# Cysteine Mediated Formation of *N*-ε-Carboxymethyllysine (CML) on Proteins

*U. SCHWARZENBOLZ* and T. HENLE*

*Institute of Food Chemistry, Technische Universität Dresden, D-01062 Dresden, Germany*
**E-mail: uwe.schwarzenbolz@chemie.tu-dresden.de*

**Abstract**: The reaction of glyoxal with nucleophilic amino acids was monitored for β-casein as well as β-lactoglobulin. As predicted from previous experiments with hippuryl amino acids, a measurable decrease of arginine can be found in the thiol-free β-casein, while the lysine content remained almost unchanged. For β-lactoglobulin, the incubation with glyoxal led to a slight decrease in the lysine content, while the arginine residues remained unmodified. Here, in accordance with nucleophilicity, it is suggested, that mainly cystein residues react with glyoxal. In solutions containing β-casein with or without glutathione, the effects were less pronounced and regarding the lysine and arginine content, the influence of thiols could hardly be recorded on a significant level. However, comparing the CML levels in the different incubations, it becomes obvious, that glutathione is favouring CML formation in a concentration depended manner. Therefore, the use of CML as an indicator, e.g. for the Maillard reaction, must be related to the composition of the reaction system.

**Keywords**: *N*-ε-carboxymethyllysine; CML; cysteine; protein modifications

## INTRODUCTION

Glyoxal is a very reactive dicarbonyl compound which is formed in the course of the Maillard reaction. It is known to react fast with nucleophilic amino acids like lysine and arginine and several reaction products have been described in literature (Glomb & Monnier 1995). Among the reaction products, beside N-ε-carboxymethyllysine (CML), mainly arginine derivatives could be found (Zyzak *et al.* 1994; Schwarzenbolz *et al.* 1997; Thornalley 2005). More recent studies have shown that thiols, due to their nucleophilic nature, are even more potent scavengers for dicarbonyls than amines or guanidines. Modifications lead, among others, to S-(carboxymethyl)cystein and crosslink amino acids, which incorporate a lysine and a cystein residue, that are crosslinked by glyoxal (Zeng & Davies 2005; Baynes *et al.* 2006). These investigations have mainly regarded isolated reaction mixtures to elucidate structures of the reaction products. For complex systems like food however, it is not possible to neglect the influence of the nucelophilic amino acids on each other. Therefore, we studied the reactivity of lysine and arginine residues when competing for glyoxal in the absence and presence of thiols.

## MATERIALS AND METHODS

β-Casein, β-lactoglobulin, reduced glutathione and glyoxal (GO) were obtained from Sigma (Deisenhofen, Germany). Hydrochloric acid (HCl) was from J.T. Baker (Deventer, the Netherlands) and cation-exchange resin Dowex 50W-X8 from Bio-Rad (Munich, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Solutions of either 1.25mM β-casein or 1.67mM β-lactoglobulin were reacted with glyoxal at a final concentration of 5mM in 0.03M phosphate buffer (pH 7.0) at 40°C for 8 days. During this incubation, samples were taken daily and analysed for residual lysine and arginine. In parallel, solutions containing 1.25mM β-casein, 5mM glyoxal and either 2.5 or 5mM reduced glutathione (GSH) were incubated for 2 days at 40°C and analysed for residual lysine and arginine, as well as for CML.

GC-MS analysis of CML was perfoemed according to Knecht *et al.* (1991). Prior to analysis,

protein hydrolysates referring to 3 mg protein were evaporated to dryness, solved in 750 µl of 1M HCl and applied to a C-18 cartridge (Strata C-18-E, Phenomenex, Aschaffenburg, Germany) as well as a cation exchanger (Strata SCX, Phenomenex, Aschaffenburg, Germany) that were coupled in line. The two columns were washed with 5 ml water and after removing the C-18 cartridge, the ion exchanger was again washed with 5 ml of water. The elution of the amino acids was performed with 4.5 ml pyridine/water (1/3, v/v). The eluate was evaporated to dryness, solved in 2 ml 1M methanolic HCl, 40 µl of 2.5mM carboxymethylornithine were added and the mixture was incubated for 30 minutes at 65°C. After evaporation to dryness the residue was solved in 0.5 ml methylene chloride and 1 ml trifluoroacetic anhydride, reacted at room temperature for 1 h, evaporated to dryness, solved in 0.3 ml methylene chloride and applied to GC-MS. Acid hydrolysis and amino acid analysis were performed according to Henle *et al*. (1991).

## RESULTS AND DISCUSSION

In previous work, we were able to show that $N^{\alpha}$-acetylcysteine added to mixtures of hippuryllysine, hippurylarginine and glyoxal resulted in a fundamental change of the spectrum of reaction products (Schwarzenbolz *et al.* 2008). While in the absence of thiol, arginine represents the main target for the reaction with glyoxal, the presence of SH-groups protects arginine and leads to an increased formation of lysine derivatives, namely CML. With mass spectrometric means it could be shown, that an intermediate consisting of a lysine residue linked to a cysteine residue via glyoxal is formed and based on this a reaction mechanism was proposed. To verify whether the situation remained similar on proteins, we incubated β-casein, which does not contain cysteine, and β-lactoglobulin with glyoxal for 8 days and analysed the residual amounts of lysine and arginine. The molar ratio between glyoxal and protein-bound arginine was 1:1. In the case of β-casein, a decrease in the amount of lysine and of arginine during incubation was measured by amino acid analysis after acid hydrolysis of the protein (Table 1). For β-lactoglobulin, the corresponding values of unmodified lysine were similar, while those of arginine were significantly higher (Table 1). On the first view, these data reflect more or less the reaction rates on model amino acids. Whereas at β-casein, glyoxal is mainly trapped by arginine, and it can be suggested that the main partner for the reaction on β-lactoglobulin was cysteine. To estimate the influence of the hydrolysis on the yield of amino acids, an enzymatic digestion of the samples was performed in parallel and the results showed that lower values of lysine, but higher levels of arginine were recovered. This indicates, that during the procedure of acid hydrolysis there is a decomposition of arginine adducts as well as a formation of lysine derivatives. This already described instability of arginine-glyoxal-adducts (Schwarzenbolz *et al.* 1997) therefore represents a protein-bound storage of glyoxal, that especially at higher temperatures is responsible for subsequent reactions on lysine, that seems to form thermodynamical more stabile products.

Table 1. Residual amounts of amino acids in % after incubation of β-casein and β-lactoglobulin with glyoxal for 8 days at 40°C and pH 7.0. All values

| Time (day) | Acid hydrolysis | | Enzymatic hydrolysis | |
|---|---|---|---|---|
| | lysine | arginine | lysine | arginine |
| **β-casein** | | | | |
| 0 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 |
| 2 | 98 ± 2 | 73 ± 3 | 95 ± 2 | 77 ± 2 |
| 8 | 97 ± 2 | 57 ± 3 | 93 ± 2 | 60 ± 2 |
| **β-lactoglobulin** | | | | |
| 0 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 |
| 2 | 100 ± 2 | 94 ± 2 | 97 ± 2 | 99 ± 2 |
| **8** | 98 ± 2 | 92 ± 2 | 96 ± 2 | 98 ± 2 |

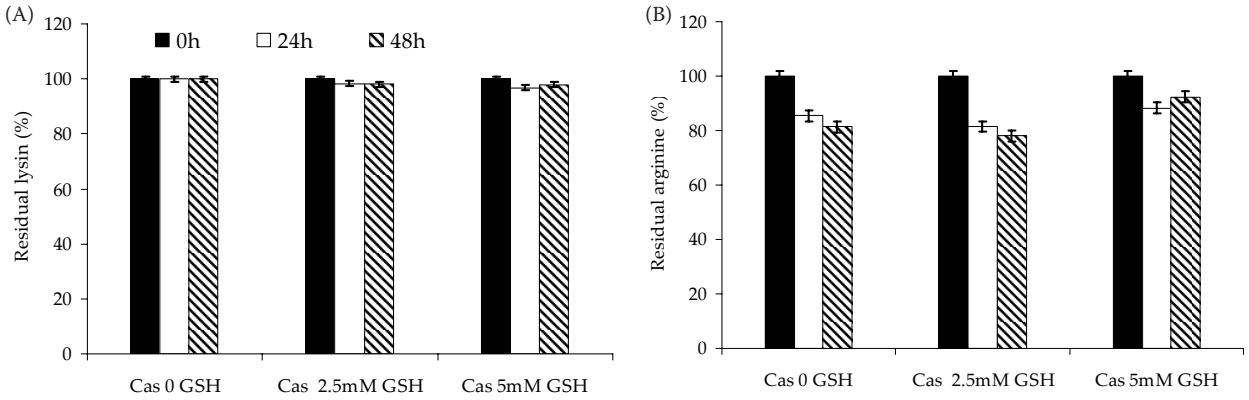


Figure 1. Incubations of β-casein with varying amounts of GSH at 40°C and pH 7.0 for 2 days: (A) residual lysine; (B) residual arginine

In contrast to model systems containing amino acids, the reactivity of the lysine residues was not increased markedly, but in the chromatograms of the amino acid analysis a peak corresponding to CML could be found qualitatively (data not shown). Therefore, to characterise the influence of thiols we incubated β-casein for 2 days with glyoxal and varying amounts of GSH. The molar ratios between arginine residues, glyoxal and GSH were chosen to be 1:1:0.5 or 1:1:1, respectively. As shown in Figure 1, the addition of GSH did not promote the reactivity of lysine, nor did it prevent arginine from reacting. The reason for this behaviour remains up to now unclear, as these results could be reproduced in equivalent experiments where GSH was replaced by $N^{\alpha}$-acetylcysteine (data not shown). As there was no lysine degradation observable on the amino acid analysis, but CML could be found qualitatively, we verified the formation of CML with a more sensitive GC-MS method and noticed increased amounts of CML with higher levels of GSH. It becomes evident, that without thiols no CML can be found, while its level can be increased through GSH addition (Figure 2).

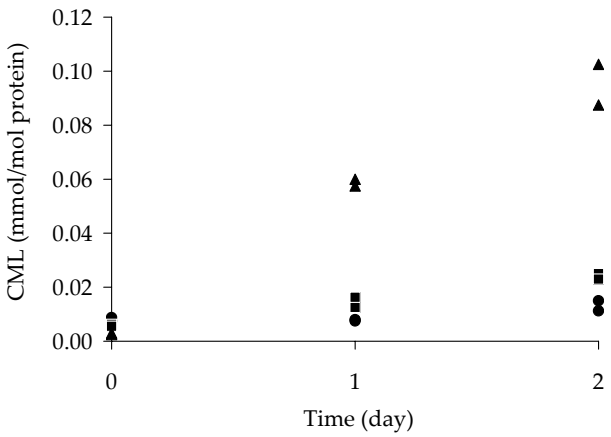


Figure 2. Concentration of CML in incubations of β-casein in the presence of 5mM (▲), 2.5mM (■) and 0mM (●) GSH

In conclusion, as CML can be found in several foods in concentrations with values that differ by a factor of 1000 (Assar *et al.* 2009), our investigation may lead to the understanding, that not only temperature, but also the chemical environment is critical for the formation of CML. In addition, as currently the uptake of advanced glycation end products like CML is discussed controversially, considering the influence of thiols on the formation of CML may offer one parameter for the optimisation of food processing.

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