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ORIGINAL ARTICLE

Ultra performance liquid chromatography-mass spectrometric determination of the site specificity of modification of β-casein by glucose and methylglyoxal

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Abstract Modification of protein by carbonyl compounds under in vitro physiological conditions is site-directed. There are few reports of the site specificity of glycation of proteins using heating conditions of relevance to food processing. The aim of this study was to determine the site specificity of modification of β-casein (βCN) by glucose and methylglyoxal (MGO). βCN (1.33 M, 3.2%) was heated with either glucose (1.345 M, 4.6%) or MGO (1 mM) at 95°C for up to 4 h. Tryptic digests were prepared and analysed by ultra performance liquid chromatography electrospray ionisation mass spectrometry (UPLC-ES/MS). The sites of formation of the Amadori product, N^{ε}-(fructosyl)lysine (FL), and the advanced glycation end-products, N^{ε}-(carboxymethyl)lysine (CML), MGO-derived dihydroxyimidazolidine (MG-DH) and MGO-derived hydroimidazolone (MG-HI), were located. FL and CML were detected at K107 and K176 residues in βCN/glucose incubations. Indigenous N^{ε}-(lactulosyl)lysine was detected at K107 only. MG-DH and MG-HI were detected at R202 and possibly R183 residues in both βCN/glucose and βCN/MGO incubations. Glycation of βCN by glucose and MGO resulted in similar site specificity for MG-DH and MG-HI formation.

Keywords Advanced glycation end-products · N^{ε}-(fructosyl)lysine · N^{ε}-(carboxymethyl)lysine · MGO-derived dihydroxyimidazolidine · MGO-derived hydroimidazolone · Ultra performance liquid chromatography-mass spectrometry

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Abbreviations

AGEs	Advanced glycation endproducts
ALEs	Advanced lipoxidation endproducts
ARP	Amadori rearrangement product
CML	N^{ε}-(carboxymethyl)lysine
FL	Fructoselysine
MR	Maillard reaction
NFPA	Nonafluoropentanoic acid
UPLC-MS	Ultra performance liquid chromatography-mass spectrometry
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid

Introduction

The Maillard reaction is a type of nonenzymic browning reaction that occurs during heating of foods that contain protein and carbonyl compounds such as reducing sugars. Typically, the ε-amino group of a lysine side chain within protein reacts with a carbonyl group to form the Amadori rearrangement product (ARP), which is N^{ε}-(fructosyl)lysine (FL) when glucose is the reactant, as shown in Scheme 1 (Ames 2008). FL undergoes further reaction to form the advanced glycation end-product (AGE) N^{ε}-(carboxymethyl)lysine (CML) (Ames 2008). Methylglyoxal (MGO) is an α-dicarbonyl that is formed during early glycation, either by oxidation of the sugar or fragmentation of the Schiff base or the ARP (Thornalley et al. 1999). MGO reacts with the guanidino group of arginine residues within protein to form a range of AGEs, notably

MGO-derived dihydroxyimidazolidine (MG-DH) and MGO-derived hydroimidazolone (MG-HI) (Brock et al. 2007a).

Bovine casein consists of four major proteins αS_1-, αS_2-, β- and κ-casein. β-Casein (βCN) accounts for 32% of casein in bovine milk (Belitz et al. 2004). βCN is a ~24 kDa protein consisting of 209 amino acids (Fig. 1). The βCN family consists of seven genetic variants (A^1, A^2, A^3, B, C, D and E), with variant A^2 predominating (Belitz et al. 2004). The different variants are the result of single or multiple amino acid substitutions. βCN is amphipathic in nature. It has a hydrophobic C-terminus and a hydrophilic N-terminus, which contains five phosphoserine residues residues, at positions 15, 17, 18, 19 and 35 (Swaisgood 2003). Circular dichroism data show that βCN has a secondary structure made up of 9% α-helix and ~25% β-sheet (Belitz et al. 2004).

There is very little information regarding the sites of modification in food proteins although the sites of lactulosylation of αS_1-casein (Scaloni et al. 2002) and βCN (Henle and Klostermeyer 1993; Scaloni et al. 2002) have been reported. In contrast, there have been several reports of the site-directed modification of a limited range of proteins, i.e., ribonuclease A, BSA and HSA, incubated under in vitro physiological conditions (Brock et al. 2003; Cotham et al. 2004; Ahmed et al. 2005; Hinton and Ames 2006; Brock et al. 2007a, b). LC-MS is an appropriate tool for this work due to its high sensitivity, reliability and reproducibility.

Ultra performance liquid chromatography (UPLC) operates at pressures of 15,000 psi (~1,000 bar) using columns packed with solid support particles of 1.7 µm diameter. Thus, compared to HPLC, UPLC offers superior resolution, speed and sensitivity of analysis, especially when coupled to a mass spectrometer (Churchwell et al. 2005).

RELEELNVPG^{10} EIVESLSSSE^{20} ESIT**R**IN**KK**I^{30} E**K**FQSEEQQQ^{40}
TEDELQD**K**IH^{50} PFAQTQSLVY^{60} PFPGPIPNSL^{70} PQNIPPLTQT^{80}
PVVVPPFLQP^{90} EVMGVS**K**V**K**E^{100} AMAP**K**H**K**EMP^{110} FP**K**YPVEPFT^{120}
ESQSLTLTDV^{130} ENLHLPLPLL^{140} QSWMHQPHQP^{150} LPPTVMFPPQ^{160}
SVLSLSQS**K**V^{170} LPVPE**K**AVPY^{180} PQ**R**DMPIQAF^{190} LLYQEPVLGP^{200}
V**R**GPFPIIV^{209}

Fig. 1 Amino sequence of β-casein (lysine and arginine residues are indicated in *bold*)

The aim of the present study was to identify the sites of modification in βCN following incubation with glucose or MGO under conditions of relevance to food processing, using UPLC-MS.

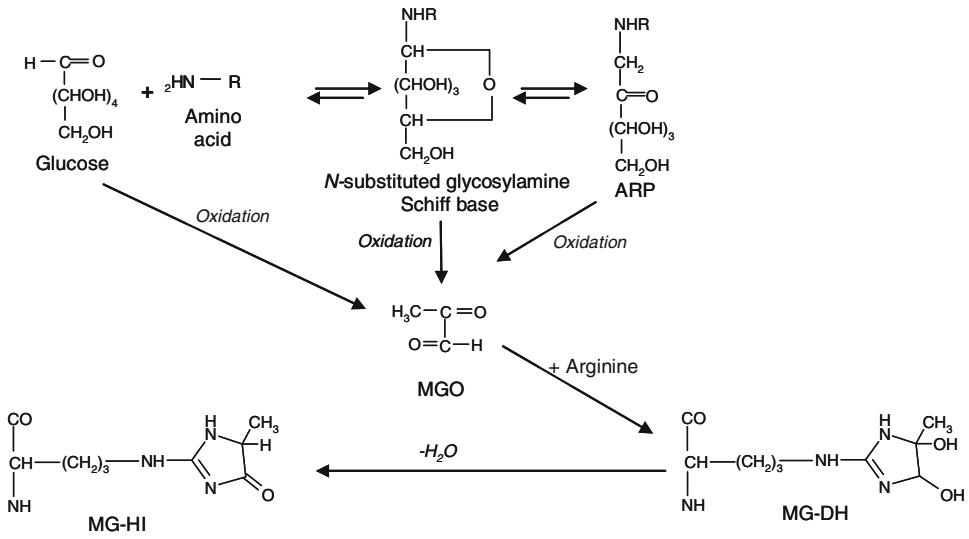
Materials and methods

Materials

The following reagents were purchased from Sigma (Gillingham, UK): β-casein (βCN) from bovine milk (≥90%), D-(+)-glucose (ACS grade), methylglyoxal (MGO) solution (40% in H_2O), trypsin (sequencing grade), nonafluoropentanoic acid (97%), ethylenediaminetetraacetic acid (EDTA, 99%), ammonium bicarbonate (≥99%), dithiothreitol (99%), 2-mercaptoethanol, 4-vinylpyridine (≥95%), morpholinepropanesulphonic acid hemisodium salt (MOPS, ≥99%), nonafluoropentanoic acid (NFPA, 97%) and HPLC grade water.

Preparation of model systems

Solutions of βCN (1.33 M, 3.2% protein) and D-(+)-glucose (0.1345 M, 4.6% sugar) in phosphate buffer (0.2 M; pH 6.7) were heated at 95°C for 0, 1, 2 and 4 h (βCN/G). A parallel set of solutions in which MGO



Scheme 1 Formation of MGO-derived dihydroxyimidazolidine (*MG-DH*) and MGO-derived hydroimidazolone (*MG-HI*) from glucose and MGO

(1 mM) replaced glucose was heated for 0, 5, 30, 60, and 120 min (βCN/MGO). βCN heated in phosphate buffer without any carbonyl compound was the control. Model systems were prepared in triplicate.

Tryptic digestion

Model systems were ultrafiltrated through a 5 kDa molecular mass cut-off ultrafiltration device (Millipore, Watford, UK) to remove residual sugar/MGO and buffer salts. Protein (0.25 mg, ~10 nmol) was digested according to Brock et al. (2003). Briefly, the protein was reduced with dithiothreitol in MOPS buffer containing urea and EDTA, derivatised with 4-vinylpyridine, and digested with trypsin at an enzyme:substrate ratio of 5:100 (w/w) at 37°C for 5.5 h. Digestion was terminated by freezing at −20°C and digests were analysed by UPLC-MS.

UPLC-MS

Samples were fractioned on a Waters (Manchester, UK) Acquity UPLC system coupled to a Waters Premier triple quadrupole mass spectrometer. Separations were conducted on a Waters UPLC® (2.1 × 50 mm) column. The stationary phase was BEH C_{18} and the particle size was 1.7 µm. The column was housed in an oven at 50°C. Samples were eluted in a liner gradient running from 90% solvent A (aqueous 5 mM NFPA) to 80% solvent B (acetonitrile) at a flow rate of 0.2 mL/min over 7.5 min. The injection volume was 7.5 µL (equivalent to 25 µg of protein, ~1 nmol). Full scan experiments were conducted by operating the MS in positive ion electrospray mode and using an *m/z* range of 200–1,800 amu. Other MS operating conditions used were: scan duration, 1 s; capillary voltage, 3 kV; cone voltage, 22 V; source temperature, 130°C; desolvation temperature, 400°C. Data were analysed using MassLynx software (version 4.1), supplied by Waters.

Semi-quantification of modified peptides

Modified and unmodified peptides were located by constructing extracted ion chromatograms at relevant *m/z* values and semi-quantified based on the percentage contribution of the peak area (% PA) of each peptide to its peptide family (Ames 2005). Briefly, peak areas of each peptide at all detected charge states were summed. The sum of the peak areas of all of the members within each peptide family gave the total peak area. The % PA of each member within a peptide family was calculated by dividing the sum of the PA of all charge states of that peptide by the total peak area and expressing it as a percentage.

Results

Site specificity of FL and CML formation in (βCN/G)

βCN incubated with glucose resulted in two of the eleven lysine residues, i.e., K107, and K176, being modified to FL and/or CML. As an example, Fig. 2 shows the location of the carboxymethylated peptide ^{170}VLPVPQKAVPYPQR^{183} with K176 modified to form CML (^{170}VK$^{176}_{CML}$R^{183}) within an extracted ion chromatogram. The ions at *m/z* 826 and 551 corresponded to the 2^+ and 3^+ charge states of this peptide (Fig. 2a, b). These ions were absent from the corresponding chromatograms for native βCN (Fig. 2c, d). Table 1 lists the peptides that were identified within the K107 and K176 families. Indigenous LL was detected only on K107, i.e., peptide ^{106}H–K$^{107}_{LL}$–K^{113}. Peptides ^{106}H–K$^{107}_{FL}$–K^{113} and ^{106}H–K$^{107}_{CML}$–K^{113} indicate modification by glucose on K107. Similarly, peptides ^{170}V–K$^{176}_{FL}$–R^{183} and ^{170}V–K$^{176}_{CML}$–R^{183} indicate modification by glucose on K176.

The kinetics of formation of FL and CML on K107 and K176 when βCN is incubated with glucose are depicted in Fig. 3. For the K107 family (Fig. 3a), the % PA of peptide ^{106}H–K$^{107}_{FL}$–K^{113} increased during the first hour of incubation (% PA = 8.8) and then its contribution to the total peak area remained constant up to 4 h. Amounts of the peptide ^{106}H–K$^{107}_{CML}$–K^{113} accounted for 5.4% of the PA after 1 h, and increased to 7.1% at 4 h. As the % PA values for peptides ^{106}HK$^{107}_{FL}$K^{113} and ^{106}HK$^{107}_{CML}$K^{113}CML increased, the contribution made by indigenous LL decreased from 15.3 to ~10% between 1 and 4 h.

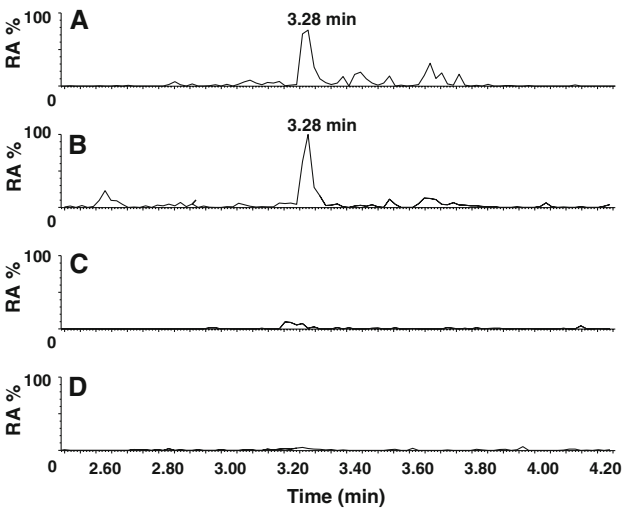


Fig. 2 Location of modified peptide ^{170}VK$^{176}_{CML}$R^{183} Extracted ion chromatograms at **a** *m/z* 826 (2^+ charge state) and **b** *m/z* 551 (3^+ charge state) for βCN incubated with glucose for 4 h show one major peak at 3.28 min. Extracted ion chromatograms at **c** *m/z* 826 and **d** *m/z* 551 for native βCN show no peak at 3.28 min. The chromatograms are normalised to the same scale

Table 1 Mass:charge (*m/z*) ratios of charge states of the detected modified and unmodified peptides within the K107 and K176 peptide families

Peptide group	Peptide	Charge state		
		1^+	2^+	3^+
K^{107} family				
Native cleaved peptide	E^{108}–K^{113}	749	375	
Native uncleaved peptide	H^{106}–K^{113}		508	339
LL peptide	H^{106}–$K^{107}{}_{LL}$–K^{113}		670	447
FL peptide	H^{106}–$K^{107}{}_{FL}$–K^{113}		589	393
CML peptide	H^{106}–$K^{107}{}_{CML}$–K^{113}		537	358
K176 family				
Native cleaved peptide	A^{177}–R^{183}	831	416	
Native uncleaved peptide	V^{170}–R^{183}		797	532
FL peptide	V^{170}–$K^{176}{}_{FL}$–R^{183}		878	586
CML peptide	V^{170}–$K^{176}{}_{CML}$–R^{183}		826	551

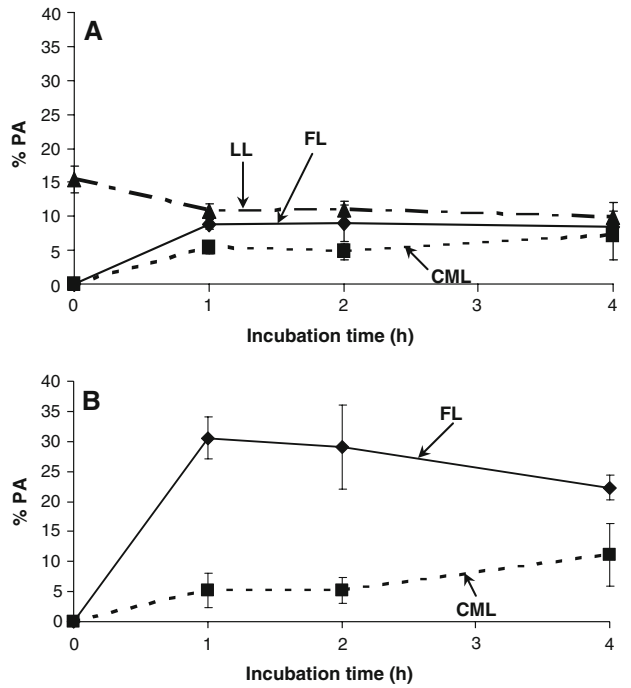


Fig. 3 K107 and K176 are the main sites of formation of FL and CML in *β*CN/G incubations. Kinetics of formation of **a** FL and CML adducts on K-107, peptide ^{106}H–K^{107}–K^{113} and **b** FL and CML adducts on K-176, peptide ^{170}V–K^{176}–R^{183}. The amounts are expressed as % PA (see "Materials and methods"). Data points represent the mean of triplicate incubations and the *error bars* represent the standard deviation

For the K176 family, peptide ^{170}VLPVPQK$^{176}{}_{FL}$AP-VYPQR^{183} (^{170}V–QR^{183}), with K-176 modified to FL (peptide ^{170}V–K$^{176}{}_{FL}$–R^{183}) accounted for 30.5% of the PA after 1 h (Fig. 3b), after which time the value slowly decreased to 22.3% after 4 h. After 4 h of incubation, the % PA for peptide ^{170}V–K$^{176}{}_{FL}$–R^{183} was ~3-fold higher compared to peptide ^{106}H–K$^{107}{}_{FL}$–K^{113}. Peptide ^{170}VK$^{176}{}_{CML}$R^{183} accounted for 5.2% of the PA after 1 h, and increased to 11% after 4 h.

Site specificity of MG-DH and MG-HI formation in *β*CN/MGO and *β*CN/G

When *β*CN was incubated with MGO or glucose, peptide ^{184}DMPIQAFLLYQEPVLGPVRGPFPIIV^{209} (^{184}D–V^{209}), with R202 modified to MG-DH (^{184}DR$^{202}{}_{MG\text{-}DH}$V^{209}) and peptide ^{184}D–V^{209} with R202 modified to MG-HI (^{184}DR$^{202}{}_{MG\text{-}HI}$V^{209}) were formed. Peptide ^{177}AV-PYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV^{209} (^{177}A–V^{209}), containing one MG-DH adduct and peptide ^{177}A–V^{209} containing one MG-HI adduct, were also detected. Both of these peptides contain arginine at positions 183 and 202 but it was not possible to distinguish which residue was modified using the methodology applied in this study, and the peptide family is designated R183/202. Peptides ^{177}A–V^{209} with modifications on both R183 and R202 were not detected. Table 2 lists the peptides resulting from modification on R202 and R183/202. Peptides ^{184}DR$^{202}{}_{MG\text{-}DH}$V^{209} and ^{184}DR$^{202}{}_{MG\text{-}HI}$V^{209} indicate modifications on R202. Peptide ^{177}A–R$^{183/202}{}_{MG\text{-}DH}$–V^{209} and ^{177}A–R$^{183/202}{}_{MG\text{-}HI}$–V^{209} indicate modifications on either R183 or R202.

The kinetics of formation of peptides ^{184}D–R$^{202}{}_{MG\text{-}DH}$–V^{209} and ^{184}D–R$^{202}{}_{MG\text{-}HI}$–V^{209} when *β*CN was incubated with MGO are depicted in Fig. 4a. The % PA of peptide ^{184}D–R$^{202}{}_{MG\text{-}DH}$–V^{209} was 6.4% after 30 min of incubation and decreased to 5% after 2 h. The % PA of peptide ^{184}D–R$^{202}{}_{MG\text{-}HI}$–V^{209} increased during the first 30 min of incubation to 8.1% and to 10% at 2 h. The kinetics of formation of peptides ^{177}A–R$^{183/202}{}_{MG\text{-}DH}$–V^{209} and ^{177}A–R$^{183/202}{}_{MG\text{-}HI}$–V^{209} are depicted in Fig. 4b. Peptide ^{177}A–R$^{183/202}{}_{MG\text{-}DH}$–V^{209} had a % PA of 5.7% after 30 min of incubation and remained at this level to 2 h. The % PA of peptide ^{177}A–R$^{183/202}{}_{MG\text{-}HI}$–V^{209} increased to 9.5% after 30 min of incubation and to 11.5% at 2 h (Fig. 4b).

The kinetics of formation of MG-HI and MG-DH on R202 and R183/202 in *β*CN/G incubations are depicted in Fig. 4c and d. Peptide ^{184}D–R$^{202}{}_{MG\text{-}DH}$–V^{209} accounted for 2.6% of the PA after 2 h of incubation and increased to 4.6% after 4 h. The % PA of peptide ^{184}D–R$^{202}{}_{MG\text{-}HI}$–V^{209} increased to 5.4% after 2 h of incubation followed by an ~2-fold increase to 4 h (% PA = 12.7) (Fig. 4c). The % PA of peptide ^{184}D–R$^{202}{}_{MG\text{-}HI}$–V^{209} was ~2-fold higher than ^{184}D–R$^{202}{}_{MG\text{-}DH}$–V^{209} after 2 h and 4 h of incubation. The kinetics of formation of peptides ^{177}A–R$^{183/202}{}_{MG\text{-}DH}$–V^{209} and ^{177}A–R$^{183/202}{}_{MG\text{-}HI}$–V^{209} are depicted in Fig. 4d. The % PA value for peptide ^{177}A–R$^{183/202}{}_{MG\text{-}DH}$–V^{209} was 5.4% after 1 h and 13.1% after 4 h. The % PA of peptide

Table 2 Mass:charge (*m/z*) ratios of the charge states of the detected modified and unmodified peptides within the R183 and R183/202 peptide families

Peptide group	Peptide	Charge state				
		1^+	2^+	3^+	4^+	5^+
R183 family						
Native cleaved peptide	D^{184}–R^{202}		1,094	730	548	
Native uncleaved peptide	D^{184}–V^{209}		1,456	971	729	
MG-HI peptide	D^{184}–$R^{202}_{\text{MG-HI}}$–V^{209}		1,483	989		
MG-DH peptide	D^{184}–$R^{202}_{\text{MG-DH}}$–V^{209}		1,492	995		
R183/202 family						
Native cleaved peptide	A^{177}–R^{183}	831	416			
Native uncleaved peptide	A^{177}–V^{209}			1,243	932	745
MG-HI peptide	A^{177}–$R^{183/202}_{\text{MG-HI}}$–$V^{209}$			1,259	945	
MG-DH peptide	A^{177}–$R^{183/202}_{\text{MG-DH}}$–$V^{209}$			1,265	949	

^{177}A–$R^{183/202}_{\text{MG-HI}}$–$V^{209}$ increased to 8.9% after 1 h of incubation and to 26.1% after 4 h. The % PA for peptide ^{177}A–$R^{183/202}_{\text{MG-HI}}$–$V^{209}$ was ~2-fold higher than ^{177}A–$R^{183/202}_{\text{MG-DH}}$–$V^{209}$ after 4 h of incubation.

The rates of formation of MG-DH and MG-HI adducts in MGO-modified βCN are highest during the first 30 min of incubation, after which levels remain reasonably constant. In the βCN/glucose incubations, the formation of MG-DH and MG-HI adducts on both peptides increases in an approximately linear fashion over the entire heating time. Regardless of the carbonyl or arginine residue, the ratio, % PA MG-HI peptide: % PA MG-DH peptide was 2–3 (Fig. 4) after 1 h heating for all of the incubations, indicating that these two adducts existed in equilibrium after this heating time.

Discussion

Semi-quantification of tryptic peptides

MS is a very powerful tool in proteomics and several studies in the past have used LC-MS to study sites of modification in different proteins. One means of monitoring levels of modified peptides is by measuring their relative abundance (RA) values. The RA approach is commonly used to account for differences in the amount of protein between different samples. The RA is calculated by dividing the sum of the peak areas of all detected charge states of a modified peptide by the sum of the peak areas of all detected charge states of C-terminal peptide of the protein (Brock et al. 2003). The drawback of this method is that the amounts of different native peptide within a protein cannot be compared due to variability in the MS response. Another approach used to overcome the differences in the MS response involves monitoring the % loss of the unmodified peptides (Hinton and Ames 2006). Alternatively, the modified and unmodified peptides within a peptide group may be expressed as a percentage sum of the PA (% PA) (Ames 2005). The residual standard deviations (RSDs) for the native peptides using the % PA approach are <5% which is better than when using the RA method or monitoring the loss of the unmodified peptide (Ames 2005).

Site specificity of modification

Our previous work investigating the site specificity of formation of FL, CML, glyoxal (GO)-derived-DH, GO-derived HI, MGO-derived DH, MGO-derived HI, tetrahydropyrimidine (THP), argpyrimidine and methionine sulphoxide (MetSO) on ribonuclease (Brock et al. 2003; Cotham et al. 2004; Brock et al. 2007a, b) and formation of FL, the Heyns rearrangement product and CML on BSA (Hinton and Ames 2006) shows modification to be a highly specific process. In the present study, we investigated the site specificity of modification of a food protein glycated under conditions relevant to food processing. Glucose and MGO were the carbonyl reactants employed. Milk is a component of a wide range of processed foods while MGO forms by oxidation of sugar and lipid. Both glucose and MGO are ubiquitous in food.

Our results demonstrate for the first time the sites of formation on βCN of FL and CML from glucose and of MG-HI and MG-DH from MGO and glucose. An earlier study (Henle and Klostermeyer 1993) monitored the reactivity of lysine residues on βCN incubated with lactose. The tryptic peptides of lactosylated βCN revealed K28/29, K32, K99 and K107 to be the favoured sites of glycation with modification occurring within 15 min of incubation at 70°C. Longer incubation times (60 min) led to gradual modification of K105, K113 and K176 while K48, and

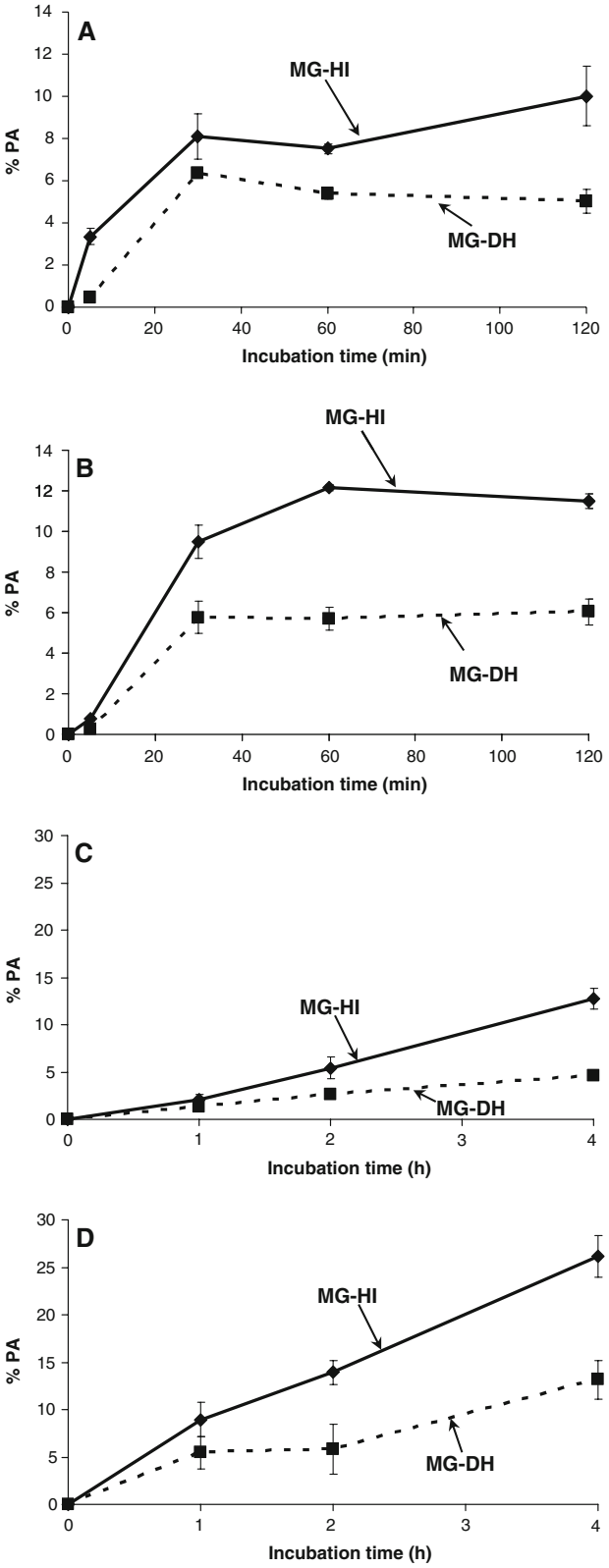


Fig. 4 R202 and R183/202 are the main sites of formation of MG-DH and MG-HI in βCN/MGO and βCN/G incubations. Kinetics of formation of **a** MG-DH and MG-HI adducts on R-202, peptide ^{184}D–R^{202}–V^{209} and **b** MG-DH and MG-HI adducts on R-183/202, peptide ^{177}A–R$^{183/202}_{\text{MG-DH}}$–V^{209} in βCN/MGO incubations. **c** MG-DH and MG-HI adducts on R-202, peptide ^{184}D–R^{202}–V^{209} and **d** MG-DH and MG-HI adducts on R-183/202, peptide ^{177}A–R$^{183/202}_{\text{MG-DH}}$–V^{209} in βCN/G incubations. The amounts are expressed as % PA (see "Materials and methods"). *Data points* represent the mean of triplicate incubations and the *error bars* represent the standard deviation

K169 remained relatively unreactive, even after 60 min at 90°C. A subsequent investigation (Scaloni et al. 2002) reported five lysines on βCN to be modified to LL in different heat-treated milks, with K107 being the favoured site of modification. Lactosylation of βCN was dependent on the degree of heat treatment with only K107 being modified in pasteurised milk, whereas K32, K48, K107, K113 and K176 were lactosylated in UHT and sterilized milk. No previous studies have reported the sites of glycation of βCN by other carbonyl compounds present in food.

In the current study, the kinetics of formation of FL on both K107 and K176 were highest during the first hour of incubation, after which time amounts either remained constant or decreased slowly until 4 h. This could be attributed to formation of CML. FL was formed to a greater extent on K176 compared to K107, while the proportion of CML on both lysines was similar. The reactivity of a lysine residue within a protein depends on various factors including location within the amino acid sequence, nature of adjacent amino acids and charge of the amino acid side chain (Henle and Klostermeyer 1993). Other factors include pH, ionic strength, presence of phosphate ions (Watkins et al. 1987), and reaction temperature (Scaloni et al. 2002). Henle and Klostermeyer (1993) observed that the location of a charged glutamic acid sidechain adjacent to a lysine (as is the case for K107 and K176) promoted lactosylation. It has also been observed that, in haemoglobin, close proximity of an acidic amino acid residue can promote glycation of lysine residues (Shapiro et al. 1980).

In the current study, neither FL nor CML adducts could be detected on the remaining nine lysine residues. This might be due to conformational changes to the protein during heating at 95°C for several hours in the presence of a carbonyl compound. Such changes might limit the accessibility to these unreactive lysine residues.

R202 (and possibly R183) was the main arginine residue undergoing modification in glycated βCN, regardless of whether the carbonyl was MGO added directly to the model system or MGO derived by oxidation of reactant glucose. MG-HI was present in higher amounts than MG-DH, regardless of the carbonyl reactant or time of heating. There are no previous reports of the formation of MG-DH or MG-HI on βCN. In both the current study and our previous work (Brock et al. 2007a) concerning the site-specificity of modification of RNase by MGO, we have shown that certain arginine residues are favoured over

others for modification. This may be attributed at least in part to the relative availability of arginine residues and their accessibility to MGO. In addition, the nature of adjacent amino acids might influence reactivity. For example, identification of R410 as the major site of modification of MGO-modified HSA has been attributed to strong electrostatic interaction between R410 and the adjacent polar Y411 (Ahmed et al. 2005). In this respect, R183 is located next to an aspartic acid residue at position 184, and might promote modification in a similar fashion to the proposed effect of glutamic acid residues on glycation of the ε-amino group of lysine residues, discussed above.

Other MGO-derived adducts, i.e., THP, argpyrimidine and CEL, were sought but could not be detected in this study. Also, methionine sulphoxide (MetSO) a marker of oxidation was not detected on any of the 6 methionine residues in either the βCN/glucose or βCN/MGO incubations. These data are in contrast to our previous findings (Brock et al. 2007b), where MetSO was formed in higher amounts than CML on RNase incubated under in vitro physiological conditions. MetSO has been identified on whey protein heated with lactose at 60°C (Meltretter et al. 2007) and in processed milks (Baxter et al. 2007). The lack of detection of MetSO in the current study may be attributed to various factors related to the temperature of heating and the composition of the system investigated. Compared to a temperature of 60°C, a temperature of 95°C may lead to lower levels of oxygen in the system and thus favour the formation of glycation products over MetSO. In processed milks, MetSO may form from oxidised lipid during storage.

In conclusion, we have demonstrated the site specificity of formation of FL, CML, MG-DH and MG-HI on βCN heated under conditions of relevance to food processing. This work has potential application to better understand the relationship between food processing operations and food functional properties and to develop metabolomics procedures for sophisticated monitoring of food processing operations.

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