moreover, only

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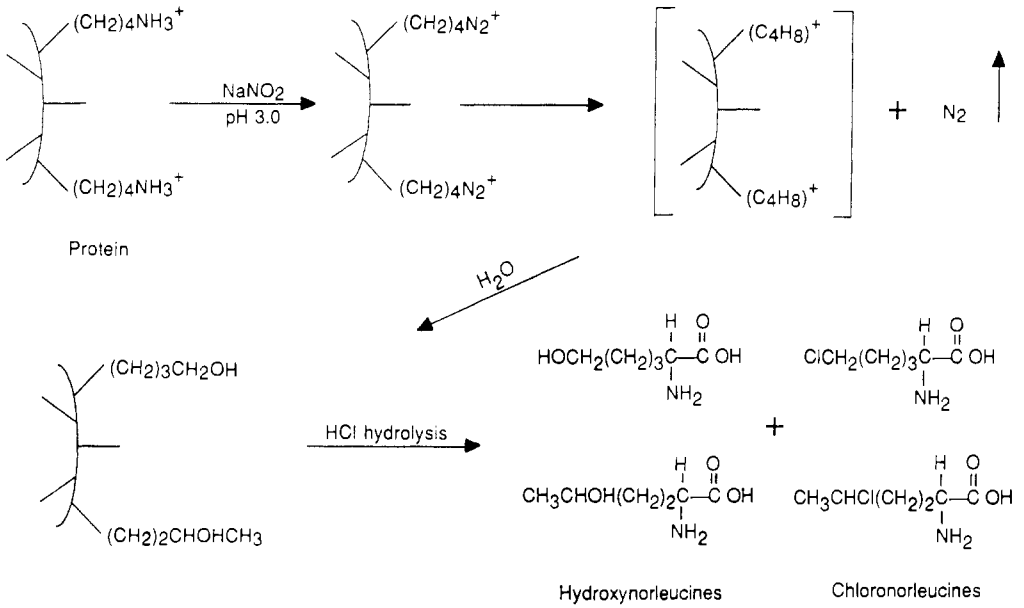

Figure 1. Reaction scheme for deamination of lysine residues in proteins and formation of chlorinated derivatives during HCl hydrolysis. Unlabeled bars projecting from the protein backbone represent neighboring amino acid residues.

a few studies have reported analyses of reactive amino acid residues after nitrite treatment of proteins. In some investigations (Knowles et al., 1974; Kurosky and Hoffman, 1972; Natake and Ueda, 1986; Philpot and Small, 1938; Shields et al., 1959) the variable reactivity of some amino acid residues with nitrite was noted, but accompanying amino acid analyses were ambiguous. Losses of some amino acids were evident in the data but were not recognized as significant; new peaks were either overlooked (Kurosky and Hoffman, 1972) or not identified (Knowles et al., 1974; Natake and Ueda, 1986).

We have investigated the deamination of lysine ϵ-amino groups using both polypeptides and proteins because of the continuing interest in nitrite as a possible precursor of nitrosamines in the diet (Hartman, 1982; McCarty, 1981). Nitrite imparts desirable color to cured meat products (Tarladgis, 1962), prevents microbial attack (Reddy et al., 1983), and protects meat lipids from oxidation reactons (Zubillaga and Maerker, 1986).

Two modified amino acids resulting from deamination of ϵ-amino groups of lysine, δ- and ϵ-hydroxynorleucines (HNLs), were isolated previously from nitrite-treated poly-L-lysine and their structures established by mass spectrometry and ^{13}C nuclear magnetic resonance (Malin and Gerasimowicz, 1987). Additional derivatives produced by the action of HCl on the HNLs during hydrolysis (chloronorleucines, CNLs) have now been characterized. The entire reaction scheme is presented in Figure 1. To determine whether lysine deamination products and their chloro derivatives can occur in nitrite-processed foods, proteins treated with $NaNO_2$ at low pH were hydrolyzed and subjected to amino acid analysis. The results provide a way to monitor changes occurring in the protein components of food products after the addition of nitrite.

EXPERIMENTAL SECTION

Polypeptides. Three proteins whose sequences have been published were investigated: horse heart cytochrome *c* (Sigma), lysine-rich histone fraction 1 (Worthington), and β-lactoglobulin A prepared by the method of Aschaffenberg and Drewry (1956). Poly-L-lysine hydrobromide samples (Sigma) had 260 or 310 monomers/polypeptide chain (molecular weights of 55 000 or 65 000). All other materials were reagent grade or the best available.

Nitrite Treatment. Protein samples (approximately 10 mg/mL) were dissolved in 0.1 M acetic acid, pH 3.0, and the resultant mixtures stirred continuously throughout the course of the reaction. Microliter amounts of redistilled glacial acetic acid were used to readjust the pH to 3.0 before and after addition of $NaNO_2$. The pH was recorded throughout the reaction and remained at 3.0 without further adjustment. The 100-fold molar excess of nitrite used was based on the total moles of amino acid residues in a protein sample expected to react with nitrite, as calculated from published sequence data for that protein (i.e., number of lysine, tryptophan, tyrosine, and cysteine residues per mole of protein times the moles of protein in the sample). This approach was adopted to ensure maximum conversion of ϵ-amino groups and other nitrite-reactive side chains. Each protein precipitated within 30 min, but the reaction was allowed to continue for 1 h. Excess nitrite was removed by centrifuging, decanting the supernatant, and washing the precipitate with deionized water repeatedly until nitrite ion peaks at 280 and 340 nm disappeared from UV–visible spectra of the supernatants. As in previous work (Malin and Gerasimowicz, 1987), poly-L-lysine hydrobromide was treated in a similar manner except that a 4-fold molar excess of $NaNO_2$ (based on the total number of lysine residues per mole of protein in the sample) was sufficient to deaminate all lysine. Throughout this paper, the term nitrite-treated refers to reaction with sodium nitrite at low pH, as described above, and implies that the reactive species is nitrous acid.

Amino Acid Analysis. Aliquots of nitrite-treated samples and untreated controls were hydrolyzed for 24 h in sealed, evacuated tubes with constant-boiling HCl containing phenol (0.05%) or with 3 N mercaptoethanesulfonic acid (MES). Replicate analyses were performed on a Beckman 119CL amino acid analyzer (W3H cation-exchange resin) with a standard 90-min single-column hydrolysate procedure, a modification of the original two-column method (Spackman et al., 1958). The elution of amino acid derivatives formed during postcolumn reaction with ninhydrin was monitored at 570 and 440 nm (for detection of proline). Signals sent to recorder pens were also sent to a Hewlett-Packard 3390A integrator attached to the analyzer.

Color constants (factors that convert integrated peak areas to moles of amino acids) for quantitating the HNLs and their chloro derivatives (CNLs) were estimated from replicate amino acid analyses of solutions of pure δ-HNL, pure ϵ-HNL, and the mixed chloro derivatives isolated by TLC (described below). Areas taken from the output of the integrator were used to calculate color constants as area/nmole.

Thin-Layer Chromatography. Hydrochloric acid hydrolysates, evaporated and redissolved in methanol, were developed on analytical silica gel G plates with use of 2-butanone/pyridine/water/glacial acetic acid (35/7.5/7.5/1). For isolation of CNLs, HCl hydrolysates were streaked on 500-μm TLC plates and developed twice. After the edges were visualized with nin-

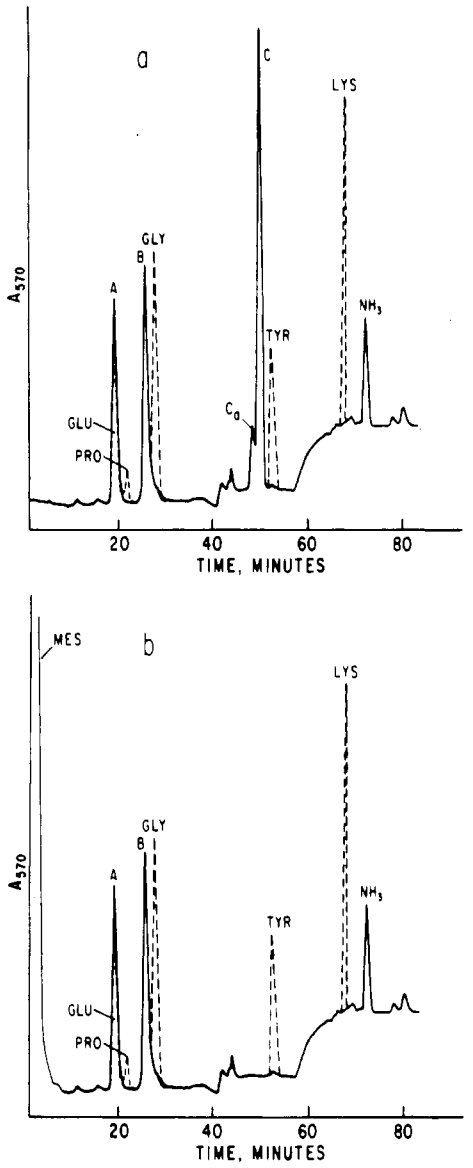

Figure 2. Amino acid analyis of deaminated poly-L-lysine hydrolysates. Peaks outlined in dashed lines indicate elution positions of amino acid neighbors of δ- and ϵ-HNLs and CNLs during a typical analysis of a protein hydrolysate. Key: a, HCl hydrolysate; b, MES hydrolysate.

hydrin, the band of interest was scraped off, extracted with methanol, evaporated, and stored frozen under nitrogen.

Hydroxynorleucines. Cation-exchange separation of amino acids on Dowex 12W-X8 using volatile buffers (Hirs et al., 1952) was adapted for isolating δ-HNL from an HCl hydrolysate of deaminated polylysine. Acidic amino acids were eluted with 0.2 M ammonium acetate, pH 5.51, evaporated to dryness, and heated in a vacuum desiccator at 40 °C to remove ammonium acetate. The dried eluate was then dissolved in 0.2 M ammonium formate/40% ethanol (pH 2.91) and the resultant mixture applied to another column of the same resin; δ-HNL eluted as a single peak when the buffer pH was increased to 3.1. Ammonium formate was sublimed from the dried sample as described above.

Authentic DL-ϵ-HNL, prepared by the method of Gaudry (1948), had previously been shown to exhibit the same TLC, amino acid analysis, cation-exchange, and mass spectral behavior as L-ϵ-HNL isolated from deaminated poly-L-lysine (Malin and Gerasimowicz, 1987).

Mass Spectrometry. For mass spectrometric analysis with sample injection via attached gas chromatograph (GC–MS), CNLs were converted to trimethylsilyl derivatives (TMS) (Liemer et al., 1977) and examined with a Hewlett-Packard 5995 electron impact instrument. The fused silica capillary column was coated with cross-linked methylsilicone (OV-1).

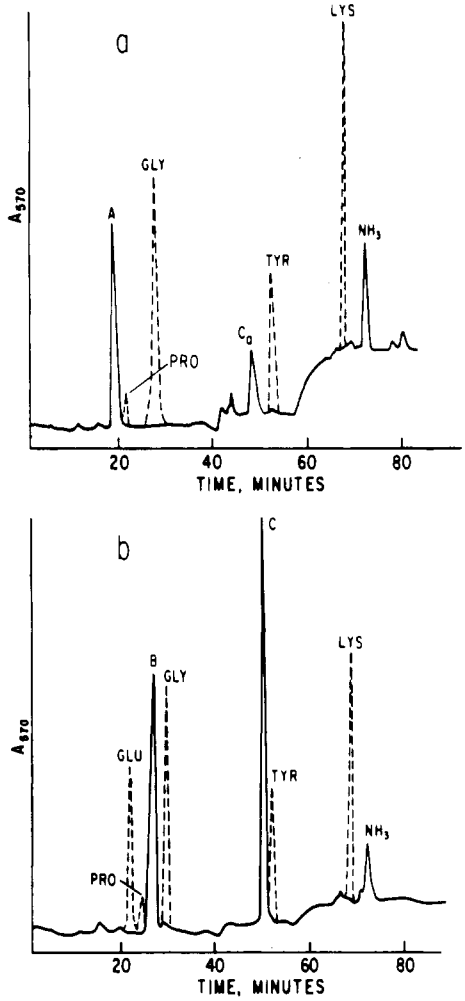

Figure 3. Amino acid analysis of pure HNLs after HCl hydrolysis. Peaks outlined in dashed lines indicate elution positions of amino acid neighbors of δ- and ϵ-HNLs and CNLs during a typical analysis of a protein hydrolysate. Key: a, hydrolysis of δ-HNL; b, hydrolysis of ϵ-HNL.

RESULTS

Nitrite-Treated Poly-L-lysine. As established previously (Malin and Gerasimowicz, 1987), amino acid analysis of polylysine treated with nitrite at low pH and hydrolyzed with HCl showed loss of all lysine and the presence of two new peaks (A and B, Figure 2a). Peak A had an elution time of 21 min, coinciding with that of glutamic acid, and was identified as δ-HNL. Peak B, identified as ϵ-HNL, eluted at 27 min between proline and glycine. Unresolved peaks C_a and C (Figure 2a) eluted at 49 and 50 min, between leucine and tyrosine, from HCl hydrolysates; these peaks were absent in MES hydrolysates (Figure 2b). This suggested that HCl reacted with the HNLs during hydrolysis to produce peaks C_a and C and that the two HNLs were the only derivatives of lysine produced directly by the deamination reaction.

When pure δ-HNL was subjected to HCl hydrolysis conditions, peak C_a appeared during amino acid analysis (Figure 3a) but peak C was absent. Similarly, peak C was the only product resulting from HCl hydrolysis of pure ϵ-HNL (Figure 3b). MES hydrolysates of each HNL did not contain either peak C_a or peak C. Based on the relative losses of δ- and ϵ-HNLs during HCl hydrolysis, conversion of ϵ-HNL to its chloro derivative (peak C) appeared to be more extensive than conversion of δ-HNL to the chloro derivative of peak C_a (see below). The chlorinated derivatives of HNLs separated as a single, unresolved band

Table I. Formation of Hydroxy- and Chloronorleucines in Poly-L-lysine and Proteins

polypeptide	products obsd, % of total		
	δ-HNL	ε-HNL	CNL[a]
poly-L-lysine[b]			
MES[c]	34.4	65.6	
HCl[d]	29.3	31.0	39.7
β-lactoglobluin[d,e]	35.6	28.9	35.5
histone 1[d,e]	32.0	36.4	31.6
cytochrome c[d,e]	17.2	43.4	39.4

[a] Isolated by TLC as an unresolvable mixture (peaks C_a + C, Figure 2a). [b] Averages of 2–12 analyses; average SD ±0.95. [c] MES hydrolysis. [d] HCl hydrolysis. [e] Based on data in Table II.

during preparative TLC of an HCl hydrolysate of deaminated polylysine and were recovered from the plate as a mixture. However, this mixture produced the overlapping peaks C_a and C during amino acid analysis (Figure 2a).

The mixture of chlorinated amino acids isolated by TLC was converted to TMS derivatives; two GC peaks (with 6.8- and 8.1-min retention times) were observed after injection into the GC–MS instrument. In the electron impact mass spectra of these peaks, the characteristic 3/1 chorine isotope ratio was present in the 100% abundance peaks (m/e 192.1 and 194.1) of the large fragments remaining after loss of the silylated carboxyl group (COOTMS). An m/e 156 fragment with a 12.8% abundance (loss of HCl from both isotopic 100% peaks) was the only other significant mass peak above m/e 100 in the spectrum of the isomer with the 6.8-min GC retention time. In contrast, the isomer with the 8.1-min retention time did not contain a significant m/e 156 peak; small mass peaks ranged downward from m/e 147.

The single m/e 156 mass peak was expected for the presence of a secondary chlorine substituent because such a fragment would have sufficient stability, and therefore lifetime, to appear in the mass spectrum. A fragment remaining after loss of HCl from a primary chloro-substituted fragment would be unstable and would break into smaller fragments immediately. These concepts have been amply demonstrated in MS studies of 1- and 2-chlorobutanes, analogues of the CNL side chain (McLafferty, 1962; ASTM, 1969). On the basis of the existence of the m/e 156 peak and the extensive literature data showing that unbranched aliphatic chains with primary chloro substituents have longer GC retention times (Hamel, 1978; Korhonen, 1982), the 6.8-min GC peak was assumed to be a secondary CNL and the later peak (8.1 min) a primary isomer.

Estimated color constants for the deaminated amino acids and their chloro derivatives were (area/nmol) as follows: δ-HNL, 3.26×10^5; ε-HNL, 3.85×10^5; mixture of CNLs, 9.15×10^5. With these estimated color constants, the average percentages of δ- and ε-HNLs and their chloro derivatives observed in HCl and MES hydrolysates of deaminated poly-L-lysine and proteins were determined (Table I). In polylysine, 5.1% less δ-HNL was observed in HCl hydrolysates compared with MES hydrolysates. In contrast, the corresponding loss of ε-HNL was 34.6%, confirming the evidence of Figure 3a,b suggesting a more facile conversion of ε-HNL to its chloro derivative compared with conversion of the δ-isomer.

Nitrite-Treated Proteins. Amino acid analyses for HCl hydrolysates of β-lactoglobulin, histone 1, and cytochrome c before and after treatment with nitrite showed almost complete loss of lysine, appearance of ε-HNL and CNLs, and increases in the glutamic acid peak at 21 min due to the coelution of δ-HNL. When the latter was calculated as glutamic acid, these increases ranged from 10 to 50% over glutamic acid content in controls not treated with nitrite. Changes in other amino acids susceptible to nitrite modification were variable, as expected. Cysteine losses were 59% in β-lactoglobulin and 39% in cytochrome c, compared with controls; histone 1 does not contain cysteine. However, cysteine losses frequently occur during protein hydrolysis. Loss of tyrosine was observed in histone 1 and cytochrome c. In chemical modifications of proteins, the reactivity of a specific class of residues is greatly affected by the microenvironments provided by neighoring side chains.

The extent of formation of HNLs and CNLs in the three proteins, calculated with the estimated color constants, is presented in Table II. Amino acid sequences reported for β-lactoglobulin (Braunitzer et al., 1972), histone 1 (DeLange, 1976), and cytochrome c (Margoliash et al., 1961) served as amino acid composition standards. The compositions of untreated samples matched those based on sequence except for histone 1, which is known to be heterogeneous (DeLange and Smith, 1976).

Because of the coelution of δ-HNL and glutamic acid, the amount of δ-HNL in the nitrite-treated samples was determined by subtracting the area of glutamic acid equivalent to that in an untreated sample from the enlarged glutamic acid peak in a nitrite-treated sample. The difference in areas was due to the presence of δ-HNL and was quantitated with the δ-HNL color constant. In each protein, the total number of HNLs, CNLs, and unreacted lysine equaled the number of lysine residues originally present. Since the percentage of ε-HNL formed in histone 1 and cytochrome c was greater than that of δ-HNL and since ε-HNL is more readily converted to CNLs (Table I), use of the single color constant for the total area of unresolved peaks C_a and C produced slight overestimates of CNL content in these two proteins (1–5%). The results of Table II indicate that the deamination of ε-amino groups in proteins is similar to the deamination reactions observed in poly-L-lysine: i.e., no products other than δ-HNL, ε-HNL, and CNLs are formed, and the reacton pathways are therefore similar.

DISCUSSION

The results reported here demonstrate the potential for lysine deamination in nitrite-treated foods and also suggest that observation of deaminated and/or chlorinated derivatives during amino acid analysis is a convenient technique for detecting the occurrence of reactions between amino acid side chains of proteins and nitrite added in processing and may also be useful in determining the fate of added nitrite. In proteins, three amino acid residues other than lysine are capable of reacting with nitrite, but products of nitrite reactions with tryptophan (Bonnet and Holleyhead, 1974; Mellet et al., 1986), tyrosine (Schnabel and Zahn, 1957), and cysteine (Byler et al., 1983) are difficult to detect and quantify by routine amino acid analysis.

Detecting losses of these amino acids or, preferably, the presence of their derivatives by amino acid analysis is not readily accomplished. Tryptophan is destroyed by HCl hydrolysis; MES hydrolysis can be used instead, but an automated program for basic amino acids must allow times for resolution of the tryptophan peak on the chromatogram since it is the first of the amino acids to elute with a basic buffer. Similarly, for detection of both *C*-nitrosotyrosine and *N*-nitrosotryptophan, modifications to automatic analyzer programs or complete manual control of buffer changes are necessary to assure resolution of peaks. *S*-Nitrosocysteine forms readily in the presence of nitrous acid (Byler et al., 1983), but its instability argues against

Table II. Amino Acid Analysis of Nitrite-Treated Proteins

amino acid	bovine β-lactoglobulin A			calf thymus histone fraction 1			horse heart cytochrome c		
	compn[a]	no NT[b]	NT[b]	compn[c,d]	no NT[b]	NT[b]	compn[e]	no NT[b,f]	NT[b,f]
Asp	16	15.8	16.4	3	7.0	6.8	8	8.1	8.4
Thr	8	7.6	7.7	7	13.1	11.0	10	9.1	9.7
Ser	7	6.7	6.5	17	12.7	12.5	0	0.5	0.1
Glu	25	24.7	27.2[g]	7	10.8	16.2[g]	12	11.4	14.8[g]
Pro	8	8.4	7.9	27	19.8	14.7	4	4.4	4.3
Gly	3	3.3	3.5	14	16.0	16.0	12	10.9	12.4
Ala	14	13.9	13.9	59	50.9	41.6	6	6.2	6.2
Cys	5	4.6	1.9	0	0.0	0.0	2	1.5	0.9
Val	10	10.0	10.8	10	9.6	9.1	3	3.6	3.1
Met	4	4.1	3.7	0	0.0	0.0	2	3.3	2.4
Ile	10	9.7	11.1	2	2.4	2.8	6	6.4	6.1
Leu	22	22.5	22.2	9	9.4	9.9	6	6.3	6.7
Tyr	4	4.6	4.6	1	1.6	0.0	4	3.9	2.2
Phe	4	4.3	4.1	1	1.6	1.5	4	4.0	4.0
His	2	2.1	2.1	0	0.9	0.7	3	3.4	3.4
Lys	15	14.8	1.7	59	57.6	0.9	19	16.5	0.2
Arg	3	3.0	3.1	4	6.0	4.4	2	1.9	2.1
av SD		±0.5	±0.7		±0.2	±0.1		±0.3	±0.2
δ-HNL			4.8[h]			18.3[h]			3.4[h]
ε-HNL			3.9			20.8			8.6
CNLs			4.8			18.1			7.8
av SD			±0.8			±2.7			±0.8

[a] Based on sequence (Braunitzer et al., 1972). [b] Averages of 2–6 analyses; NT = nitrite treatment. [c] Based on sequence (DeLange, 1976). [d] The histone 1 (lysine-rich) fraction is heterogeneous and contains molecules with several differences in amino acid composition (DeLange and Smith, 1979). [e] Based on sequence (Margoliash et al., 1961). [f] Results calculated by the method of Babul and Stellwagen (1972). [g] Total area calculated as glutamic acid. [h] The increase in area of the glutamic acid peak due to the coelution of δ-HNL was found by deducing the area of glutamic acid in the non-NT control from total peak area. The difference was the area of δ-HNL.

basing detection of nitrite–protein reactions on appearance of a new peak. Moreover, cysteine losses can also occur through other reactions during hydrolysis and subsequent analysis; a decrease in cysteine content therefore cannot be considered definite proof of a nitrite–protein reaction.

Nitrite reactions reported for methionine (Meyer and Williams, 1983) and arginine (Ishibashi and Kawabata, 1981) cannot occur in proteins because cyclization steps involve the α-amino group. However, losses of methionine accompanied by the formation of methionine sulfoxide or methionine sulfone have been observed in amino acid analyses of protein fractions isolated from bovine muscle after treatment at 5 °C for 14 days with levels of nitrite typical of those used in food processing (Malin, unpublished data). These methionine derivatives appear very early in the amino acid analysis, and analyzer programs must permit the recording of early elution times if they are to be observed on the chromatogram. In the present study, methionine losses were not observed in β-lactoglobulin and were negligible in cytochrome *c*; methionine sulfoxide and methionine sulfone were not detected in the amino acid analysis of either protein.

Although the nitrite deaminations described here were conducted at pH 3.0, similar treatments of poly-L-lysine at pH 5.5–6.4 resulted in deamination of 13% of the ε-amino groups during a 24-h period (Malin, unpubished data). These results suggest that addition of nitrite at pH levels just below neutral has the potential to lower the content of available lysine in a food protein.

One of the advantages in using deamination of lysine ε-amino groups as the basis for monitoring protein–nitrite reactions is that there is a higher probability of detecting the lysine deamination products reported here than of observing nitroso derivatives of tyrosine and tryptophan. The total content of these two amino acids often comprises a smaller fraction than lysine of the total amino acids present. Furthermore, tyrosine and tryptophan are more likely to reside within the hydrophobic interior domains of a protein, where the folded structure would tend to inhibit access of nitrous acid, even at low pH; lysine residues are usually located near or on the exterior protein surface exposed to the chemical environment. Deamination and nitrosation may not be detectable at very low nitrite concentrations, but when reactions with nitrite do occur, deaminated amino acids (HNLs) and their chloro derivatives (CNLs) elute within the range of the acidic and neutral amino acids during routine amino acid analysis of protein hydrolysates and are thus more readily observed.

ABBREVIATIONS USED

GC–MS, gas chromatography–mass spectrometry; CNL, chloronorleucine; δ-HNL, δ-hydroxynorleucine = 2-amino-5-hydroxyhexanoic acid; ε-HNL, ε-hydroxynorleucine = 2-amino-6-hydroxyhexanoic acid; MES, mercaptoethanesulfonic acid; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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Registry No. δ-HNL, 114298-96-3; ε-HNL, 305-77-1; δ-CNL, 86941-60-8; ε-CNL, 14380-91-7; nitrite, 14797-65-0; poly-L-lysine hydrobromide, 25988-63-0; L-lysine, 56-87-1; cytochrome *c*, 9007-43-6.

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