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ORIGINAL ARTICLE

Faox enzymes inhibited Maillard reaction development during storage both in protein glucose model system and low lactose UHT milk

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Abstract Fructosamines, also known as Amadori products, are formed by the condensation of glucose with the amino group of amino acids or proteins. These compounds are precursors of advanced glycation end products (AGEs) that can be formed either endogenously during aging and diabetes, and exogenously in heat-processed food. The negative effects of dietary AGEs on human health as well as their negative impact on the quality of dairy products have been widely described, therefore specific tools able to prevent the formation of glycation products are needed. Two fructosamine oxidase enzymes isolated from *Aspergillus* sp. namely, Faox I and Faox II catalyze the oxidative deglycation of Amadori products representing a potential tool for inhibiting the Maillard reaction in dairy products. In this paper, the ability of recombinant Faox I and II in limiting the formation of carboxy-methyl lysine (CML) and protein-bound hydroxymethyl furfurol (b-HMF) in a commercial UHT low lactose milk and a beta-lactoglobulin (β-LG) glucose model system was investigated. Results show a consistent reduction of CML and b-HMF under all conditions. Faox effects were particularly evident on b-HMF formation in low lactose commercial milk. Peptide analysis of the β-LG glucose system identified some peptides, derived from cyanogen bromide hydrolysis, as suitable candidates to monitor Faox action in milk-based products. All in all data suggested that non-enzymatic reactions in dairy products might be strongly reduced by implementing Faox enzymes.

Keywords Faox · Maillard reaction · CML · b-HMF · AGEs · Milk

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Introduction

Initial steps of Maillard reaction (MR) involved reversible reactions between carbohydrates or lipids carbonyl moiety and amines to form a Schiff base which undergoes rearrangements producing the Amadori product (Yaylaylan et al. 1994). Stable covalent adducts or cross-links called advanced glycation end products (AGEs) are formed by dehydration and fragmentation both in human body and in foods (dietary AGEs). It has been hypothesized that dietary AGEs represent a consistent risk factor (Vlassara et al. 2002; Cai et al. 2002) due to their ability to induce oxidative stress and inflammation (Uribarri et al. 2010) by binding to cell surface receptors (Vlassara 2001; Schmidt et al. 1999), thus posing severe risk in particular for some categories (Šebeková et al. 2008). The formation of dietary AGEs in dairy products largely occurring under severe heat treatments, should be always prevented for nutritional and sensorial reasons (Pischetsreider and Henle 2012; Schwambach and Peterson 2006).

Several tools have been developed to limit glycation and thus the formation of dietary AGEs either by chemical approaches, using carbonyl traps, such as rutin (Pashikanti et al. 2010), natural antioxidants (Peng et al. 2011; Wu and Lin 2011), sugar autoxidation inhibition (Cervantes-Laurean et al. 2006), reactant encapsulation technology (Fiore et al. 2012) and

bivalent cation addition (Gökmen and Şenyuva 2007), or by enzymatic approaches employing enzymes, such as fructosamine oxidase (Capuano et al. 2007), fructosamine-3-kinase, and FN3K-related proteins (Van Schaftingen et al. 2012).

Two fructosamine oxidase enzymes, hereafter referred to as Faox I and Faox II, which have been isolated by Monnier and co-workers from fungi, capable of using the Amadori products as substrates (Takahashi et al. 1997a, b), catalyzed the oxidative deglycation of low molecular weight fructosamine or Amadori product to yield glucosone and aminoacids. After the catalytic cycle, reduced FAD is oxidized by molecular oxygen with the concomitant release of one molecule of hydrogen peroxide (Wu et al. 2000). Faox I and II have been reported to react differentially with AP on free amino acids and AP formed on small peptides. In contrast, glycated lysyl residues bound to globular proteins, such as bovine serum albumin (BSA) are poor substrates for the enzyme (Monnier and Wu 2003). The reason for the differential activity towards variably sized glycated substrates was explained by the resolution of Faox X-ray structure by Monnier and co-workers. Faox II is a two domain FAD-enzyme with an overall topology similar to that of monomeric sarcosine oxidase, where the catalytic site is buried in a 12 Å deep pocket which is not easily accessible to glycated globular proteins (Collard et al. 2008).

It has been shown that when Faox I was added during the glycation reaction of small globular proteins, such as insulin or beta-lactoglobulin (β-LG), the development of MR was significantly reduced (Capuano et al. 2007; Mennella et al. 2005). Although any clear mechanism was not identified to explain this effect, and it has been hypothesized that it was due to a temporary conformational changes occurring during glycation making the Amadori product accessible to the enzyme catalytic site.

In this paper, we aimed at clarifying this point, investigating the capacity of Faox I and Faox II to reduce AGEs formation when added during the glycation of milk and globular proteins. The effects of Faox I and II were evaluated using both a UHT low lactose commercial milk (LLM) and a β-LG-glucose model system. Glycated peptides as well as non-enzymatic glycation markers such as N^{ε}-(Carboxymethyl)lysine (CML) and bound 5-hydroxymethylfurfural (b-HMF) were quantified while glycated β-LG peptides were characterized by MALDI-TOF analysis. The results showed a reduction in glycated peptides CML and b-HMF in all systems investigated.

Materials and methods

Expression and purification of Faox I and II

Both Faox I and II cDNAs were received as a kind gift from Prof Monnier. They were both PCR amplified and cloned in the *Age*I–*Hin*dIII site and *Age*I–*Xho*I site, respectively of pET28-SUMO3GFP (a kind gift from EMBL, Heidelberg) using site-specific primers: Faox I: 5′-T CATC TACCGGTGGCATGGCGCCTTCAATTTTGAG C-3′ and 5′-GTCTCGAAGCTTTTATCACGG ACCTCTG CTCTCTCCAATC-3′; FaoxII: 5′-TCATCTACCGGTGGC ATGGCGGTAACCAAG TCATCTTC-3′ and 5′-CGCGC GCTCGAGTTATCATAACTTGGAAATATCTCTATAT TGTAC ATTTGTCC.

Both recombinant Faox I and II constructs were expressed in BL21(DE3) cells (Novagen) 16 h at 20 °C in the presence of 0.1 mM IPTG, yielding soluble protein.

Faox I was lysed in: 20 mM Tris (pH 8.0), 10 mM imidazole, 250 mM NaCl, 0.1 % Triton X-100, in the presence of PMSF, DNase I and lysozyme, clarified and purified by FPLC, using an ÄKTA system with a 1 mL His Trap FF column (GE Healthcare) by stepwise elution. The protein eluted >90 % pure in 110 mM Imidazole, 20 mM Tris (pH 8.0), 250 mM NaCl and the fusion protein was removed with SenP2 protease (prepared in-house from pETM11 SenP2 a kind gift from EMBL, Heidelberg) at a dilution 1:800, 16 h at 20 °C. The cleaved protein was concentrated on Amicon 10 kDa MWCO to 10 mg mL^{-1} and polished on a size exclusion column, Superdex 75 10/30.

Faox II lysate was prepared as Faox I, but at pH 8.5 and in the initial purification step the recombinant protein eluted at 250 mM imidazole. The fusion protein was cleaved as before, concentrated, with a change of buffer (20 mM Tris, 50 mM NaCl, pH 8.5) on Amicon 10 kDa MWCO and isolated from the SUMO/6×His Tag on a 1 mL MonoQ column, eluting cleanly using a linear salt gradient.

Enzymatic activity

The activity of recombinant Faox I and Faox II was evaluated following the production of H_2O_2 in a peroxidase—coupling reaction at 25 °C by monitoring the formation of a quinone dye at 505 nm (ε 7,210 mol^{-1} L cm^{-1}) (Nicell and Wright 1997). The reaction mixture contained 10 mM KPO_4 pH 7.0, 0.38 mM aminoantipyrine, 0.6 U horseradish peroxidase, 0.5 mM phenol, in a final buffer volume of 1 mL (Nicell and Wright 1997). Synthetic Fruct-Lys (ε-Fructosyl-lysine) was used as substrate. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of quinine dye per minute.

Circular dichroism

All CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier temperature control system [Model PTC-423-S]. Molar ellipticity per mean residue, $[\theta]$ in deg $cm^2 \times dmol^{-1}$, was calculated from the

equation: $[\theta] = [\theta]_{obs} \times mrw/10 \times l \times C$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, C is the protein concentration in mg $\times$ mL^{-1}, and l is the optical path length of the cell in cm. Far-UV measurements (183–250 nm) were carried out at 20 °C, at time constant of 4 s, 2 nm band width, scan rate of 10 nm min^{-1}, using a 0.1 cm optical path length cell and a protein concentration of 0.2 mg $\times$ mL^{-1} in 6.6 mM buffer phosphate pH 8.0. CD spectra were signal averaged over at least three scans, and baseline was corrected by subtracting a buffer spectrum. CD of Faox II at different pHs were performed after dialysis in different buffers, such as 10 mM Tris, 10 mM NaCl, pH 9.0; 15 mM KPO_4, 10 mM NaCl, pH 6.0; 15 mM NaAc, 10 mM NaCl, pH 5.2; 15 mM NaAc, 10 mM NaCl, pH 4.0. Before registering the CD spectra, dialyzed samples were diluted in water. Thermal unfolding curves were determined by recording the molar ellipticity at 222 nm, using a scanning rate of 1 °C min^{-1} ranging from 20 to 90 °C.

Multiple-angle light-scattering (MALS) analysis

The monomeric form of recombinant Faox I was determined by combining size exclusion chromatography (SEC) with a MALS instrument. Experiments were run at 0.5 mL min^{-1} in 20 mM Tris, 150 mM NaCl, pH 7.0 buffer loading 300 μg enzyme on a Superdex 75 10/30 size exclusion column (GE Healthcare) connected to an FPLC ÄKTA purifier system which in turn was connected to a refractive index (Shodex RI 101) followed by a Mini Dawn Treos (Wyatt Technology, USA) light scattering instrument. The online measurement of the intensity of the Rayleigh scattering as a function of the angle as well as the differential refractive index of the eluting peak in SEC was used to determine the weight-average molecular mass (Mw) eluted proteins, using the Astra 5.3.4.14 software (Wyatt Technologies) (Ascione et al. 2012).

Experimental systems

The experiments were performed both on a commercial low lactose UHT milk (LLM) and on a β-LG glucose model system. All samples were prepared under a sterile hood to avoid bacterial contamination, being filtered before use through 0.45 μm (Faox I and Faox II) or 0.22 μm cutoff (β-LG and glucose) membranes. In brief, 10 mg mL^{-1} β-lactoglobulin (Sigma) was added to glucose (0.5 M) in 200 mM phosphate buffer at pH 7.2 with a final volume of 1 mL. UHT low lactose commercial milk was not filtered before usage to leave its physicochemical properties unaltered, and enzymes were added at a protein ratio of 1:1,000. After Faox enzymes addition, samples were stored at 37 °C for 17 days. When the enzymes were not mixed to samples, the equivalent volume of buffer was added. Following incubation, samples were frozen at −20 °C before analysis.

Evaluation of Maillard reaction development in the model system

The MR development was assessed by measuring b-HMF and CML. Glycation of specific peptides of the protein–glucose system was evaluated by MALDI TOF MS analysis.

Quantification of b-HMF

The extraction procedure was performed according to Morales and Jiménez-Pérez (1998) with slight modifications: 500 μL of low lactose commercial UHT milk or β-LG glucose model system were transferred to a 3 kDa regenerated cellulose centrifugal filter unit (Amicon Ultra, Millipore Ireland) and centrifuged three times at 4,200 rpm for 30 min. 200 μL distilled water was added following each cycle. The final volume of the protein concentrate was adjusted to 1 mL.

A 500 μL were mixed with an equal volume of 0.3 N oxalic acid in an eppendorf tube covered with parafilm to prevent evaporation. The tube was heated in a water-bath system at 100 °C for 60 min. After cooling to room temperature 1 mL of 40 % (w/v) trichloroacetic acid (TCA) solution was added. The mixture was stirred for 5 min and centrifuged 15 min at 14,800 rpm. The supernatant was passed through a 0.45 μm regenerated cellulose filter and injected on to an UPLC system that consisted of two LC-20AD class VP pumps and a SPD-20A UV/VIS detector equipped with an SIL-20A autosampler, all from Shimadzu (Kyoto, Japan). The mobile phase was a mixture of acetonitrile in water (5 % v/v) at a flow rate of 1 mL min^{-1} under isocratic conditions and a Synergi 4 μm Hydro-RP 80 Å, 250 $\times$ 4.6 mm column (Phenomenex, Torrance, CA) was used for the chromatographic separation. The UV/VIS detector was set at 280 nm and HMF was quantified using the external standard method. A calibration curve was built within the range 0.1–10 μg mL^{-1}, and the coefficient of determination r^2 was 1 after three replicates. The limit of detection (LOD) was 0.050 μg mL^{-1}, whereas the limit of quantification (LOQ) was 0.150 μg mL^{-1}. All of the analyses were performed in quadruplicate by injecting 20 μL of milk or model system extracts in the system and the results expressed as micrograms per mL sample. To calculate the effect exerted by the Faox enzymes (percentage of reduction), the amount of b-HMF present in the sample at time zero was subtracted from those find at the end of the incubation time.

Quantification of N^{ε}-(carboxymethyl)lysine (CML)

The analysis of CML was performed according to Delatour et al. (2009) and Fenaille et al. (2006) with slight modifications. Briefly, 60 μL of LLM (corresponding to 1.92 mg of protein) or 192 μL of β-LG glucose model system (corresponding to 1.92 mg of b-LG) were diluted in 450 μL of 0.2 M sodium borate (pH 9.2).

An aliquot of 500 μL of sodium borohydride 1.0 M in 0.1 M NaOH was added in the mixture, and the solution was incubated overnight at room temperature to achieve the complete reduction in Amadori compound or Schiff's base. Subsequently 1 mL of TCA was added to the mixture and the samples were centrifuged for 10 min at 4,000 rpm (4 °C). After careful removal of the supernatant, the protein pellet was diluted in 2 mL of 6 N HCl. The mixture was incubated for 24 h at 110 °C in an air forced circulating oven, and 1,000 μL was evaporated under a gentle flow of nitrogen. The samples were reconstituted in 990 μL of water and 10 μL of the internal standard *d2*-CML were added to obtain a final concentration of 238 ng mL^{-1}. Samples were loaded on to equilibrated Oasis HLB 1 cc cartridges (Waters, Wexford, Ireland) and eluted according to the method described by Delatour et al. (2009), finally 20 μL were injected onto the LC/MS/MS system. Identification and quantification of CML and *d2*-CML were performed on API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) coupled to a Turboionspray (TIS) interface, equipped with an HPLC binary micropump series 200 (Perkin-Elmer, USA).

CML and *d2*-CML separation was achieved on a reversed-phase HPLC column (TSKgel-amide 80, 2.0 mm × 25 cm, Tosoh Bioscience) using the following mobile phases: A, 0.1 % formic acid and B, acetonitrile. Compounds were eluted at 200 μL min^{-1} through the following gradient of solvent B (*t* in [min]/[%B]): (0/10), (4/10), (8/90), (10/10), (12/10). With the above-described chromatographic conditions, typical retention times of CML and *d2*-CML were 3.5 min. Positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage: 5.0 kV; capillary temperature: 350 °C, dwell time 100 ms. The chromatographic profile was recorded in multiple reaction monitoring mode and the characteristic transitions were monitored to improve selectivity. CML was quantified using a linear calibration curve built with specific solutions of CML and *d2*-CML dissolved in water (50–1,000 ng mL^{-1}). The LOD and LOQ were, respectively, 10 and 30 ng mL^{-1} for CML, and the coefficient of determination r^2 was 0.9998. The internal standard was used for the recovery test, varying 70–85 %. All of the analyses were performed in quadruplicate, and the results expressed as nanograms per milligram sample.

To calculate the effect exerted by the Faox enzymes (percentage of reduction) the amount of CML present in the sample at time zero was subtracted from those find at the end of the incubation time.

Evaluation of protein glycation on the β-LG-glucose model system

Reduction and carboxymethylation procedure

Purified β-LG (5 mg) was dissolved in 300 μL of 0.3 M Tris–HCl, pH 8.0, containing 6 M—guanidine–HCl, 1 m M-EDTA, and treated with dithiothreitol (10:1 molar excess with respect to cysteinyl residues) at 37 °C for 2 h. Carboxymethylation was carried out with a fivefold molar excess of iodoacetic acid with respect to dithiothreitol, at pH 8.0, at room temperature for 30 min in the dark. The sample was desalted by gel filtration through a PD-10 G-25 column (Bio-Rad) in 50 m M—ammonium bicarbonate, pH 8.5, and freeze-dried.

Protein hydrolysis

Cyanogen bromide (CNBr) hydrolysis of β-LG was performed in 70 % (w/w) trifluoroacetic acid at room temperature overnight using a ratio of 40 mol of CNBr per mole of methionine. The volatile side products of the reaction, methyl-thiocyanate, excess cyanogen bromide, and trifluoroacetic acid were removed by freeze drying.

MALDI-TOF analysis

MALDI-TOF MS analyses were performed on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with an N_2 laser ($\lambda = 337$ nm). DHB was used as matrix for analyzing peptides resulting from protein hydrolysis,. The matrix was prepared by dissolving 10 mg of DHB in 1 mL of aqueous 50 % (v/v) acetonitrile containing 1 % (v/v) PA. The instrument operated with an accelerating voltage of 20 kV, a grid voltage of 95 % of the accelerating voltage, a guidewire of 0.05 % and a delayed-ion extraction time of 175 ns, for peptides.

External mass calibration was performed with the signal of the matrix dimer at $[M + H^+] = 379.05$ and with the monoisotopic masses of peptide standards, including angiotensin I ($[M + H^+] = 1{,}296.68$) and bovine insulin ($[M + H^+] = 5{,}730.61$), thereby achieving an accuracy in the measurement of the peptide mass better than 80 ppm. The mass spectra were acquired in positive linear ion mode. Raw data were elaborated using the software program Data Explorer version 4.0 (Applied Biosystems).

Signals of mass spectra were identified according to the expected molecular mass from the known milk protein sequences, taking into consideration CNBr specificity according to bioinformatics tools, such as MASCOT 2.3 software (Matrix Science, London, UK) or other online resources, such as ExPASy FindPept or Protein Prospector.

Statistical analysis

Statistical analyses were performed at least in triplicate and analysis of variance using Duncan's new multiple range test was performed, using XLStat Pro v.11.3 (Addinsoft, Germany).

Results and discussion

Biochemical characterization of Faox I and Faox II

pET28SUMO-Faox I and pET28SUMO-Faox II were obtained by cloning PCR-amplified cDNAs in the *Age*I–*Hin*dIII and *Age*–*Xho*I sites, respectively, of pETM28SUMO. The resulting clones were verified by bidirectional sequencing. Bacterial expression and purification allowed us to obtain high yields of proteins with >98 % purity, as assessed by LC–ESI–MS and SDS-PAGE. Enzyme-specific activity using Fruc–Lys as substrate was in agreement, as previously described (Collard et al. 2008). A study of the enzymes in solution by SEC-MALS analysis showed that Faox I and Faox II are monomeric proteins with a MW value of 46,030 ± 46 and 45,570 ± 45 kDa, respectively, with a Rh of 3.20 ± 0.01 nm. The recombinant enzymes were also characterized in solution by far-UV circular dichroism (CD) spectroscopy. Spectra suggest that native Faox I and Faox II display a high degree of secondary structure with 31 % alfa helix and 23 % beta strand for both enzymes according to the variable selection method (CDSSTR), using DICHROWEB. These data obtained in solution are in agreement with Faox I, whose three-dimensional structure was characterized by X-ray crystallography by Monnier and co-workers (Collard et al. 2008). To investigate the pH stability of Faox II, CD spectra at four different pH: 9.0, 6.0, 5.2, and 4.0 were acquired (Fig. 1a). Data show that changes in pH do not affect CD spectra of Faox II that preserves almost all of its secondary structure as well as its specific activity towards Fruct–Lys substrate as shown in Fig. 1b). In particular, 82, 86, 55 % of relative-specific activity was preserved at pH 6.0, 5.2 and 4.0, respectively. The melting points at the different pHs ranged from 52.0 to 58.8 °C after which the enzyme aggregated (data not shown). These events were irreversible under the conditions used, likely due to cross-linking between the six Cys present within the amino acid sequence (Tornatore et al. 2008).

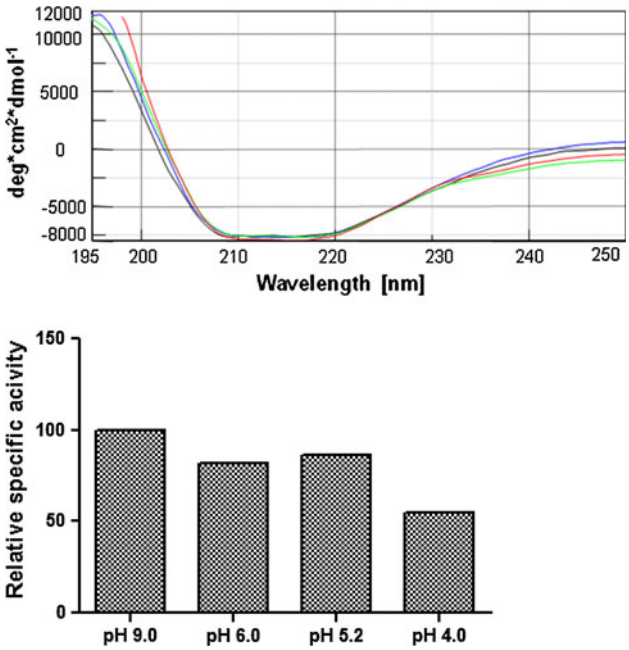


Fig. 1 *Upper panel* overlay of CD spectra of Faox II at four different pHs: pH 4.0 (*black line*), pH 5.2 (*blue line*), pH 6.0 (*green line*), pH 9.0 (*red line*); **b** *bottom panel*, relative specific activity of Faox II at four different pHs: 4.0, 5.2, 6.0, 9.0 (color figure online)

Faox enzymes reduce the formation of CML in low lactose milk (LLM) and protein glucose model system

The two experimental MR-sensitive systems used in this work were incubated in the presence of Faox enzymes at 37 °C for 17 days. The aim of this prolonged incubation time was to investigate the enzymes ability to limit glycation during product shelf life. At the end of the storage time CML, which is a common and well characterized marker of non-enzymatic glycation was measured (Van Boekel 1998). As shown in Fig. 2 in the β-LG-glucose system, the addition of Faox I or Faox II determined a decrease in CML formation of 24 and 38 %, respectively.

A similar set of experiments were also carried out on UHT low lactose commercial milk. In this milk, very reactive carbohydrates, such as glucose and galactose are formed by lactose hydrolysis, thus determining a fast browning and off flavor development (Yaylaylan et al. 1994; Van Boekel 2006) and possibly a decrease of milk nutritional properties (Henle 2007). The CML concentration found in a commercial low lactose milk was 14 ng CML mg^{-1} of protein and this value almost doubled after storage at 37 °C for 17 days. As shown in Fig. 3, when Faox I and Faox II were added a reduction of CML concentration of 65 and 58 %, respectively, was detected. It is worth noting that in the conditions applied, the effect of Faox I and Faox II on the CML formation was more evident in low lactose commercial milk than in the protein

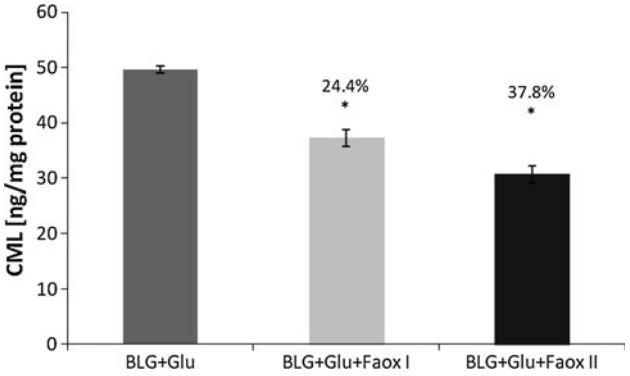


Fig. 2 CML content in a milk-like model system after storage at 37 °C for 17 days. Mean change in CML reduction was significant in relationship to the control after incubation using Duncan's test ($*P < 0.05$); $n = 4$

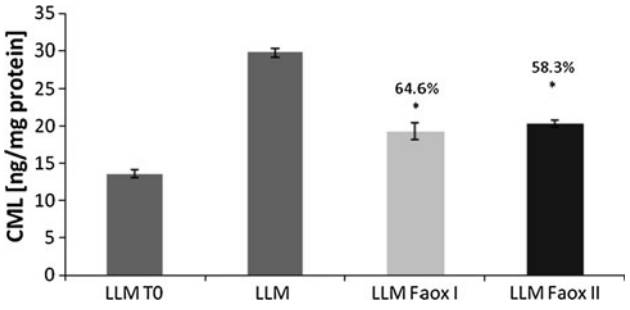


Fig. 3 CML content in a UHT low lactose commercial milk at time zero (LLM T0) after storage at 37 °C for 17 days (LLM). Mean change in CML reduction was significant in relationship to the control after incubation using Duncan's test ($*P < 0.05$); $n = 4$

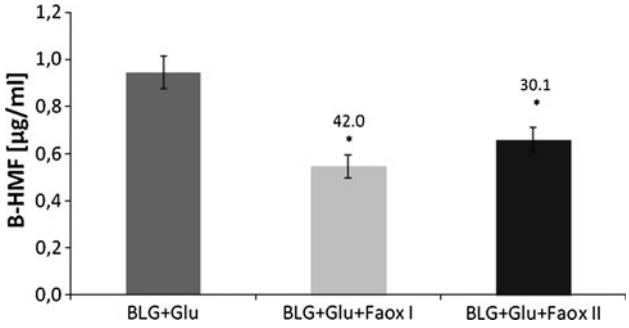


Fig. 4 Bound-HMF content in a milk-like model system after storage at 37 °C for 17 days. Mean change in b-HMF reduction was significant in relationship to the control after incubation using Duncan's test ($*P < 0.05$); $n = 4$

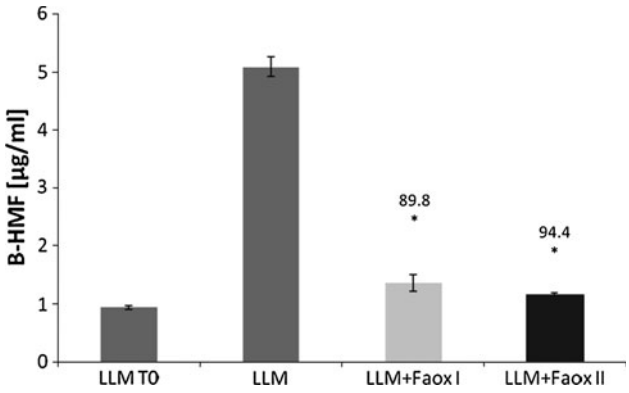


Fig. 5 Bound-HMF content in a UHT low lactose commercial milk at time zero (LLM T0) and after storage at 37 °C for 17 days (LLM). Faox I or II were added immediately before storage in a 1:1,000 ratio. Mean change in bound HMF reduction was significant in relationship to the control after incubation using Duncan's test ($*P < 0.05$); $n = 4$

glucose model system. One possible explanation for this finding might be that part of the milk protein fraction is formed by small peptides and free amino acids that could be suitable substrates for Faox I and Faox II action.

Faox enzymes reduce the formation of bound HMF (b-HMF) in low lactose commercial milk and protein glucose model system

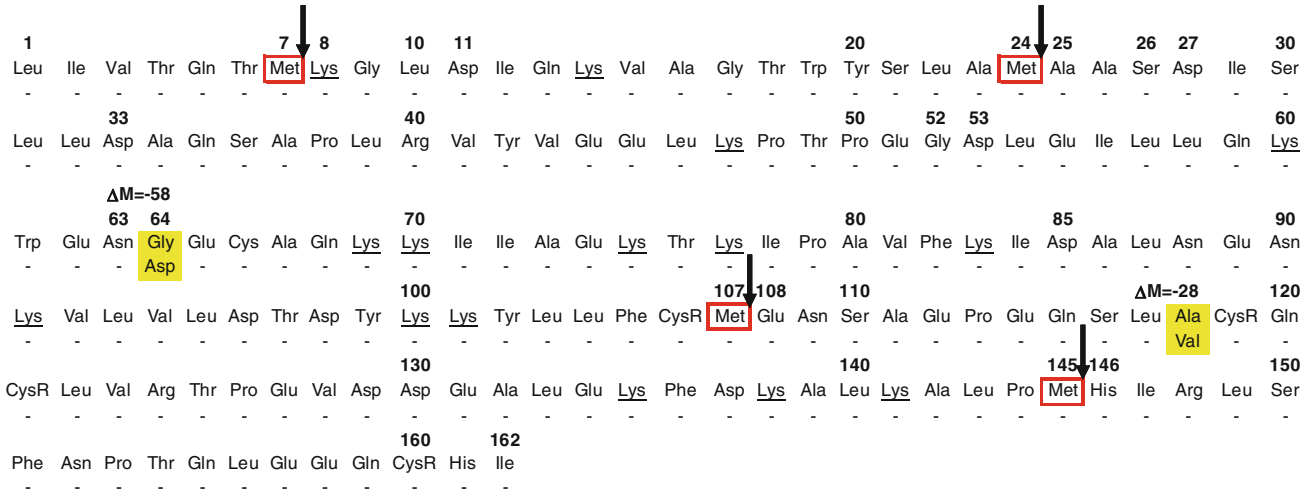
The effect of Faox I and II was also assessed by measuring the levels of b-HMF which is formed during the early stages of the Maillard reaction. b-HMF can be considered as a reliable index of the extent of the MR providing an accurate estimation of glycated protein lysyl residues (Morales et al. 2000; Chávez-Servín et al. 2005). As shown in Fig. 4, the addition of Faox enzymes to protein glucose system just before thermal incubation lowered its level by 42 and 30 %, respectively.

Data of Fig. 5 shown that, as already observed for CML, the effect of Faox enzymes was much more evident in low lactose commercial milk samples than in the β-LG glucose system. Commercial milk at time zero of the experiment had a b-HMF concentration of 0.94 µg mL^{-1}. By the end of incubation, this concentration had increased to 5.88 µg mL^{-1}. The samples incubated in the presence of Faox I and II showed a decrease in the b-HMF concentration of 90 and 94 %, respectively.

When comparing the effect of Faox on the two different markers of the MR development it could be hypothesized that the more pronounced effect found on b-HMF as compared to that observed on CML might be due to the different formation pathways of these two compounds. In fact, CML could be formed reacting with lysine from both carbohydrates and lipids during autoxidation reactions (Requena et al. 1997), whereas b-HMF can only be derived from the Maillard reaction path (Morales and Jiménez-Pérez 1998).

The peptides of β-LG represent a marker to monitor the Faox activity

To verify the specificity of the enzyme action the samples of the β-LG-glucose model system at the end of the



Fragment	Expected molecular mass (Da)		Aminoacid sequence	Measured molecular mass (Da)		
		Red, Carb, Homoserine		Control	Heat treated	FAOX
(1-7)	756		LIVTQT**M**	757	757	757
(8-24)	1833		**K**GLDIQ**K**VAG TWYSLA**M**	1849	nd	1850
(25-107)Var B	9332	9407(G_{64})	AAS-DISLL-DAQSAPLRVYVEE L**K**PTPEG-DLEILLQ**K**WENGECAQ**KK**IIAE**K**T**K**IPAVF**K**I-DALNEN**K**VLVL-DT-DY**KK**YLL FCM	nd	nd	nd
(25-107)Var A	9388,9	9464(D_{64})				
(108-145)B (A_{118})	4169,0	4286		4286	4287	4288
(108-145)B (A_{118})1Gluc			ENSAEPE QSL **AC**Q**C**LVRTPE VDDEALE**K**FD **K**AL**K**ALP**M**		4449	4450
(108-145)B (A_{118})2Gluc					4612	4612,1
(108-145)A (V_{118})	4198	4315		4315	4315	4317
(108-145)B (A_{118})1Gluc			ENSAEPE QSL **VC**Q**C**LVRTPE VDDEALE**K**FD **K**AL**K**ALP**M**		4477	4479
(108-145)B (A_{118})2Gluc					4637	4640
(146-162)	2064	2122	HIRLSFNPTQ LEEQ**C**HI	2124	2125	2125

Fig. 6 *Panel A* amino acid sequence of bovine β-LG. *Black arrows* indicate CNBr hydrolysis sites. In *yellow* the amino acid substitution in variant A and B, putative glycated lysine residues are underlined. *Panel B* expected and measured molecular mass after CNBr hydrolysis

incubation time were subjected to cyanogen bromide hydrolysis and MS investigations. As showed in Fig. 6, the hydrolysis of β-LG can lead to the formation of 5 peptides that were all found in the spectra (data not shown). Among them the peptides 1–7, 8–24 and the 108–145, which is present in two isoforms due to the two genetic variants β-LG-A and β-LG-B, were the most affected by Faox action. It is worth to be noticed that these target peptides, 8–24 and 108–145, contain lysine residues having high solvent accessibility, as reported previously (Fogliano et al. 1998).

In Fig. 7, the mass spectra of the region 730–2100 uma is showed highlighting the two smallest glucosylated peptides formed upon the cyanogen bromide hydrolysis namely the β-LG(f1–7) and β-LG (f8–24). Data of Table 1 showed that the amount of glucosylated form is reduced by 20 and 30 %. Similar results were also observed on the peptide β-LG (f108–145). This peptide is present in two isoforms related to the two genetic variants of β-LG (β-LG-A and β-LG-B), therefore the use of smaller and unique peptide to monitor the Faox action was preferred. Interestingly, the reduction in glucosylation reaction caused by Faox measured on the glucosylated peptides of this model system is in line with that measured considering CML and bound HMF.

Conclusions

In previous work by our group (Mennella et al. 2005; Capuano et al. 2007), the ability of Faox enzymes to inhibit MR development has already been shown. Data demonstrated that when the enzyme is added together with glucose (i.e. during the glycation reaction) a reduction of protein glycation is observed by LC–ESI–MS. This effect, although no clear mechanisms have been demonstrated, could be due to a temporary local unfolding of the protein which allows the glycated site to fit into the catalytic cavity of the enzyme. As expected in fact, when enzyme is added

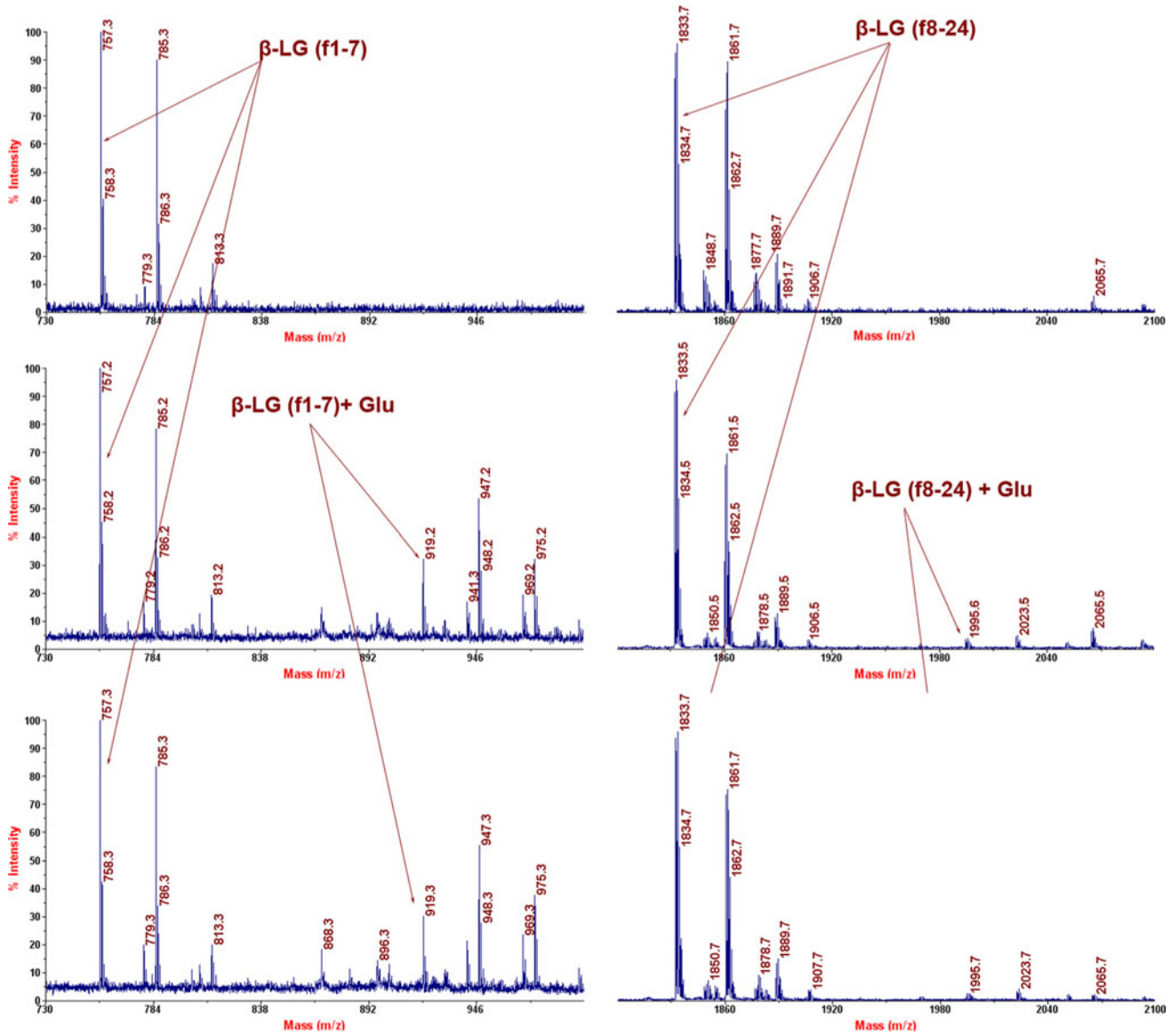


Fig. 7 MALDI spectra of the region 730–2100 uma after CNBr hydrolysis β-Lg after reduction and alkylation. *Panels on the left* showed the region of the fragment 1–7, *panels on the right the region* of the fragment 8–24. *Top panel* control samples without glucose; *middle panel* β-LG-glucose; *bottom panel* β-LG-glucose added with Faox I. Quantitative data are summarized in Table 1

Table 1 Relative intensity values of CNBr peptides β-LG (1–7) and β-LG (8–24) from the MALDI-TOF analysis showed in Fig. 7

β-Lg Peptides	Molecular mass (Da)	Panel b (control) Relative intensity	Panel c (Faox) Relative intensity	Reduction of glycated peptides by Faox (%)
β-LG (f1–7)	757.3	18.9	18.9	–
β b-LG (f1–7) + Glu	919.3	8.5	6.8	20
β-LG (f8–24)	1832.7	100.0	100.0	–
β-LG (f8–24) + Glu	1995.7	5.2	3.5	30

on already glycated β-LG, no effect is observed (Capuano et al. 2007). This hypothesis is supported by the observation that using the enzyme at lower concentrations such as 1:2,000 and 1:5,000 the de-glycation action is severely reduced. This finding strengthened the hypothesis that Faox should react with the glycated protein before its refolding

making the glycated site not accessible to the enzyme catalytic cavity.

This paper is the first one comparing the effects of Faox I and Faox II both on a protein-glucose model systems and a real food substrate, such as the low lactose milk, considering MR markers, such as CML and b-HMF. Data presented here clearly showed that these enzymes can be used for limiting protein glycation that occurs during milk storage opening the way for a possible practical use during milk storage.

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