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ORIGINAL ARTICLE

N-ε-fructosyllysine and *N*-ε-carboxymethyllysine, but not lysinoalanine, are available for absorption after simulated gastrointestinal digestion

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Abstract Food processing leads to a variety of chemical modifications of amino acids in food proteins. Recent studies have shown that some modified amino acids resulting from glycation reactions can pass the intestinal barrier when they are bound in dipeptides. In this study, we investigated as to what extent modified amino acids are released from post-translationally modified casein during simulated gastrointestinal digestion. Casein was enriched with *N*-ε-fructoselysine, *N*-ε-carboxymethyllysine, and lysinoalanine, in different degrees of modification. The casein samples were subjected to a two-step proteolysis procedure, simulating gastrointestinal digestion. The digestibility of modified casein as measured by analytical size-exclusion chromatography (SEC) decreased with increasing degree of modification especially after enrichment of fructoselysine and lysinoalanine. Semi-preparative SEC of digested casein samples revealed that fructoselysine and carboxymethyllysine are released bound in peptides smaller than 1,000 Da, which is comparable to native amino acids. The glycation compounds should, therefore, be available for absorption. Lysinoalanine as a crosslinking amino acid, however, is mostly released into longer peptides of at least 30–40 amino acids which should strongly impair its absorption availability.

Keywords Maillard reaction · Glycation · Advanced glycation end product (AGE) · Lysinoalanine (LAL) · Carboxymethyllysine (CML) · Fructoselysine · Simulated gastrointestinal digestion

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Abbreviations

AAA	Amino acid analysis
AGE	Advanced glycation end product
Bis-CML	*N*,*N*-bis(carboxymethyl)-L-lysine
BW	Body weight
CML	*N*-ε-carboxymethyllysine
FL	*N*-ε-fructoselysine
HPLC	High pressure liquid chromatography
LAL	Lysinoalanine
MG-H1	Methylglyoxal-derived hydroimidazolone 1
MRP	Maillard reaction product
MW	Molecular weight
PBS	Phosphate buffered saline
SEC	Size-exclusion chromatography
WRF	Tryptophan-rich fraction
YRF	Tyrosine-rich fraction

Introduction

Food processing includes various types of treatment which lead to the modification of food constituents, especially of proteins. Alkali treatment, e.g., during isolation of proteins, favors amino acid racemisation and crosslinking of proteins due to the formation of lysinoalanine (LAL) (Bohak 1964; Friedman 1999). The Maillard reaction implies the covalent attachment of sugars and their degradation products to lysine and arginine residues, thereby giving rise to Amadori products like fructoselysine (FL) and lactuloselysine and advanced glycation end products (AGEs) like *N*-ε-carboxymethyllysine (CML), methylglyoxal-derived hydroimidazolone 1 (MG-H1), and pyrraline (Ledl and Schleicher 1990; Henle 2005) (Fig. 1). With the daily diet, between 500 and 1,200 mg of Amadori products and between 25 and 75 mg of AGEs have been estimated to be

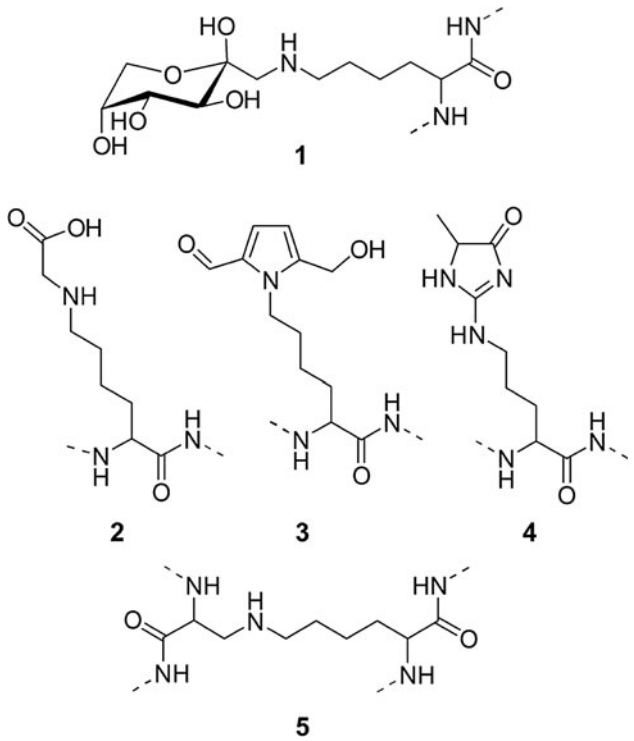

Fig. 1 Chemical structures of the Amadori product *N*-ε-(1-deoxyfructosyl)-lysine (fructoselysine, FL, **1**), the advanced glycation end products *N*-ε-carboxymethyllysine (CML, **2**), 6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-norleucine (Pyrraline, **3**), *N*-δ-(5-methyl-4-oxo-5-hydro-2-imidazolonyl)-ornithine (methylglyoxal-derived hydroimidazolone 1, MG-H1, **4**), and the crosslinking amino acid *N*-ε-(2-amino-2-carboxy-1-ethyl)-lysine (lysinoalanine, LAL, **5**). *Dashed bonds* indicate the peptide chain

ingested mainly from milk and bakery products (Henle 2003). LAL has been analyzed in heated milk products and sodium caseinates in concentrations up to 2,200 mg/kg protein (Henle et al. 1993). Both alkaline treatment and the Maillard reaction contribute to the reduction of the nutritional quality of proteins, because crosslinking impairs proteolysis by intestinal peptidases (Friedman et al. 1981; Krause and Freimuth 1985), and lysine as an essential amino acid is blocked and unavailable for the organism (Finot and Magnenat 1981).

Maillard reaction products (MRPs) have also been detected in human tissue and plasma proteins (Thornalley et al. 2003), but the concentrations circulating in vivo are much lower than the amounts taken up with the daily diet (Henle 2003). The occurrence of the Maillard reaction in vivo has long been linked to the consequences of diabetes due to irreversible protein modification and crosslinking (Brownlee et al. 1984; Ledl and Schleicher 1990). Moreover, dietary MRPs are discussed to impose a risk on human health by enhancing oxidative stress and initiating inflammatory responses ultimately leading to atherosclerosis (Šebeková and Somoza 2007; Uribarri et al. 2007). Controversially, positive effects of dietary MRPs such as their antioxidant capacity have been reported (Ames 2007; Henle 2007; van Boekel et al. 2010). LAL is nephrotoxic to rats fed the free amino acid, but this effect has not been verified for other species (Friedman 1999).

Possible in vivo effects of any dietary modified amino acid depend on the question whether these products are available for absorption and then taken up into the circulation. In balance studies, human subjects ingested meals with known contents of MRPs, which were subsequently measured in plasma or urine (Lee and Erbersdobler 1994; Förster et al. 2005). Such studies are indicative of the release from proteins and epithelial transport of MRPs if the concentrations in physiological fluids like blood or urine are influenced by changes in the dietary intake. The urinary excretion of FL and LAL is only poorly dependent on the dietary intake, but more than 60 % of pyrraline from foods is excreted renally (Förster et al. 2005). While some authors claim that dietary CML strongly contributes to the concentrations in vivo (Uribarri et al. 2007), two recent studies could find no such contribution in humans (Semba et al. 2012) and rats, respectively (Alamir et al. 2012). In rats, the metabolic transit of CML, FL, and LAL, amounted to 30.4, 31.2, and 5.6 %, respectively, with CML being mainly excreted via the urine and FL accumulating in the kidneys (Somoza et al. 2006). When moderate amounts of protein-bound CML (0.2–1.2 mg/kg BW/d) were applied to rats, between 23 and 38 % of the compound were recovered in the urine and between 37 and 48 % in the feces (Alamir et al. 2012).

Balance studies are valuable approaches to gain first insights into the metabolic fate of post-translationally modified amino acids. However, they do not readily allow estimations on the bioavailability of substances since they represent the product of different physiological factors, at least (i) the release from proteins during digestion, (ii) the transit across the intestinal barrier, and (iii) metabolizations in vivo. For Maillard reaction products such as CML or pyrraline, we were able to show that they are actively transported into epithelial cells when bound in dipeptides, but not as the free amino acids, while FL was neither transported in free nor in peptide-bound form (Hellwig et al. 2011). In food, however, the predominant part of modified amino acids is present in a protein-bound form (Hegele et al. 2008).

With a typical western diet, 70–100 g of protein is ingested daily (Daniel 2004). Dietary proteins are first digested by the gastric endopeptidase pepsin followed by five pancreatic endo- and exopeptidases. Luminal digestion yields a mixture of short-chain peptides and free amino acids with the peptides predominating (Adibi and Mercer 1973; Daniel 2004). These peptides can pass the mucus

layer on intestinal cells and reach the gut epithelium, where brush-border peptidases cleave peptides longer than three amino acids into absorbable units, since only free amino acids and di- and tripeptides are available for intestinal absorption, i.e., they can be taken up by specific transporters (Brandsch and Brandsch 2003; Daniel 2004; Bröer 2008).

Protein-bound LAL and casein-bound *N*-ε-(γ-glutamyl)-L-lysine are only poorly released during simulated gastrointestinal digestion (Friedman et al. 1981; Krause and Freimuth 1985; Savoie et al. 1991; Hellwig et al. 2009). These results, however, cannot directly be transferred to products such as FL or CML since (i) LAL is a crosslinking amino acid and (ii) racemisation occurs during the alkaline treatment which also hinders proteolysis. Amino acids which are chemically modified without crosslinking such as homoarginine seem to be as well digestible as proteinogenic amino acids (Qiao et al. 2004), but this has not been shown for any defined MRP until now. Therefore, we enriched casein with LAL, FL, and CML. Proteins were subjected to gastrointestinal digestion. The course of the digestion was monitored by size-exclusion chromatography (SEC). Subsequently, the digested proteins were fractionated by semi-preparative SEC to analyze whether the products are enriched in peptide fractions which can possibly be transported.

Materials and methods

Chemicals

Glyoxylic acid monohydrate was obtained from Fluka (Steinheim, Germany) and D-glucose from Alfa Aesar (Karlsruhe, Germany). Sodium cyanoborohydride was purchased from Merck (Darmstadt, Germany). All chemicals used for simulated gastrointestinal digestion, namely pepsin (E.C. 3.4.23.1, 4,230 U/mg protein), mucin, pancreatin, trypsin (E.C. 3.4.21.4, 15,700 U/mg protein), and bile extract were from porcine sources and purchased from Sigma-Aldrich (Steinheim, Germany). The SEC and amino acid analysis calibration standards were also obtained from Sigma-Aldrich, except dextran blue (Serva, Heidelberg, Germany), LAL, Lys-Ala, and Ala-Lys (Bachem, Bubendorf, Switzerland). All other chemicals were of the highest purity available. The water used for the preparation of buffers and solutions was obtained using a Purelab plus purification system (US-Filter, Ransbach-Baumbach, Germany). Preparations of FL (Krause et al. 2003), furosine (Henle et al. 1995), CML (Hellwig et al. 2011), and sodium caseinate from raw cow's milk (Hellwig and Henle 2010) were performed according to the literature stated.

Preparation of samples for analysis

Acid hydrolysis of modified proteins and fractionated digestion mixtures was always performed in the presence of 6N HCl at 110 °C in a drying oven for 23 h. Aliquots of samples for amino acid analysis (AAA) were then evaporated to dryness in vacuo and suspended in a suitable volume of AAA loading buffer (0.12 N lithium citrate, pH 2.20). For the determination of furosine, portions of 500 µL of acid hydrolyzates were subjected to solid-phase extraction (Resmini et al. 1990). Aliquots of 1,000 µL were evaporated to dryness and reconstituted in 0.4 % acetic acid prior to HPLC measurement. All samples were membrane filtered (0.45 µm) before analysis.

High-pressure liquid chromatography

Furosine analysis was performed according to Resmini et al. (1990) using a high-pressure gradient system from Amersham Pharmacia Biotech (Uppsala, Sweden), consisting of a pump P-900 with an online degasser (Knauer, Berlin, Germany), a column oven, a UV detector UV-900, and an 8-way outlet valve PV-908. A stainless steel column (250 mm × 4.6 mm) filled with Econosphere RP-8 material of 5 µm particle size with a guard column (5 mm × 4 mm) of the same material was used (Alltech, Deerfield, IL). The injection volume was between 20 and 50 µL and the separation was performed at a column temperature of 34 °C and a flow rate of 1.2 mL/min. The mobile phases were 0.4 % aqueous acetic acid (solvent A) and 0.3 % potassium chloride in 0.4 % aqueous acetic acid (solvent B). Isocratic elution was performed for 11.5 min at 100 % A, and then a linear gradient to 50 % B in 7 min was applied. The proportion of solvent B was held at 50 % for 2.5 min. The absorbance was read at 280 nm.

Amino acid analysis (AAA)

Measurements of proteinogenic amino acids, CML and LAL were performed with the amino acid analyzer S 433 (Sykam, Fürstenfeldbruck, Germany) using a PEEK column filled with the cation exchange resin LCA K07/Li (150 × 4.6 mm, 7 µm). Lithium buffers ready for use were purchased from Sykam and employed for different gradient programs utilized previously (Hellwig et al. 2011). Post-column derivatization with ninhydrin was applied, and VIS detection was performed with an integrated two-channel photometer simultaneously working at 440 and 570 nm, respectively. External calibration was performed with the synthesized standards and an amino acid mixture (Sigma-Aldrich, Steinheim, Germany). The injection volume was between 10 and 100 µL. Tyrosine, which is not modified by the methods applied in this study, was taken as an

internal standard for the evaluation of amino acid chromatograms. The lysine blockage was calculated as the quotient of the lysine-to-tyrosine ratios of modified and native casein.

Analytical size-exclusion chromatography (SEC) and calculation of digestibility parameters

Analytical separation of peptide mixtures was performed using the HPLC system mentioned above. The column used was a Superdex peptide HR 10/30 (Pharmacia Biotech, Uppsala, Sweden). For analytical runs, the column was eluted at ambient temperature for 70 min with phosphate-buffered saline (PBS, 0.32 M Na^+, 4.4 mM K^+, 10 mM PO_4^{3-}, 0.3 M Cl^-, pH 7.4) at a flow rate of 0.5 mL/min. All samples were membrane filtered (0.45 µm) before analysis. The injection volume was 50 µL. Detection was performed at 220 and 280 nm. The following standards were used for molecular mass calibration: dextran blue (2,000 kDa), hemoglobin (64.5 kDa), ovalbumin (42.8 kDa), lysozyme (14.6 kDa), insulin (5,733 Da), insulin B chain (3,496 Da), neurotensin (1,673 Da), bradykinin (1,060 Da), leupeptin (426.6 Da), Val-Tyr (280.3 Da), Lys-Ala, and Ala-Lys (217.3 Da).

Digestibility was defined as the percentage of peptides and amino acids smaller than 1,000 Da ($\Delta_{1,000}$) after gastric and intestinal digestion. For the calculation of $\Delta_{1,000}$, the peak areas of the fractions with peptides smaller than 1,000 Da ($A_{<1,000}$) were summarized for digested casein (A_{Prot}) and the respective enzyme blanks (A_{bl}) and put in the following equation:

$$\Delta_{1,000} = \frac{A_{<1,000,\text{Prot}} - A_{<1,000,\text{blank}}}{A_{\text{total,Prot}} - A_{\text{total,blank}}} \times 100\,\%$$

Similarly, the liberation of free tryptophan (Δ_{Trp}) was calculated from the respective areas of the tryptophan-rich fraction (WRF):

$$\Delta_{\text{Trp}} = \frac{A_{\text{WRF,Prot}} - A_{\text{WRF,blank}}}{A_{\text{total,Prot}} - A_{\text{total,blank}}} \times 100\,\%$$

As a third parameter, the relative progress of digestion after 60 min (Π_{60}) was estimated to recognize a possible delay in the digestion under intestinal conditions. This was calculated from the percentage of peptides ($\Delta_{1,000}$) liberated after 60 min of intestinal digestion divided by $\Delta_{1,000}$ at the end of digestion:

$$\Pi_{60} = \frac{\Delta_{1000,\,60\text{min}}}{\Delta_{1000,\,360\,\text{min}}} \times 100\,\%$$

Semi-preparative SEC

The same SEC system as described above was used. 100 µL of sample was injected after membrane filtration (0.45 µm), and the eluates of two consecutive runs were combined. The eluate was fractionated into seven fractions (10–20, 20–25, 25–30, 30–35, 35–40, 40–55, 55–65 min). The fractions were lyophilized and hydrolyzed in the presence of 5 mL 6N HCl and further worked up as described above. For the estimation of the recovery of furosine after hydrolysis of FL in the presence of PBS, 15 mL of a 3 µM FL solution in PBS was lyophilized and further processed like the semi-preparative SEC fractions. The recovery of FL was 98 %.

Preparation of modified proteins

Three samples of casein were enriched with FL by incubation of the protein with glucose in dry state in a defined humidified atmosphere. 1,000 mg of casein and 180 mg of glucose were suspended in 7 mL of water. The mixtures were lyophilized and stored in a desiccator over a saturated solution of magnesium nitrate ($a_W = 0.52$) (Rockland 1960) for 5 days. One of the mixtures (Glu-1) was then suspended in water and dialyzed against distilled water for 4 days using cellulose dialysis tubings (MWCO ca. 12 kDa, Sigma-Aldrich, Steinheim, Germany). The other two mixtures were heated in a drying oven for 4 h (Glu-2) and 24 h (Glu-3), respectively, and then dialyzed as well. The retentates were lyophilized and stored at −20 °C.

Enrichment of CML was performed by reductive alkylation (Glorieux et al. 2004). Casein (2,000 mg) and different amounts of glyoxylic acid (CML-1, 10 mg; CML-2, 60 mg; CML-3, 490 mg) were dissolved in 100 mL of 0.2 M sodium phosphate buffer, pH 7.4. The solutions were stirred for 20 h at 40 °C in a water bath after the addition of 1.1 g $NaBH_3CN$. Dialysis and lyophilization were performed as described above until cyanide was no more detectable. Casein was enriched with lysinoalanine (LAL) following the method of Sternberg and Kim (1977). Casein (100 mg/mL) was dissolved in 0.2N sodium borate buffer, pH 10.0, and the pH value was adjusted to 10.0 by addition of sodium hydroxide. The solution was heated to 100 °C under reflux. Samples of 10 mL were removed with a syringe at the end of the preheating phase (LAL-1), and after 15 min (LAL-2), and 180 min (LAL-3), of constant heating at 100 °C. The samples were dialyzed and lyophilized as described above.

Simulated gastrointestinal digestion

A method combining different literature methods (DIN 2004; Hernández-Ledesma et al. 2004) was applied. 100 mg of casein was suspended in 1.9 mL of synthetic gastric juice (2.90 g/L NaCl, 0.70 g/L KCl, 0.27 g/L KH_2PO_4, 3.00 g/L mucin), and the pH value was adjusted to 2.0 ± 0.1 by addition of 3 N HCl. Then, 100 µL of a

pepsin solution (20 mg/mL in synthetic gastric juice) was added and the mixture incubated under constant stirring in a water bath at 37 °C for 2 h. After 10 and 120 min, respectively, 200 µL of the suspensions was removed and diluted with 800 µL of PBS. The enzymes were immediately inactivated by heating the samples in a boiling water bath for 10 min. The samples were deep-frozen until SEC analysis.

After the stomach phase, 1.5 mL of synthetic pancreatic juice (0.30 g/L KCl, 0.50 g/L $CaCl_2 \cdot 2\ H_2O$, 0.20 g/L $MgCl_2 \cdot 6\ H_2O$, 1.00 g/L $NaHCO_3$, 9.00 g/L lyophilized porcine bile, 0.30 g/L urea) was added. The pH value was adjusted to 7.5 ± 0.1 by addition of solid $NaHCO_3$. Then, 100 µL of pancreatic enzyme suspension containing 14.4 mg pancreatin and 0.5 mg trypsin in synthetic pancreatic juice was added and the suspension stirred for further 6 h. Every 60 min, the pH value was adjusted to 7.5 ± 0.1 by addition of 3N HCl. After 10, 60, 180, and 360 min, 400 µL of the solutions was removed and diluted with 327 µL PBS. The enzymes were inactivated as described above and the samples deep-frozen until SEC analysis. The remaining solution was also heated for 10 min in a boiling water bath and used for SEC fractionation.

Statistical analysis

Comparison of means of digestibility values was performed by one-way ANOVA using the software PASW Statistics 18.

Results and discussion

Preparation of modified proteins

A large number of lysyl and arginyl dipeptides, glycated at their amino or guanidino side chains, can pass a barrier of cultured epithelial cells most probably via the peptide transporter PEPT1 (Hellwig et al. 2011). In food, however, glycated amino acids are bound in proteins from which they have to be released into di- or tripeptides prior to any transport. We, therefore, wanted to examine how far modified proteins are digested by measuring their overall digestibility and the length of peptides in which modified amino acids are enriched. Based on this, a conclusion can be drawn as to whether modified amino acids are available for intestinal absorption. Casein was taken as the model protein, because due to lack of pronounced tertiary structure concomitant denaturation could not have a major influence on the digestibility. The casein used for this study was isolated from raw cow's milk by isoelectric precipitation using sodium acetate buffers and was free from any lysine modification. Casein was then selectively enriched with three representatives of post-translational modifications, namely LAL as the main product resulting from alkaline treatment of proteins, FL as an example for the quantitatively important Amadori products, and CML as an AGE. The investigation of LAL should principally offer the possibility to compare our results with results from the literature, because the digestion of alkali-treated proteins has already been studied (Friedman et al. 1981; Krause and Freimuth 1985; Savoie et al. 1991). The three types of modification resulted in ascending lysine blockage (Table 1), which can fully be explained by the target modifications with the exception of the casein preparation CML-3. A difference between lysine blockage and CML content was noted together with the occurrence of an unknown peak in the amino acid chromatogram eluting even before aspartic acid (Fig. 2). Since this peak should represent a very acidic compound, we suppose that it could be *N*,*N*-bis(carboxymethyl)-L-lysine (Bis-CML). Quantification using the CML calibration curve revealed that the unassigned 30 % of lysine modification in CML-3 could fully be explained by this substance. Further studies will have to show if bis-carboxymethylation might be a new phenomenon in Maillard chemistry which could have been overlooked until now. The putative formation of Bis-CML as a by-product is not considered disadvantageous for our investigations, because Bis-CML strongly resembles CML in terms of the side-chain properties.

The casein modifications can thus be considered very selective with regard to the enrichment of the target substances. Casein preparations with lysine modifications of low-, medium-, and high grade were obtained. In processed food proteins, the lysine modification mostly accounts for up to 2 % (Henle et al. 1993, 1995; Hull et al. 2012), thus to the lowest degree of modification studied here. However, in samples like heated milk powder or bread crust, more than 10–20 % of lysine can be modified to the Amadori product (Henle et al. 1995). The casein samples with low-grade modification in this study can thus be considered a suitable model for food proteins. The lysine modification differs between the high-grade modified casein preparations, because the modifiability of lysine residues is limited, e.g., for LAL, which requires the reaction of two protein-bound amino acids in close vicinity. Moreover, it has to be stated that racemisation reactions will surely have occurred during the alkaline treatment. All casein preparations were free from short-chain peptides as measured with the analytical SEC system prior to digestion.

Simulated gastrointestinal digestion

Protein digestibility and release of amino acids is often measured in in vitro assays using pepsin and mixtures of

Table 1 Modification and digestibility parameters of native and modified casein samples

Casein sample	Lysine modification (mol-%$_{Lys}$)	Content of target modification (mol-%$_{Lys}$)	$\Delta_{1,000}$ (%)	Δ_{Trp} (%)	Π_{60} (%)
Native casein	–	–	78.3 ± 4.4^{a}	11.4 ± 0.5^{a}	91.2 ± 1.4^{a}
Glucose-modified casein		FL			
Glu-1	8.1 ± 5.3	7.2 ± 0.3	74.3 ± 1.3^{a,b}	10.1 ± 0.3^{a,b}	89.9 ± 0.5^{a,b}
Glu-2	24.3 ± 4.9	22.9 ± 2.2	72.0 ± 2.6^{a,b}	10.6 ± 0.7^{a,b}	90.9 ± 2.0^{a}
Glu-3	68.2 ± 4.1	61.1 ± 2.9	61.4 ± 0.2^{c}	8.5 ± 0.3^{c}	87.0 ± 2.4^{a,b}
Carboxy-methylated casein		CML			
CML-1	3.5 ± 2.4	4.7 ± 1.1	71.5 ± 0.7^{b}	11.4 ± 0.4^{a}	90.3 ± 0.5^{a,b}
CML-2	20.4 ± 2.1	18.5 ± 1.1	68.9 ± 0.6^{b}	11.0 ± 0.8^{a,b}	88.5 ± 0.6^{a,b,*}
CML-3	90.6 ± 1.2	60.3 ± 1.7	57.6 ± 1.1^{c}	10.9 ± 0.2^{a,b}	85.6 ± 1.7^{b}
Alkali-treated casein		LAL			
LAL-1	2.8 ± 2.1	1.7 ± 0.5	71.1 ± 0.4^{b}	10.6 ± 0.2^{a,b}	89.5 ± 0.8^{a,b}
LAL-2	5.1 ± 4.8	5.4 ± 3.3	68.6 ± 0.1^{b}	9.9 ± 0.3^{b}	88.7 ± 0.6^{a,b}
LAL-3	21.0 ± 2.1	17.7 ± 1.1	56.4 ± 0.4^{c}	7.6 ± 0.4^{c}	86.6 ± 0.3^{a,b}

In each column, means with equal superscript letters are not significantly different ($P < 0.05$) as evaluated by one-way ANOVA and Scheffé's post hoc test using PASW Statistics 18.0. Data are mean ± SD, $n = 3$

* $n = 2$

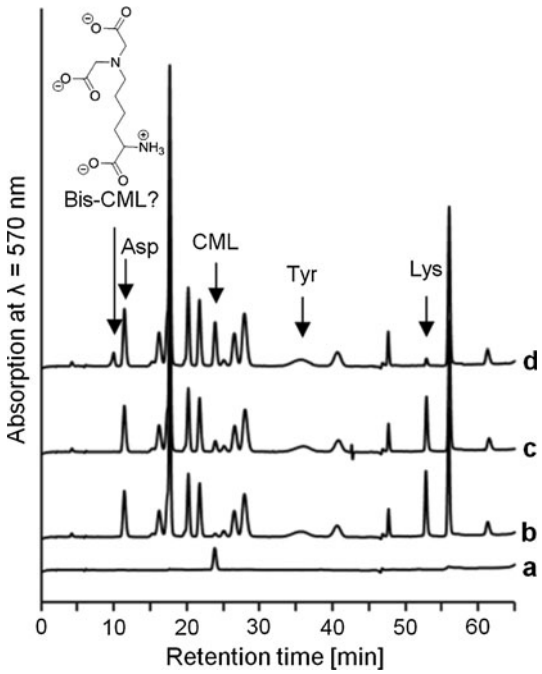

Fig. 2 Amino acid analysis with VIS detection after post-column ninhydrin derivatization (λ = 570 nm) of (*a*) a CML standard, and the carboxymethylated casein samples CML-1 (*b*), CML-2 (*c*), and CML-3 (*d*) after acidic hydrolysis

pancreatic proteases, e.g., pancreatin (Savoie et al. 1991; Hack and Selenka 1996; Qiao et al. 2004; DIN 2004; Hernández-Ledesma et al. 2004; Agudelo et al. 2004). Absorption is implemented in dynamic digestion models by concomitant dialysis or in static models by removal of aliquots from the digestion mixture. As in our previous study (Hellwig et al. 2009), we used a static digestion system (DIN 2004), which adheres as close as possible to the physiological conditions in terms of the composition of gastric and intestinal juices, pH adjustment, enzyme-to-substrate ratios, and transit time (Adibi and Mercer 1973; Savoie et al. 1991; Madsen 1992; Qiao et al. 2004; DIN 2004; Hernández-Ledesma et al. 2004). Casein samples are first incubated as a suspension in simulated gastric juice at pH 2.0 in the presence of pepsin (84.6 U/mg protein) for 2 h. During the incubation, the pH value rose to no more than 3.0, which is still within the physiological limits. Mucins as highly glycosylated proteins which protect the stomach epithelium from enzymatic actions and the acidic conditions were added to the gastric juice in a concentration of 0.3 %, because they could also have an impact on the protein–peptidase interactions. The digestion mixture was then diluted by the addition of intestinal juice, and the pH was set to 7.5, which terminates pepsin action in the assay. Besides pancreatin, which is a mixture of proenzymes from porcine pancreas, trypsin was added to the mixture to guarantee the activation of proenzymes. During digestion, full solubility of the digested peptides was achieved under simulated intestinal conditions after 60 min at the latest. The enzymatic activity in the samples taken for SEC analysis was terminated by incubation in a boiling water bath. Application of this treatment did not lead to a decomposition of FL, which is the least stable compound tested here, as examined by incubation and HPLC measurements of hippuryl-fructoselysine (Krause et al. 2003) as a model of peptide-bound FL (data not shown).

The progress of digestion was monitored by analytical SEC with UV-detection at 220 nm. We utilized a SEC column with a high selectivity for small peptides. As shown in Fig. 3, the correlation between the elution time and the logarithm of the molecular weight was linear between 0.25 and 40 kDa. Below 250 Da, effects of retention due to interactions of the analytes with the column predominated over size-exclusion, since free tyrosine and tryptophan eluted much later than expected. As could directly be seen from the chromatograms (Fig. 3), peptide lengths gradually declined during digestion. After 120 min of gastric digestion, peptides have an average molecular mass of 1,000 Da, while after 360 min of intestinal digestion most peptides are smaller than 1,000 Da, and tyrosine and tryptophan are released as the free amino acids as well.

Influence of casein modification on gastric and intestinal digestibility

In order to give a measure for the digestibility of casein after gastric and intestinal digestion, we evaluated the accumulation of peptides and amino acids in seven molecular mass fractions as shown in Fig. 3. The fractions from MW-1 to MW-4 can be attributed to distinct molecular weight ranges. The peptides in fraction MW-5 must be small peptides. The last two fractions are a tyrosine-rich fraction (YRF) and a tryptophan-rich fraction (WRF). As one measure for the digestibility of proteins, we calculated the percentage of peptides with molecular masses smaller than 1,000 Da ($\Delta_{1,000}$) which are released during digestion. The release of such peptides is often taken as a measure for the digestibility of proteins in the literature (Savoie et al. 1991). This is not synonymous with absorbability, since only free amino acids, di- and tripeptides can be absorbed by intestinal carriers (Brandsch and Brandsch 2003; Daniel 2004; Bröer 2008). However, the shorter the peptides the more easily they can cross the epithelial mucus layer and reach the membrane-bound peptidases by which they can be degraded to absorbable products.

The digestibility parameters are compiled in Table 1. The digestibility of unmodified casein amounts to 78.3 ± 4.4 % ($\Delta_{1,000}$), which is higher than literature values measured after dialysis and nitrogen determination [e.g., 63 ± 6 % (Savoie et al. 1991)]. This can originate from the relatively high impact on absorption at 220 nm of tyrosine and tryptophan (Kuipers and Gruppen 2007), two amino acids which are released relatively easily (Savoie et al. 1988; Vorob'ev et al. 1996; Agudelo et al. 2004). It also has to be taken into consideration that dialysis of small-molecular peptide mixtures with a continuous molecular-mass distribution as that resulting from digestion might be incomplete and that digestibility values based on dialysis studies might underestimate true digestibility. However, differences between the digestibility of native and modified caseins should be discernable using either of the systems. 91.2 % of the resulting peptides (A_{220}) smaller than 1,000 Da are already liberated after 60 min of digestion (Π_{60}). 11.4 % of the whole peptide and amino acid peak area are due to free tryptophan (Δ_{Trp}).

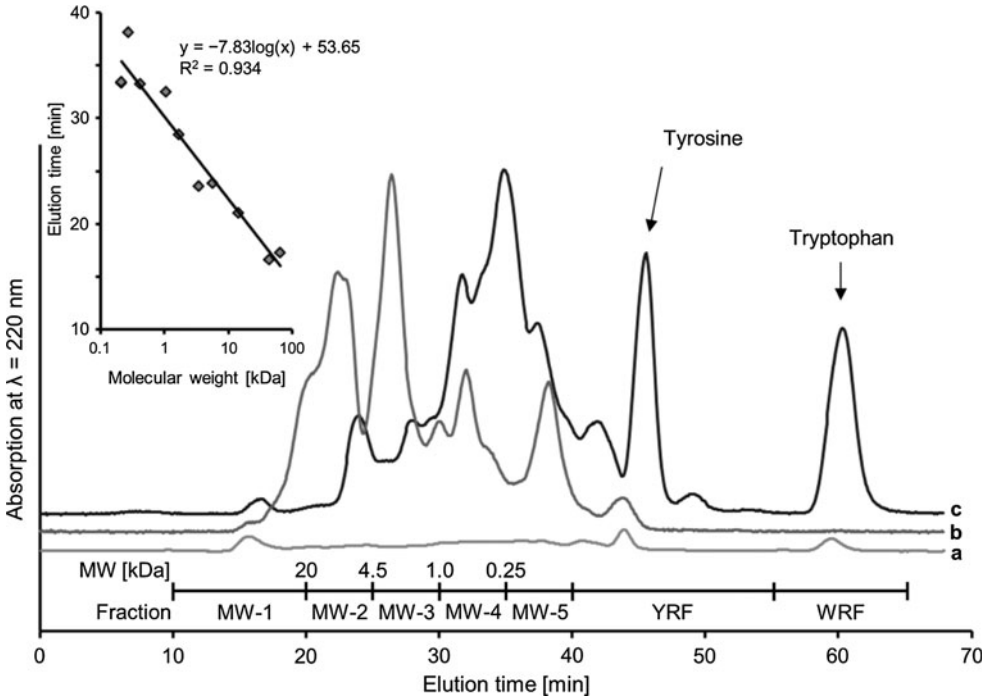

Fig. 3 Size-exclusion chromatography of *a* the enzyme blank after 120 min of gastric and 360 min of intestinal digestion, *b* a native casein sample after 120 min of gastric digestion, and *c* a native casein sample after 120 min of gastric digestion and 360 min of intestinal digestion. Calibration of the molecular mass scale (*inset*) and visualization of the fractionation limits for semi-preparative size-exclusion chromatography using PBS as the eluent with the respective molecular masses

The digestibility $\Delta_{1,000}$ of all modified casein preparations was lower than that of native casein, indicating that an improvement of digestion by, e.g., alkaline treatment (Krause and Freimuth 1985) mainly results from denaturation reactions which improve the accessibility by pancreatic peptidases. The reduction of casein digestibility in this study should thus reflect a reduction in the accessibility and cleavability of the protein backbone. This can result from (i) covalent modification of amino acid side-chains, (ii) crosslinking reactions in the case of LAL-casein, which can also take place as side reactions in Glu-caseins, and (iii) racemisation reactions due to alkaline treatment. The influence of side-chain modifications obviously is not very high since $\Delta_{1,000}$ of all modified samples is not lowered by more than 25 % despite the high lysine modification of, e.g., the caseins CML-3 and LAL-3. Moreover, Δ_{Trp} of all CML-casein samples is not significantly lowered compared with the native casein, indicating that the interplay of peptidases can guarantee sufficient cleavage of proteins and sufficient release of individual amino acids. The parameters $\Delta_{1,000}$ and Δ_{Trp} tend to decrease only when crosslinking and racemisation reactions occur concomitantly. All modifications slow down the digestion process as can be interpreted from the parameter Π_{60}. This delay, however, is significant only for the strongly carboxymethylated casein CML-3. Peptidases might still be able to recognize the modified lysine residues, but the rate of the cleavage reaction decreases as was shown for alkali-treated protein (Krause and Freimuth 1985). During food processing, proteins can be modified with FL, CML, and LAL in lysine modification ranges of the lowest degree studied here. The impact of food processing on the digestibility of food proteins should, therefore, be relatively small, as long as crosslinking reactions remain at a low level. However, the impact of severe heating effects of food proteins, e.g., from coffee, bread crust, cookies, or beer, needs to be further explored.

Influence of casein modification on the release of amino acids

The digestibility values (Table 1) do not allow predictions concerning the distribution of FL, CML, and LAL in the seven molecular mass fractions because these products cannot be measured by UV-absorption. The eluate of 2 mg of digested casein was, therefore, collected and fractionated into the respective fractions (Fig. 3). The amino acid composition of the fractions was analyzed after acid hydrolysis. The results of the distribution of four proteinogenic amino acids into six molecular weight fractions are shown in Fig. 4. The fraction WRF has been omitted since it contained only tryptophan, which could not be quantified due to its acid lability. While the digestibility $\Delta_{1,000}$ of native casein is at 78.3 % as measured by SEC, the release of individual amino acids into peptides smaller than 1,000 Da strongly differs (Fig. 4), amounting to 52.0 ± 0.5 % of total Asx, 76.4 ± 0.8 % of total Leu, 95.2 ± 0.8 % of total Tyr, and 84.3 ± 1.2 % of total Lys. This semi-preparative SEC system, therefore, could corroborate the reported releasing behavior of amino acids from casein (Savoie et al. 1988; Vorob'ev et al. 1996; Agudelo et al. 2004). Acidic amino acids tend to remain in longer peptides, while basic and aromatic amino acids are released into shorter peptides or free amino acids. This can be explained by the substrate specificity of the five intestinal peptidases. Three of them are endopeptidases cleaving peptide chains after cationic amino acid residues (trypsin), aromatic residues (chymotrypsin), and aliphatic residues (elastase), respectively. In the so cleaved peptides, cationic, aromatic, and aliphatic residues prevail at the carboxy termini. Concomitantly, the carboxypeptidases A and B, respectively, acting as exopeptidases, cleave aromatic and cationic amino acids, respectively, from the carboxy termini of peptide chains. There is no luminal peptidase recognizing anionic side chains.

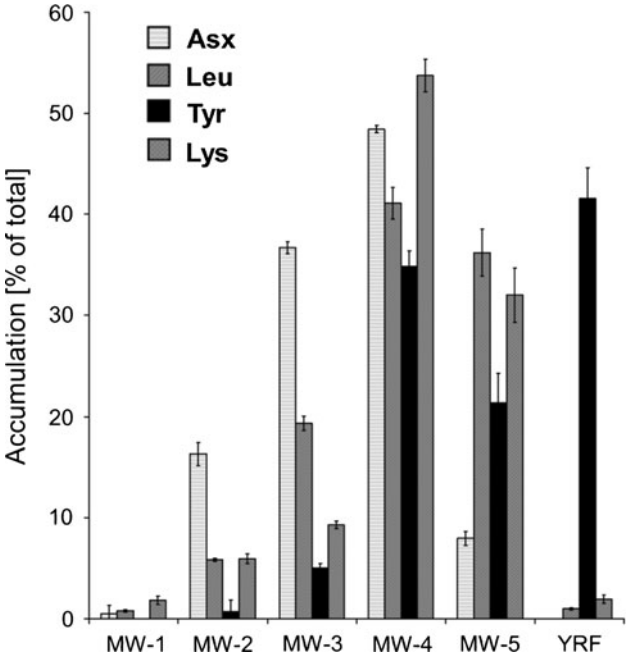

Fig. 4 Relative amounts of selected native amino acids in different molecular mass fractions (cf. Fig. 3) after 120 min of gastric and 360 min of intestinal digestion of native casein. Fractionation was performed by semi-preparative size-exclusion chromatography using PBS as the eluent. The fraction WRF is not shown because it was found to contain only tryptophan

We then fractionated digested samples of modified proteins. The distribution of FL, CML, and LAL into six molecular-weight fractions is presented in Fig. 5. The absorption availability of the amino acids expressed as the release of amino acids into peptides smaller than 1,000 Da

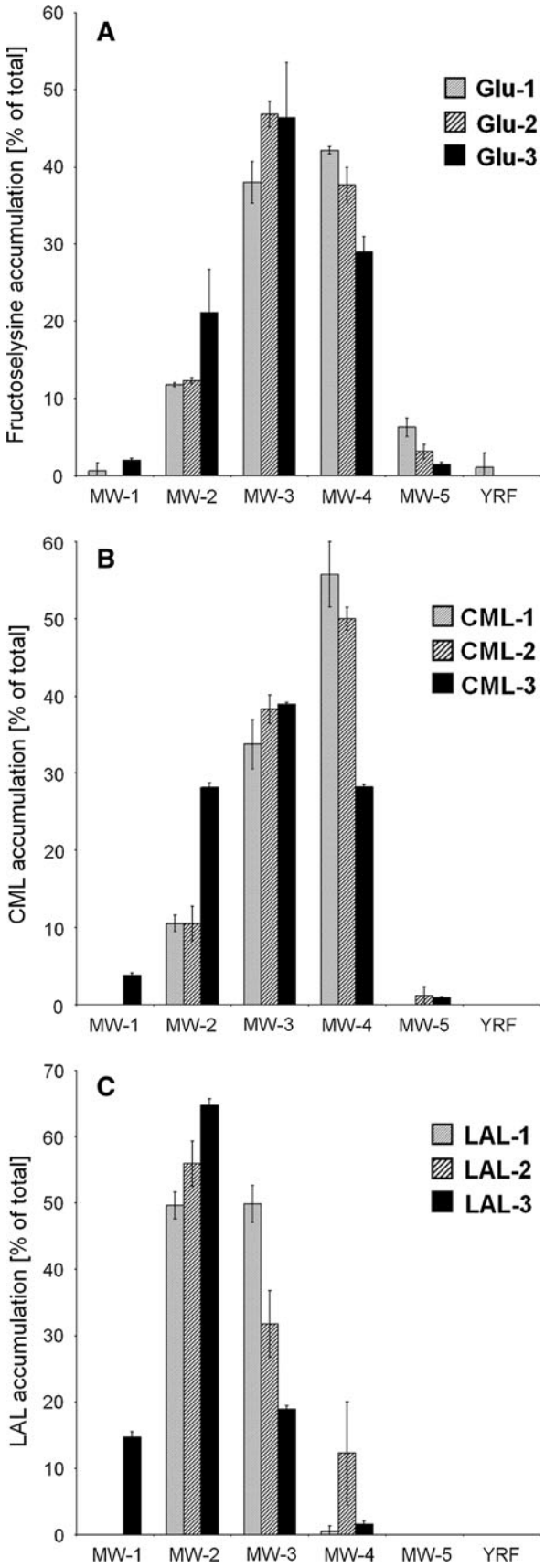

Fig. 5 Relative amounts in different molecular mass fractions (cf.► Fig. 3) of **A** fructoselysine from glucose-modified casein, **B** CML from carboxymethylated casein, and **C** LAL from alkali-treated casein after 120 min of gastric and 360 min of intestinal digestion. Fractionation was performed by semi-preparative size-exclusion chromatography using PBS as the eluent. The fraction WRF is not shown because it was found to contain only tryptophan

is compiled in Table 2. The length of the peptides into which the modified amino acids are released increases with increasing lysine blockage. This has already been described for FL (Finot and Magnenat 1981), which is mostly distributed into peptides between 1 and 4.5 kDa in this study. Like most of the proteinogenic amino acids, CML accumulates predominantly in peptides between 250 and 1,000 Da. LAL, however, is practically absent from peptides smaller than 1,000 Da. Savoie et al. (1991) also found a release of only 3–6 % LAL into peptides smaller than 1,000 Da during simulated digestion. At the end of digestion, LAL strongly accumulates in peptides between 4.5 and 20 kDa with a length of at least 30–40 amino acids. This non-appearance of peptides available for absorption must entail the poor availability of LAL to humans and its transport into the large intestine (Lee and Erbersdobler 1994). On the contrary, between 30 and 60 % of FL, and CML, respectively, are released into peptides smaller than 1,000 Da. The occurrence of a small amount of FL, but not of CML, in the fraction MW-5 could possibly point to a release of FL in the free form which can result from the substrate specificity of the intestinal peptidases discussed above. Unfortunately, we failed to analyze the free adducts by amino acid analysis due to strong matrix effects from the synthetic gastric and intestinal juices. However, free amino acids should accumulate in the fractions MW-5, YRF, and WRF, where, interestingly, 10–40 % of the native amino acids occur, while the fraction nearly completely lacks modified amino acids. This could mean that modified amino acids are predominantly present in small peptides like those utilized in our previous study (Hellwig et al. 2011). However, FL dipeptides are not substrates of the intestinal peptide transporter PEPT1 and can, therefore, not be transported across the epithelium (Hellwig et al. 2011). Instead, FL should get into the large intestine like LAL. The important difference between LAL and FL, however, is that the microbiota gets the Amadori product in the form of small peptides, while LAL rather remains bound in longer peptides. It would thus be interesting to know if LAL is available to the microbiota at all, especially in light of the high recovery of intact LAL in feces samples in balance studies (Lee and Erbersdobler 1994; Somoza et al. 2006). The difference in the release from food proteins might determine the differences in the recovery of modified amino acids in feces.

Table 2 Estimation of the absorption availability of native and modified amino acids from proteins after simulated gastrointestinal digestion using semi-preparative size-exclusion chromatography

Casein sample	Released amino acid	Absorption availability (% of total)
Native	Asx	52.0 ± 0.4
Native	Leu	76.4 ± 0.8
Native	Tyr	95.2 ± 0.8
Native	Lys	84.3 ± 1.2
Glu-1	FL	49.5 ± 3.2
Glu-2	FL	40.8 ± 1.8
Glu-3	FL	30.4 ± 1.9
CML-1	CML	55.8 ± 4.2
CML-2	CML	51.2 ± 1.2
CML-3	CML	29.1 ± 0.5
LAL-1	LAL	0.5 ± 0.9
LAL-2	LAL	12.3 ± 7.8
LAL-3	LAL	1.6 ± 0.5

The absorption availability is calculated as the summarized percentage of the respective amino acid in the fractions MW-4, MW-5, YRF, and WRF (cf. Fig. 3). Data are mean ± SD, $n = 3$

The example of CML shows that the release of individual amino acids from proteins and the role that this plays for absorption must be discussed with respect to the molecular structure. Using the SEC fractionation system, the release also of native amino acids differs strongly and never reaches 100 %, nor are the native amino acids completely bound in peptides smaller than 1,000 Da after luminal digestion. It is well established, however, that all native amino acids can be taken up into the body after digestion. If luminal digestion does not directly produce absorbable units, it must be the brush-border peptidases which further degrade longer peptides into absorbable amino acids, di- and tripeptides. Thus, if an acidic amino acid such as CML is released as well as Asx in the semi-preparative fractionation systems, the absorption availability of CML should be as good as that of Asx. The poor release measured for LAL corroborates findings known from the literature (Savoie et al. 1991). It is also consistent with our recent findings for glutamyllysine (Hellwig et al. 2009). Cross-linking amino acids are generally poorly liberated from proteins during luminal digestion.

In summary, we were able to show that the chemical modification of amino acids tends to reduce the digestibility of casein, especially when racemization and cross-linking side reactions occur. LAL is poorly released from modified casein and should not be available for intestinal absorption. FL and CML, however, are available for absorption, because they appear in small peptides similar to proteinogenic amino acids having similar chemical characteristics. The missing transportability of FL either free or bound in dipeptides (Hellwig et al. 2011) illustrates that luminal digestion should have a strong impact on the form in which the intestinal microbiota gets in contact with modified amino acids.

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