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Detection and identification of a protein-bound imidazolone resulting from the reaction of arginine residues and methylglyoxal

Thomas Henle[1], Axel W. Walter[1], Rainer Haeßner[2], Henning Klostermeyer[1]

[1] Lehrstuhl für Milchwissenschaft, Technische Universität München, Vöttinger Strasse 45, D-85350 Freising-Weihenstephan, Germany
[2] Lehrstuhl für Organische Chemie II, Technische Universität München, Lichtenbergstrasse 4, D-85748 Garching, Germany

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Nachweis und Identifizierung eines proteingebundenen Imidazolons aus der Reaktion zwischen Argininresten und Methylglyoxal

Zusammenfassung. In Säurehydrolysaten verschiedener Laugenbackwaren (Brezeln, Knabberartikel) wurde eine im Aminosäurechromatogramm unmittelbar nach Pyridosin eluierende ninhydrinpositive Verbindung nachgewiesen. Sie konnte nach präparativer Isolierung aus einer Lebensmittelprobe sowie nach unabhängiger Synthese mittels FAB-MS, ^{1}H- und ^{13}C-NMR eindeutig als proteingebundenes Imidazolon identifiziert werden, welches in den zwei tautomeren Formen N^δ-(5-Methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithin und N^δ-(4-Methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithin vorliegt. Das säurelabile Aminosäurederivat entsteht durch direkte Kondensation der Guanidinogruppe von Arginin mit dem Zuckerabbauprodukt Methylglyoxal und ist Vertreter einer neuen Form posttranslationaler Proteinmodifikationen. Für eine Anzahl handelsüblicher Laugenbackwaren lagen die nach enzymatischer Totalhydrolyse bestimmbaren Gehalte des Imidazolons zwischen 900 und 1300 mg pro 100 g Protein. Während des Backprozesses werden somit zwischen 20 und 30% der Argininreste mit Methylglyoxal umgesetzt.

Abstract. A ninhydrin-positive compound was detected in acid hydrolysates of various alkali-treated bakery products (pretzels, snack bars), eluting immediately after pyridosine in amino acid chromatograms. Following preparative isolation from a food sample and independent synthesis, the compound was unequivocally identified by fast-atom bombardement-mass spectrometry, ^{1}H- and ^{13}C-nuclear magnetic resonance as a protein-bound imidazolone, existing in two tautomeric forms, namely N^δ-(5-methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine and N^δ-(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine. The acid-labile amino acid derivative is formed by direct condensation of the guanido group of arginine and methylglyoxal, a sugar degradation product, and represents a previously unknown post-translational protein modification. For a number of commercially available alkali-treated bakery products, the amounts of the imidazolone after complete enzymic digestion ranged between 900 and 1300 mg per 100 g protein, indicating that between 20 and 30% of the arginyl residues might react with methylglyoxal during the bakery process.

Correspondence to: T. Henle

Introduction

During heating or storage of carbohydrates in the presence of alkali, methylglyoxal (pyruvaldehyde, 2-oxopropanal) is formed via enolization, β-elimination of water and subsequent cleavage of the sugar carbon chain [1, 2]. Methylglyoxal, a mutagenic [3] and cytotoxic [4] compound, has been found in foods such as bread [5], coffee [6], wine and various beverages [7, 8], vegetable oils [9], as well as in cigarette smoke [10]. The α-dicarbonyl is known to be highly reactive towards α-amino acids [11] and proteins [12]. The comparable phenylglyoxal has been used in protein chemistry for a selective derivatization of arginyl residues [13].

Recently, the occurrence of methylglyoxal in the human body, where it is thought to be formed predominantly from triose phosphates [14], gave rise to suggestions concerning the involvement of methylglyoxal in the chronic complications of diabetes mellitus [15]. However, chemistry of the modification of proteins by methylglyoxal and other dicarbonyls still remains unclear, as no corresponding protein-bound reaction products have been identified unequivocally up to now.

In the present paper, detection of a previously unknown imidazolone derived from protein-bound arginine is described. The amino acid derivative was isolated from alkali-treated bakery products as well as from model mixtures of N^α-acetylarginine and methylglyoxal, and its structure was elucidated by means of fast-atom bombardment-mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR).

Materials and methods

Chemicals. N^{α}-Acetyl-L-arginine, methylglyoxal (40% aqueous solution) and dimethyl-d_6 sulphoxide (DMSO) were obtained from Sigma (Deisenhofen, Germany). Pepsin and pronase were from Merck (Darmstadt, Germany). The suspension of aminopeptidase M was from Boehringer (Mannheim, Germany). Prolidase was from Serva (Heidelberg, Germany). Hydrochloric acid was from J.T. Baker (Deventer, The Netherlands). Dowex AG 50W-X8 (200–400 mesh, H^+ form) ion-exchange resin was from Bio-Rad (München, Germany). All other chemicals were from Merck (Darmstadt, Germany). Bakery products were obtained from local retail stores.

Acid hydrolysis. Samples (100 mg) of various bakery products (corresponding to 15 mg protein) were hydrolysed in the presence of 15 ml of 6 *M* HCl as described in [16].

Enzymic hydrolysis. Samples were hydrolysed using four enzymes (pepsin, pronase E, aminopeptidase and prolidase) as described in [17].

Amino acid analysis. This was performed on an Alpha Plus amino acid analyser (LKB Biochrom, Cambridge, UK), using a stainless steel column (150×4 mm, Alltech, Unterhaching, Germany) filled with ion-exchange resin DC4A-spec, sodium form (Benson, Reno, Nev., USA). The composition of buffers and reagent as well as running conditions are described in [16] and [18].

Synthesis of the protein-bound imidazolone ***1****.* N^{α}-Acetyl-L-arginine (1081 mg; 5 mmol) and methylglyoxal (900 µl of a 40% aqueous solution; 5 mmol) were dissolved in 50 ml of 0.2 *M* sodium phosphate, pH 7.0, and heated under reflux at 90° C for 3 h. After cooling, 50 ml of 4 *M* HCl was added and the mixture was boiled under reflux for 2 h. The solution was dried under reduced pressure at room temperature. The residue was dissolved in 10 ml water. After adjusting the pH to 2.0 with 1 *M* HCl, the solution was applied to a column (16×120 mm), filled with cation-exchange resin Dowex 50W-X8, previously equilibrated with 200 ml of 0.07 *M* sodium citrate, pH 3.25. The column was eluted with 0.16 *M* sodium citrate, pH 5.35, at a flow rate of 0.6 ml/min. Fractions (1 ml) were collected and 10-µl-aliquots run on the amino acid analyser: **1** was detected in fractions 43–65. These fractions were pooled and freeze dried. For desalting, the residue was dissolved in 10 ml water and applied to the chromatography column, previously equilibrated with 75 ml of 2 *M* HCl and 25 ml water. Citrate ions were eluted with 50 ml water, sodium ions with 50 ml 1 *M* HCl. Compound **1** (hydrochloride) was finally eluted with 30 ml of 4 *M* HCl and evaporated to dryness (**1**: 250 mg). Elementary analysis gave: $C_9H_{16}O_3N_4{\times}2\ HCl{\times}H_2O$ [relative molecular mass (M_r) 319.2]; calculated: C=33.86%, H=6.32%, N=17.55%; found: C=33.33%, H=6.54%, N=17.65%. – MS (FAB): 229.4 (MH^+).

Isolation of ***1*** *from pretzel crust.* For isolation of protein, 50 g crust was boiled for 2 h under reflux in 1000 ml of 50% aqueous isopropanol containing 0.5% thioglycerol. The filtrate was collected and the residue extracted as described above. The filtrates were pooled and dried under reduced pressure at room temperature. The dried residue was hydrolysed with 2000 ml of 6 *M* HCl under reflux for 23 h. After filtration, the hydrolysate was evaporated to dryness. The residue was dissolved in 40 ml water. Aliquots (20 ml) of the membrane-filtered (0.8 µm) solution were applied to the chromatography column, previously equilibrated with 75 ml 2 *M* HCl and 25 ml water. The column was eluted with 2.6 *M* HCl and 5-ml-fractions were collected. **1** was detected together with hydrophobic and basic amino acids in fractions 18–28. Fractions were pooled and evaporated to dryness. The residue was dissolved in 10 ml water and again applied to the chromatography column, equilibrated with 200 ml of 0.07 *M* sodium citrate, pH 3.25. The column was eluted with 0.13 *M* sodium citrate, pH 5.35, and 1-ml-fractions were collected: **1** was detected together with basic amino acids in fractions 30–46. Final isolation of **1** was achieved via rechromatography of the pooled fractions as described above, eluting the column with 0.09 *M* sodium citrate, pH 5.35. Pure **1** was detected in fractions 87–147. Desalting was performed as described above (**1**: 6.4 mg).

NMR analysis. ^1H- and ^{13}C-NMR spectra (solvent d_6-DMSO, external standard tetramethylsilane) were recorded with a 500 AMX (500 MHz) instrument (Bruker, Rheinstetten, Germany).

Results and discussion

In the course of routine amino acid analysis, an unknown ninhydrin-positive compound, designated C, was detected in acid hydrolysates of various bakery products (e.g. pretzels, snack bars), eluting immediately after pyridosine in the ion-exchange chromatogram (Fig. 1a). The food samples investigated had been treated with alkali immediately before the baking process, in order to obtain their typical flavour, taste and colour. Comparative analysis showed significantly higher amounts of C in the crust compared to the corresponding crumb (Fig. 1a, b). The formation of C correlated with a decrease in the amount of the basic amino acid arginine. For industrial samples produced under similar heating conditions but without the use of alkali (e.g. bread rolls, salt crackers), no C or only very small amounts of the compound were determined.

Treatment of carbohydrates with alkali leads to a degradation of the carbon chain and the formation of highly reactive α-dicarbonyls via retroaldol reaction [1, 2]. These highly reactive degradation products are known to react with amino acids and proteins, predominantly with the guanidino group of arginine. After heating a reaction mixture, consisting of equimolar amounts of N^{α}-acetylarginine and methylglyoxal, and subsequent removal of the acetyl moiety with diluted hydrochloric acid, one major peak (Peak 1) was detected after

Table 1. Nuclear magnetic resonance (^1H- and ^{13}C-NMR) data of N^{δ}-(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine (**1a**) and N^{δ}-(5-methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine (**1b**)

1a		**1b**	
Atomic assignment	δ (ppm)	Atomic assigment	δ (ppm)
^1H			
(C-5)CH_3	1.34 (d, 3H, $J_{CH_3,H}$=7.1 Hz)	(C-4)CH_3	1.31 (d, 3H, $J_{CH_3,H}$=7.1 Hz)
(C-β)H_2 } (C-γ)H_2 }	1.60–1.90 (4H)	(C-β)H_2 } (C-γ)H_2 }	1.60–1.90 (4H)
(C-δ)H_2	3.37 (q, 2H)	(C-δ)H_2	3.34 (q, 1H)
(C-α)H	3.89 (m, 1H)	(C-α)H	3.89 (m, 1H)
(C-5)H	4.35 (dq, 1H, J_{H,CH_3}=7.1 Hz $J_{H,NH}$=1.7 Hz)	(C-4)H	4.28 (q, 1H, J_{H,CH_3}=7.1 Hz)
NH_3^+	8.54 (br, 3H)	NH_3^+	8.54 (br, 3H)
(N-δ)H	9.44 (t, 1H, J_{NH,CH_2}=6.0 Hz)	(N-1)H	9.54 (s, 1H, $w_{1/2}$=5.56 Hz)
(N-1)H	10.64 (br, 1H)	(N-δ)H	9.89 (t, 1H, J_{NH,CH_2}=6.0 Hz)
COOH	12.26	COOH	12.64
^{13}C			
CH_3	15.89	CH_3	16.08
C-γ	23.90	C-γ	24.69
C-β	26.97	C-β	26.84
C-δ	41.21	C-δ	41.99
C-α	51.41	C-α	51.41
C-5	54.62	C-4	54.34
C-2	155.94	C-2	157.03
COOH	170.59	COOH	170.59
C-4	174.95	C-5	176.00

Values of δ are given relative to tetramethylsilane

amino acid analysis, eluting with the identical retention time to compound C and showing the same ratio of absorption at 570 nm versus 440 nm (Fig. 1c). Besides some minor compounds, only small amounts of unreacted arginine were present, indicating a nearly complete derivatization of arginine during the reaction with methylglyoxal. Sufficient amounts of Peak 1 were isolated from the reaction mixture via preparative ion exchange chromatography. Compound C was isolated in a similar manner from an acid hydrolysate of the crust of an alkali-treated pretzel. For a complete separation of C from the hydrophobic and basic amino acids present in the hydrolysate, repeated chromatography, using sodium citrate buffers with varying ionic strength, was necessary.

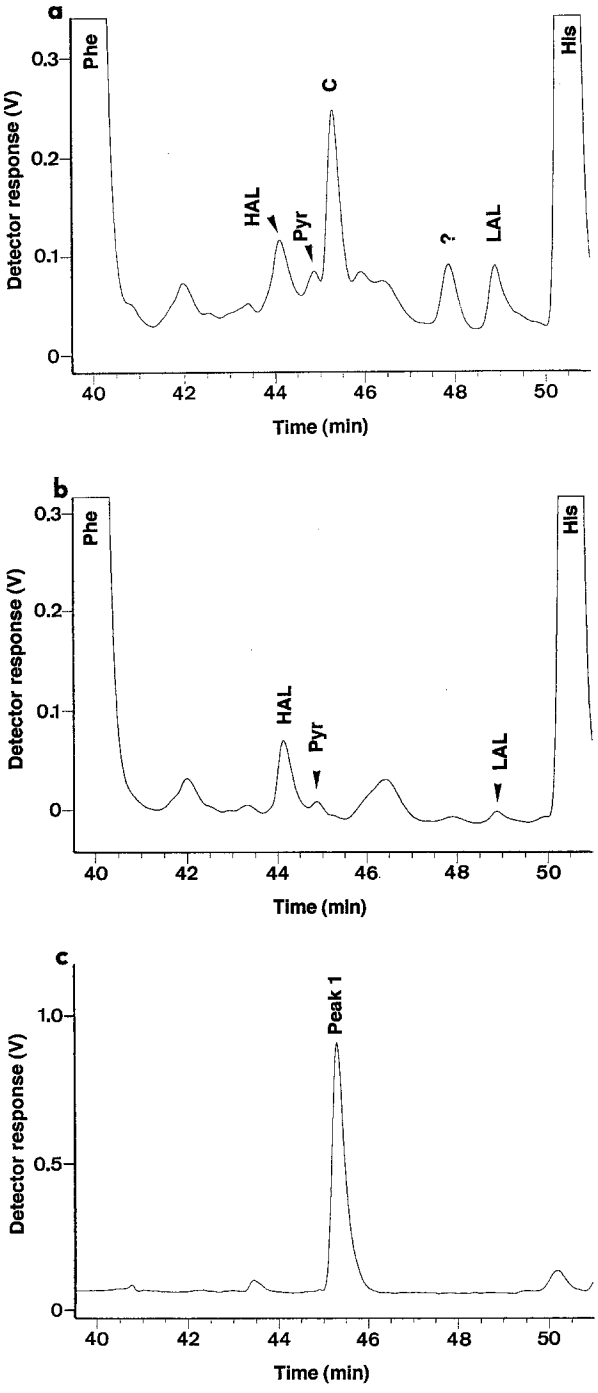


Fig. 1 a–c. Chromatograms (detail) of acid hydrolysates: HAL, histidinoalanine, possibly co-eluting with an unknown compound; Pyr, pyridosine; LAL, lysinoalanine.
a Alkali-treated pretzel (crust).
b Alkali-treated pretzel (crumb).
c Model mixture of N^{α}-acetylarginine and methylglyoxal (90° C, 3 h)

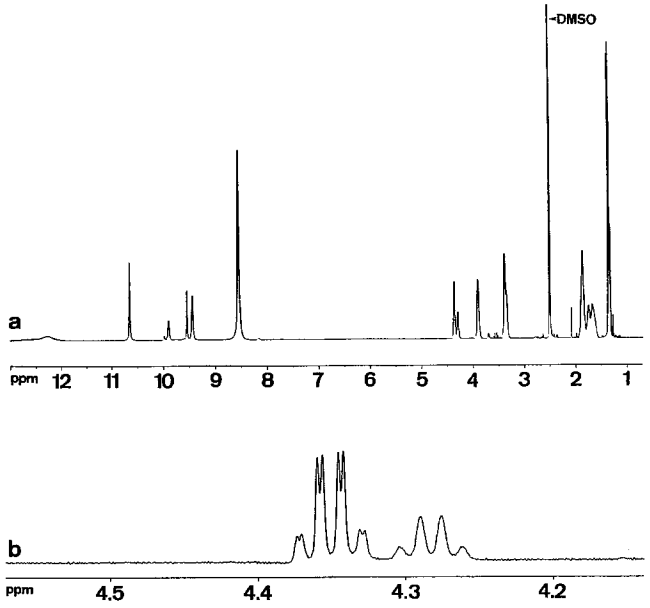
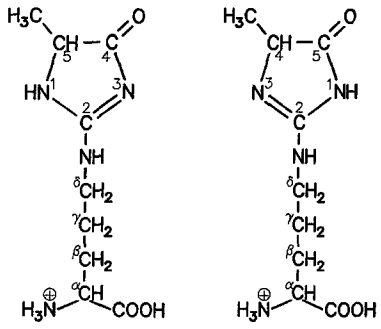


Fig. 2a, b. ^{1}H-Nuclear magnetic resonance (NMR) of compound C: DMSO, dimethyl sulphoxide. **a** Whole spectra. **b** Detail

FAB-MS of Peak 1 as well as of C showed a pseudomolecular ion (MH^+) at m/z=229.4, indicating the formation of a compound with the empirical formula $C_9H_{16}O_3N_4$ (calculated M_r=228.24) from a condensation reaction between one molecule each of arginine (M_r=174.20) and methylglyoxal (M_r=72.06) and loss of one molecule of water (M_r=18.01).

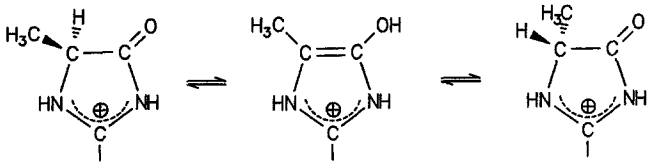
For unequivocal structure elucidation, ^{1}H- and ^{13}C-NMR analysis was performed. Chemical shifts and coupled multiplicities are listed in Table 1. It could be shown from the signal intensities in the ^{1}H- as well as in the ^{13}C-spectra, that Peak 1 and C consisted of a mixture of two compounds in a ratio of 2 to 1 (Fig. 2a, b). An imidazolone, existing in the two tautomeric forms N^{δ}-(5-methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine, **1a**, and N^{δ}-(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine, **1b**, was postulated (Fig. 3). The conjugated keto-imine structure **1a** represents the major form. In the ^{1}H-NMR spectra, the C-5 of the heterocyclic ring appeared at 4.35 (1H, q, J=7.1 Hz) *with* a vicinal coupling ($J_{H,NH}$=1.7 Hz). In contrast, for the keto-amide **1b** the corresponding proton at C-4 appeared at 4.28 (1H, q, J=7.1 Hz) *without* a vicinal coupling (Fig. 2b). In the ^{13}C-NMR spectra, C-4 of **1a** was at 174.95 and the corresponding C-5 of **1b** was at 176.00. This proved the existence of the imidazolone structure for both tautomers. A possible enol structure of **1b** could be ruled out, as this would result in a chemical shift for C-5 of about 160 ppm.

The C-5 of **1a** and C-4 of **1b** are chiral centres. Thus, two diastereomers for **1a** and **1b** each are possible. Unexpectedly, in the ^{13}C-NMR spectra, only one diastereomer was found for each tautomer. Acid- or base-catalysed epimerization



1a 1b

Fig. 3. N^{δ}-(5-Methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine (**1a**) and N^{δ}-(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine (**1b**)



[S] [R]

Fig. 4. Epimerization of **1** via an enolic intermediate, leading to [R] an [S] configurated diastereomers

must be responsible for the favoured formation of one diastereomer (Fig. 4). During additional ^{1}H-NMR analysis in the presence of D_2O, an exchange of the protons at the chiral C-atoms was observed, resulting in a decrease in the corresponding signals. This proved the existence of an enolic intermediate and the proposed mechanism shown in Fig. 4. Evaluation of the real configuration of the epimeric C-atoms has not yet been possible.

Numerous suppositions for a direct reaction between α-dicarbonyls and the guanidino group of arginine can be found in the literature [13, 19, 20]. Structures of possible reaction products have been postulated and various attempts were made for their isolation [21]. However, to date no individual protein-bound derivatives have been characterized. Thus, the two tautomers of the imidazolone **1** represent a new type of post-translational protein modification formed during food processing.

Investigations on pure **1** showed that the arginine derivative is unstable during acid hydrolysis. Under the conditions routinely used for complete protein hydrolysis (incubation for 23 h at 110° C in the presence of 6 *M* HCl), between 50 and 70% of **1** is degraded to arginine and some unknown breakdown products. Consequently, correct quantification of **1** is limited to enzymic hydrolysis. For a number of commercially available alkali-treated bakery products, the amounts of **1** after complete enzymic digestion ranged between 900 and 1300 mg/kg protein of the food samples. These values indicate that between 20 and 30% of the arginyl residues might react with methylglyoxal during the bakery process. Further research concerning the conditions that influence the formation of methylglyoxal and its reaction with arginine, as well as concerning the physiological properties of the uncommon amino acid **1** is necessary.

Besides their formation during alkali-induced degradation of carbohydrates, α-dicarbonyls might also be formed within the pH range normally present in foods and biological samples (pH 4.0–7.4) through advanced stages of the Maillard reaction [22]. It seems likely that **1** represents a basic structure for numerous products arising from the reaction of such sugar derivatives (mainly 1- and 3-deoxyosones) and protein-bound arginine in foods as well as in the human body. Investigations to confirm this hypothesis are now in progress.

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