



# Functional properties of milk proteins as affected by enzymatic oligomerisation

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## ABSTRACT

Milk proteins were enzymatically modified by transglutaminase, lactoperoxidase, laccase as well as glucose oxidase and analysed for changes in molar mass distribution, techno- and tropho-functional properties.

The study revealed that high degrees of protein cross-linking were not only detected upon enzymatic modification of milk proteins by transglutaminase, but also upon incubation with oxidoreductases like lactoperoxidase, laccase as well as glucose oxidase. Due to different reaction mechanisms, oligomeric (20,000–200,000 g/mol) and polymeric (>200,000 g/mol) reaction products with different functional property profiles were synthesised. In contrast to transglutaminase-treated milk proteins, e.g., lactoperoxidase-treated milk proteins performed outstanding interfacial properties and glucose oxidase-treated milk proteins higher *in vitro* digestibility. Oxidoreductase-treated proteins were shown to exhibit increased antioxidative capacity. Laccase was demonstrated to generate oligomeric as well as polymeric protein/protein and protein/phenolic acid reaction products.

The study characterises enzymatically cross-linked proteins and makes it possible to specifically select modified proteins for industrial applications according to the requirements towards food proteins, weighing changes in techno- and tropho-functional protein properties.

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## 1. Introduction

Enzymatic modification probably belongs to the most innovative tools in food protein processing to optimise the sensory, techno-functional as well as tropho-functional properties proteins perform in food products (Gerard, 2002; Koppelman & Wijngaards, 2000; Lorenzen, 2000a, 2000b). Industrial applications of enzymes on food proteins have particularly been described for hydrolases (e.g. pepsin (Schmelzer et al., 2007), rennet (Friedenthal, Siimer, & Köstner, 1981) or trypsin (Jost & Monti, 1977)) and transferases (transglutaminase (Jaros, Partschfeld, Henle, & Rohm, 2006; Lorenzen, 2007; Yokoyama, Nio, & Kikuchi, 2004)), whereas only little is known about protein modification by enzymes of further enzyme classes like oxidoreductases, lyases, isomerases and ligases.

Recently, transglutaminase [EC 2.3.2.13] has been intensively studied for the ability to modify food proteins by deamidation, acyl-transfer and especially enzymatic cross-linking of protein side chains. Gerard (2002), Jaros et al. (2006), Motoki and Seguro (1998), Özrenk (2006), Yokoyama et al. (2004), and Zhu, Rinzeau, and Tramper (1995) reviewed that transglutaminase forms  $\epsilon$ -( $\gamma$ -glutamyl)-lysine cross-linked protein polymers; their improved

techno-functional (e.g. increased heat stability, water- and fat-binding as well as gel forming properties) and tropho-functional properties (e.g. partly reduced allergenic potential) constitute the relevance of industrial applications of transglutaminase in the production of meat, fish and dairy products.

However, certain protein substrates can not efficiently be cross-linked by transglutaminase due to limited steric availability of glutamyl and lysyl residues in the protein structure (Færgemand, 1998), enzyme inhibitors in the product (e.g. raw milk) (De Jong, Boumans, Wilhelmus, & Wijngaards, 2005) or thermodynamic incompatibilities of protein molecules at the enzyme's active site (Han & Damodaran, 1996). Moreover, applications of transglutaminase are limited by the functional property profiles of its protein reaction products; whereas interfacial stabilisation behaviour was demonstrated to increase, foaming and emulsifying properties of proteins were shown to negatively correlate with their increasing molar masses during incubation with transglutaminase (Færgemand, Otte, & Qvist, 1998b; Liu & Damodaran, 1999; Lorenzen, 2000a, 2000b; Özrenk, 2006; Sharma, Zakora, & Qvist, 2002; Tang, Yang, Chen, Wu, & Peng, 2005). More complex techniques such as post-emulsification enzymatic cross-linking (Færgemand & Murray, 1998; Sharma et al., 2002), combined proteinase/transglutaminase treatment (Flanagan & Fitzgerald, 2003) or cross-linking of proteins with N-fatty-acyl-peptides (Wada et al., 2001) had to be considered in consequence.

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Thus, it might be relevant to analyse whether enzymes of other enzyme classes may catalyse cross-linking of protein molecules through different, more unspecific mechanisms. Singh (1991) and Koppelman and Wijngaards (2000) reviewed on enzymes which might be used as potential transglutaminase substitutes, but only few studies have as yet been performed to characterise molar mass, structure and functional properties of their enzymatically generated protein reaction products. Enzymatic oligomerisation reactions have been reported for peroxidase (Aeschbach, Amado, & Neukom, 1976; Færgemand, Otte, & Qvist, 1998a; Matheis & Whitaker, 1984; Stahmann, Spencer, & Honold, 1977), laccase (Lantto, Puolanne, Katina, et al., 2007; Primo-Martin, Valera, & Martinez-Anaya, 2003; Shotaro, 2002), monoamine oxidase (Færgemand et al., 1998a), glucose oxidase (Primo-Martin et al., 2003) as well as tyrosinase (Lantto, Puolanne, Kruus, Buchert, & Autio, 2007; Thalmann, 2003). Thalmann (2003) reported about superior foam forming properties of tyrosinase-treated whey proteins, Shotaro (2002) about improved gel forming abilities of laccase-treated proteins.

Lacking comprehensive data about enzymatic oligomerisation of proteins by other enzymes than transglutaminase, their mechanisms of action and their reaction products, this study aims at presenting a qualitative and quantitative overview over the enzymatic oligomerisation of milk proteins by oxidoreductases (lactoperoxidase, laccase as well as glucose oxidase) in relation to transglutaminase. Milk proteins of open-chain, random-coil tertiary structure (sodium caseinate), of compact, globular structure (whey protein isolate), and of a more complex protein system (total milk protein in skim milk powder) were chosen to present an overview over changes in molar mass, techno-functional and tropho-functional properties of proteins upon enzymatic oligomerisation.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Proteins

Sodium caseinate was donated by Fonterra New Zealand's Dairy Company (Rellingen, Germany). Whey protein isolate was purchased from MILEI GmbH (Stuttgart, Germany). Total milk protein in low heat skim milk powder was obtained from Nordmilch (Bremen, Germany).

#### 2.1.2. Enzymes/cofactors

Transglutaminase [EC 2.3.2.13] from *Streptococcus* sp. (enzyme activity: 100 U/g) was donated by Ajinomoto (Tokyo,

Japan). Lactoperoxidase [EC 1.11.1.7] from bovine milk (enzyme activity: 950 U/mg) was donated by DMV International (Veghel, Netherlands). Laccase [EC 1.10.3.2] from *Myceliophthora thermophila* (enzyme activity: 10,500 PCU/mL) was from Novozymes A/S (Bagsværd, Denmark). Glucose oxidase [EC 1.1.3.4] from *Aspergillus niger* (enzyme activity: 10,000 U/g) was supplied by Novozymes A/S (Bagsværd, Denmark). Hydrogen peroxide was from Merck (Darmstadt, Germany) and chlorogenic acid from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Pancreatin from porcine pancreas (enzyme activity: 8× USP) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

#### 2.1.3. Further material

All further chemicals were analytical grade and purchased from Merck (Darmstadt, Germany). Demineralised water was prepared with the Ultra Clear Water System (Barsbüttel, Germany).

### 2.2. Methods

#### 2.2.1. Enzymatic modification of proteins

Sodium caseinate, whey protein isolate and total milk protein (w/w = 5% protein in demineralised water) were incubated with transglutaminase, lactoperoxidase, laccase or glucose oxidase under the reaction conditions given in Table 1.

#### 2.2.2. Characterisation of molar mass distribution

**Size exclusion chromatography analysis.** Size exclusion chromatography analysis was performed with a Fast Protein Liquid Chromatography 2000 System (Pharmacia Biotech AB, Uppsala, Sweden). 25 µL sample solution was filtered [0.2 µm filter membrane] and injected into a Superdex 200 10/300 GL analytical column (Tricorn, No. 17517401). Analytes were isocratically eluted with FPLC eluent [0.1 mol/L tris, 0.15 mol/L sodium chloride, 8 mol/L urea, pH 8.0] at a flow rate of 0.3 mL/min at room temperature and spectro-photometrically detected at  $\lambda = 280$  nm. Gel Filtration Calibration Kits Nos. 170441201 and 170441101 (GE Healthcare, Freiburg, Germany) were used as protein standards. The amount of protein molecules with molar masses of (I)  $>2 \times 10^5$  g/mol, (II)  $2 \times 10^4$ – $2 \times 10^5$  g/mol, (III)  $1 \times 10^4$ – $2 \times 10^4$  g/mol and (IV)  $<1 \times 10^4$  g/mol was calculated from the FPLC chromatograms and expressed in per cent (%) of the total protein. Determinations were performed in duplicate. The coefficient of variation in the present studies varied between 0.4% and 1.2%.

#### 2.2.3. SDS-PAGE

SDS-PAGE was performed according to VDLUFA method book VI, 30.6.4. (VDLUFA – Verband Deutscher Landwirtschaftlicher

**Table 1**  
Reaction conditions of the enzymatic modification of milk proteins.

|           | Reaction conditions      |     |                           |                   |                                          |
|-----------|--------------------------|-----|---------------------------|-------------------|------------------------------------------|
|           | U enzyme/mg milk protein | pH  | Reaction temperature (°C) | Reaction time (h) | µmol cofactor/mg milk protein            |
| SC + TG   | 0.050                    | 6.7 | 6                         | 16                | –                                        |
| WPI + TG  | 0.050                    | 7.0 | 6                         | 16                | –                                        |
| TMP + TG  | 0.050                    | 7.0 | 40                        | 4                 | –                                        |
| SC + LPO  | 47.500                   | 6.8 | 40                        | 16                | 1.80 µmol H <sub>2</sub> O <sub>2</sub>  |
| WPI + LPO | 9.500                    | 7.2 | 50                        | 1                 | 18.00 µmol H <sub>2</sub> O <sub>2</sub> |
| TMP + LPO | 9.500                    | 7.0 | 50                        | 1                 | 1.80 µmol H <sub>2</sub> O <sub>2</sub>  |
| SC + PPO  | 0.011                    | 6.8 | 40                        | 16                | 0.02 µmol CA                             |
| WPI + PPO | 0.011                    | 7.2 | 40                        | 1                 | 0.08 µmol CA                             |
| TMP + PPO | 0.011                    | 7.0 | 40                        | 1                 | 0.08 µmol CA                             |
| SC + GOX  | 0.500                    | 6.8 | 40                        | 16                | –                                        |
| WPI + GOX | 0.500                    | 7.2 | 40                        | 16                | –                                        |
| TMP + GOX | 0.010                    | 7.0 | 20                        | 16                | –                                        |

SC, sodium caseinate; WPI, whey protein isolate; TMP, total milk protein in skim milk powder; TG, transglutaminase; LPO, lactoperoxidase; PPO, laccase; GOX, glucose oxidase; CA, chlorogenic acid.

Untersuchungs- und Forschungsanstalten (Ed.). VDLUFA method books III (1997), 1996) on SDS–PAGE gradient gels (4–23%) under non-denaturing conditions (Thalmann, 2003). Electrophoresis unit LKB Multiphor Electrophoresis Cell Model 2117 (LKB, Bromma, Sweden), Electrophoresis Constant Power Supply 300/150 (Pharmacia Biotech AB, Uppsala, Sweden) and Cryostat Julabo 725 (JULABO Labortechnik GmbH, Seelbach, Germany) were used. Molar mass distributions of protein samples were assessed densitometrically (TotalLab2.1 Software, Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom), using Rotimark Standard® (Carl Roth GmbH, Karlsruhe, Germany) as molar mass marker. The amount of protein molecules with molar masses of (I)  $>2 \times 10^5$  g/mol, (II)  $2 \times 10^4$ – $2 \times 10^5$  g/mol, (III)  $1 \times 10^4$ – $2 \times 10^4$  g/mol and (IV)  $<1 \times 10^4$  g/mol was calculated and expressed in per cent (%) of the total protein. Determinations were performed in duplicate. The coefficient of variation in the present studies varied between 2.4% and 3.6%.

#### 2.2.4. Determination of soluble amino nitrogen and sulfhydryl groups

Soluble amino nitrogen was assessed using OPA reagent as described by Frister, Meisel, and Schlimme (1986). Sulfhydryl groups were determined with DTNB according to Ellman (1959). Determinations were performed in triplicate; mean values and standard deviations were calculated.

#### 2.2.5. Amino acid analysis

Amino acid analysis was performed according to VDLUFA method book III, 4.11.1. (1997) by cation exchange chromatography analysis. Amino Acid Analysis Unit Alpha Plus 4151 with cation exchange resin 80-2038-45 (Pharmacia Biotech, Freiburg, Germany) was applied. Protein samples were hydrolysed (6 mol/L HCl, 1% phenol, 24 h, 110 °C), lyophilised and resolubilised in sodium citrate buffer (pH 2.20) (Pharmacia Biotech, Freiburg, Germany). Samples were injected into the column and eluted with sodium citrate buffers (Pharmacia Biotech, Freiburg, Germany), stepwisely increasing the pH-value from pH 2.20 to pH 3.35 to pH 4.24 to pH 9.35. The absorbance of the eluate was monitored by ninhydrin reaction of eluting amino acids at  $\lambda = 570$  nm and  $\lambda = 440$  nm. Amino acid standard kit (Pharmacia Biotech, Freiburg, Germany) was used for identification. Determinations were performed in duplicate; mean values were calculated.

#### 2.2.6. Characterisation of functional properties

Untreated as well as enzymatically treated milk proteins (w/w = 5% protein in demineralised water (pH 7.0)) – unless otherwise noted – were tested for their functional properties as follows:

**Heat stability** was assessed by heating milk protein solutions ( $v = 5$  mL in glass test tubes) at 140 °C. Heat stability was defined as the time span until milk proteins visibly coagulated.

**Surface hydrophobicity** was determined spectro-photometrically by SDS binding method (Kato, Matsuda, Matsudomi, & Kobayashi, 1984) with slight modifications (Hiller & Lorenzen, 2008).

**Surface tension** of protein solutions was assessed with a tensiometer (Krüss, Hamburg, Germany) by drop shape analysis. The silhouette of a pendant, capillary generated protein solution drop was continuously recorded via digital camera for 20 min at 20 °C. Surface tension was calculated (Drop shape analysis software DSA 1.80; Krüss, Hamburg, Germany) by Young Laplace equation from the radius of the curvature (as extracted from the drop contour) as well as the density difference between air and protein solution.

**Emulsifying properties** of proteins were assessed according to Pearce and Kinsella (1978) as the size of the liquid/lipid interface between an aqueous protein phase and an oil phase. Fourty millilitre of protein solution (w/w = 0.1% protein in demineralised water) and 10 mL triheptanoin (Sasol GmbH, Witten, Germany)

were mixed (Bühler Mixing System; Bühler, Freiburg, Germany) at 35,000 U/min for 30 s. Emulsions were diluted ( $v/v = 1/100$ ) with an aqueous solution of sodium chloride (w/w = 0.6%) plus sodium dodecyl sulphate (w/w = 0.1%). The size of the liquid/lipid interface was assessed turbidimetrically at a wave length of  $\lambda = 500$  nm (spectro-photometer Libra S12, Biochrom, Bremen, Germany) and calculated as the quotient  $Q = [4.6 \times \text{Extinction} (\lambda = 500 \text{ nm})] / [\Phi \times c \times l]$ .  $\Phi$  was defined as the volume fraction of the dispersed phase ( $\Phi = 0.2$ ),  $c$  as the protein concentration of the emulsion ( $c = 1000$  g/m<sup>3</sup>) and  $l$  as the path length of the cuvette ( $l = 0.01$  m).

Emulsifying activity was expressed as the size of the liquid/lipid interface [m<sup>2</sup>/g] of freshly prepared emulsions. Emulsion stability was expressed as the size of the liquid/lipid interface [m<sup>2</sup>/g] of emulsions after storage [16 h, 20 °C] and centrifugation [10 min, 4000g, 15 °C].

**Foaming properties** were assessed by introducing air into protein solutions (w/w = 1% protein in demineralised water) by an Aero-Latte stirrer (5 min, 1000 turns/min, 40 °C). Foam formation was expressed as the overrun in per cent, foam stability as the time span until half the foam volume elapsed.

**Viscosity** was measured by shearing protein solutions between 0/s and 1000/s at 20 °C (rotation viscosimeter UDS 200, Anton Paar GmbH, Ostfildern, Germany) using cone-plate geometry. Viscosity measurement values at 800/s, 850/s, 900/s, 950/s and 1000/s were averaged to calculate the average apparent viscosity of protein solutions.

**Antioxidative capacity** was determined by ABTS radical cation method (Pellegrini, Re, Yang, & Rice-Evans, 1999). Ten microlitre of protein solution (w/w = 0.1% protein in demineralised water) were incubated with 1 mL ABTS<sup>+</sup> solution for 30 min at 30 °C. The extinction of this sample at  $\lambda = 734$  nm ( $E_{\text{probe}}$ ) was continuously assessed (spectro-photometer Libra S12, Biochrom, Bremen, Germany). ABTS<sup>+</sup> solution served as solvent blanc ( $E_{\text{standard}}$ ). Antioxidative capacity (AC) was calculated as  $AC = 100 - [(E_{\text{standard}} - E_{\text{probe}}) / E_{\text{standard}}] \times 100$ .

Determinations of techno-functional properties were at least performed in triplicate. Mean values and standard deviations are given.

**In vitro digestibility** was determined by incubating milk proteins (w/w = 1% in demineralised water) with pancreatin (enzyme/substrate ratio = 1/500) at 37 °C for 30 min at pH 7.8 (pH-stat system; Metrohm, Herisau, Switzerland). Every 5 min samples were withdrawn and subsequently analysed for changes in molar mass distribution and soluble amino nitrogen. *In vitro* digestibility experiments were performed in triplicate.

### 3. Results and discussion

#### 3.1. Changes in molar mass distribution of milk proteins

Milk proteins were incubated with transglutaminase, lactoperoxidase, laccase as well as glucose oxidase and analysed for changes in molar mass distribution upon enzymatic treatment.

Fig. 1 illustrates changes in molar mass distribution which were assessed for sodium caseinate during a 24-h incubation time with laccase. Fig. 1 shows that casein monomers ( $\sim 2 \times 10^4$  g/mol) rapidly diminished upon enzymatic modification. Intermediately (0–7 h incubation time), reaction products with molar masses of  $2 \times 10^4$ – $2 \times 10^5$  g/mol occurred. The quantitative amount of these oligomeric intermediates subsequently ( $>7$  h incubation time) decreased again and mainly polymeric reaction products  $>2 \times 10^5$  g/mol were formed (Fig. 1).

Table 2 summarises changes in molar mass distribution of milk proteins upon enzymatic modification by transglutaminase,

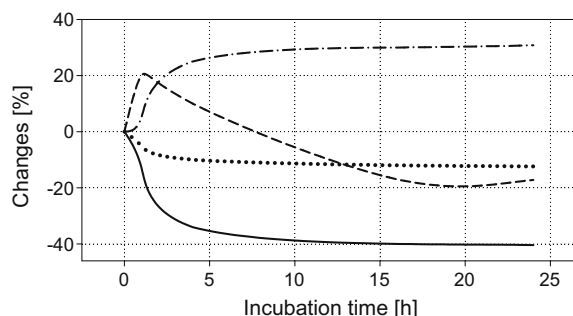


**Fig. 1.** Changes (assessed by densitometric SDS-PAGE analysis; mean values of duplicate determination) in molar mass distribution (casein monomers (—); molar mass fraction  $< 2 \times 10^4$  g/mol (....); molar mass fraction of  $2 \times 10^4$ – $2 \times 10^5$  g/mol (---); molar mass fraction  $> 2 \times 10^5$  g/mol (— · —)) of sodium caseinate during incubation with laccase (0.53 U/mg milk protein and 0.02  $\mu$ mol chlorogenic acid, 24 h, 40 °C, pH 7.0).

lactoperoxidase, laccase as well as glucose oxidase, expressed quantitatively (as the amount of modified milk protein monomers (%)) as well as qualitatively (as the description whether oligomeric or polymeric reaction products were generated). Table 2 shows that – under optimal reaction conditions – high quantitative degrees of protein cross-linking were achieved by transglutaminase, but also by enzymes like lactoperoxidase, laccase or glucose oxidase. Especially laccase induced high degrees of protein monomer modification (Table 2). In most cases, oligomeric ( $2 \times 10^4$ – $2 \times 10^5$  g/mol) to polymeric ( $> 2 \times 10^5$  g/mol) reaction products were released upon enzymatic modification. Table 2 indicates that higher quantitative and qualitative degrees of protein cross-linking were achieved as a result of enzymatic modification of sodium caseinate and total milk protein, whereas protein monomers in whey protein isolate were modified to a lesser extent. This aspect is especially true for glucose oxidase-treated whey protein isolate; only 2% of the whey protein monomers were modified and no densitometrically significant cross-linking products were detected (Table 2).

Concerning protein modification by transglutaminase, it was reported that milk protein oligomers and polymers of high molar masses were formed upon an intermolecular isopeptide-bonding

of side chains of lysyl and glutaminy residues (Flanagan, Gunning, & FitzGerald, 2003; Jaros et al., 2006; Liu & Damodaran, 1999; Lorenzen, 2000a, 2000b; Tang et al., 2005; Yokoyama et al., 2004). In contrast, protein modification and cross-linking by oxidoreductases was discussed to result from a less specific, enzyme-induced oxidation of aromatic and sulfur-containing amino acid residues and a spontaneous isopeptide-bonding of the oxidised protein groups (Færgemand et al., 1998a; Matheis & Whitaker, 1984; Thalmann, 2003). The formation of isodityrosine-, ditryptophane- or disulfide-bonded protein reaction products was reported in the literature (Aeschbach et al., 1976; Stahmann et al., 1977). Laccase was shown to cross-link proteins by bonding protein and phenolic acid molecules (Færgemand et al., 1998a; Shotaro, 2002). Besides the formation of oligomeric and polymeric reaction products of milk proteins and chlorogenic acid in this study (Table 2), further experiments revealed that laccase also cross-linked milk proteins and phenolic acid molecules from plant extracts, such as from wine, blackberry, green tea and black currant extracts (results not shown).

The aspect that the cross-link inducing activity of transglutaminase targets different functional protein groups than the activities of oxidoreductases was confirmed by analyses of the amounts of soluble amino nitrogen and sulfhydryl groups in milk proteins pre and post enzymatic modification. Table 3 shows that an incubation of milk proteins with transglutaminase caused decreased amounts of soluble amino nitrogen, whereas the amount of sulfhydryl groups remained unchanged. These observations correspond to an isopeptide-bonding of the amino groups of lysyl and glutaminy residues by transglutaminase (Jaros et al., 2006; Zhu et al., 1995). Concerning lactoperoxidase-, laccase- as well as glucose oxidase-treated milk proteins, an increased amount of soluble amino nitrogen was detected post enzymatic modification, whereas a minor amount of sulfhydryl groups was observed for lactoperoxidase- and laccase-treated milk proteins (Table 3). A decrease in the amount of sulfhydryl groups upon incubation with oxidoreductases could be explained by the theory that the sulfhydryl groups serve as electron donating substrates which can easily be oxidised to form e.g. intermolecular disulfide bonds (Dean, Shanlin, Stocker, & Davies, 1997; Matheis & Whitaker, 1984; Özer, Grandison, Robinson, & Atamer, 2003). Higher amounts of soluble amino nitrogen in lactoperoxidase-, laccase- as well as glucose oxidase-treated

**Table 2**

Quantitative and qualitative comparison<sup>a</sup> of the enzymatic modification of milk proteins.

|                               |     | Enzymatic modification      |                             |                             |                             |
|-------------------------------|-----|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                               |     | TG                          | LPO                         | PPO                         | GOX                         |
| Modified protein monomers (%) | SC  | 98.2 $\pm$ 1.2 <sup>b</sup> | 39.8 $\pm$ 1.4 <sup>c</sup> | 90.5 $\pm$ 0.3 <sup>d</sup> | 70.3 $\pm$ 2.1 <sup>e</sup> |
|                               | WPI | 50.6 $\pm$ 2.3 <sup>f</sup> | 45.9 $\pm$ 0.8 <sup>g</sup> | 51.3 $\pm$ 1.8 <sup>h</sup> | 2.0 $\pm$ 0.2 <sup>i</sup>  |
|                               | TMP | 96.3 $\pm$ 0.4 <sup>j</sup> | 57.9 $\pm$ 2.4 <sup>k</sup> | 92.0 $\pm$ 1.0 <sup>l</sup> | 86.1 $\pm$ 0.8 <sup>m</sup> |
| Reaction products             | SC  | p <sup>b</sup>              | p <sup>c</sup>              | p <sup>d</sup>              | p <sup>e</sup>              |
|                               | WPI | O/p <sup>f</sup>            | O <sup>g</sup>              | O <sup>h</sup>              | – <sup>i</sup>              |
|                               | TMP | p <sup>j</sup>              | O/p <sup>k</sup>            | p <sup>l</sup>              | p <sup>m</sup>              |

TG, transglutaminase; LPO, lactoperoxidase; PPO, laccase; GOX, glucose oxidase; SC, sodium caseinate; WPI, whey protein isolate; TMP, total milk protein in skim milk powder; O, oligomeric reaction products ( $2 \times 10^4$  g/mol– $2 \times 10^5$  g/mol); P, polymeric reaction products ( $> 2 \times 10^5$  g/mol).

<sup>a</sup> Assessed by PLC and/or SDS-PAGE; results of at least duplicate determination.

<sup>b</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 6.7.

<sup>c</sup> 47.5 U/mg milk protein and 1.8  $\mu$ mol H<sub>2</sub>O<sub>2</sub>, 16 h, 40 °C, pH 6.8.

<sup>d</sup> 0.011 U/mg milk protein and 0.02  $\mu$ mol chlorogenic acid, 16 h, 40 °C, pH 6.8.

<sup>e</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 6.8.

<sup>f</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 7.0.

<sup>g</sup> 47.5 U/mg milk protein and 18.0  $\mu$ mol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.2.

<sup>h</sup> 0.011 U/mg milk protein and 0.08  $\mu$ mol chlorogenic acid, 1 h, 40 °C, pH 7.2.

<sup>i</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 7.2.

<sup>j</sup> 0.05 U/mg milk protein, 4 h, 40 °C, pH 7.0.

<sup>k</sup> 4.8 U/mg milk protein and 1.8  $\mu$ mol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.0.

<sup>l</sup> 0.011 U/mg milk protein and 0.08  $\mu$ mol chlorogenic acid, 1 h, 40 °C, pH 7.0.

<sup>m</sup> 0.01 U/mg milk protein, 16 h, 20 °C, pH 7.0.

**Table 3**

Changes in the amount of sulfhydryl groups and soluble amino nitrogen of milk proteins upon enzymatic modification.

|                                  |     | Enzymatic modification |                |                |                |
|----------------------------------|-----|------------------------|----------------|----------------|----------------|
|                                  |     | TG                     | LPO            | PPO            | GOX            |
| Amount of sulfhydryl groups      | SC  | 0 <sup>a</sup>         | — <sup>b</sup> | — <sup>c</sup> | 0 <sup>d</sup> |
|                                  | WPI | 0 <sup>e</sup>         | — <sup>f</sup> | — <sup>g</sup> | 0 <sup>h</sup> |
|                                  | TMP | 0 <sup>i</sup>         | — <sup>j</sup> | — <sup>k</sup> | 0 <sup>l</sup> |
| Amount of soluble amino nitrogen | SC  | — <sup>a</sup>         | + <sup>b</sup> | + <sup>c</sup> | + <sup>d</sup> |
|                                  | WPI | — <sup>e</sup>         | + <sup>f</sup> | + <sup>g</sup> | + <sup>h</sup> |
|                                  | TMP | — <sup>i</sup>         | + <sup>j</sup> | + <sup>k</sup> | + <sup>l</sup> |

+, Increase; —, decrease; 0, no change; SC, sodium caseinate; WPI, whey protein isolate; TMP, total milk protein in skim milk powder; TG, transglutaminase; LPO, lactoperoxidase; PPO, laccase; GOX, glucose oxidase.

<sup>a</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 6.7.

<sup>b</sup> 47.5 U/mg milk protein and 1.8 μmol H<sub>2</sub>O<sub>2</sub>, 16 h, 40 °C, pH 6.8.

<sup>c</sup> 0.011 U/mg milk protein and 0.02 μmol chlorogenic acid, 16 h, 40 °C, pH 6.8.

<sup>d</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 6.8.

<sup>e</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 7.0.

<sup>f</sup> 47.5 U/mg milk protein and 18.0 μmol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.2.

<sup>g</sup> 0.011 U/mg milk protein and 0.08 μmol chlorogenic acid, 1 h, 40 °C, pH 7.2.

<sup>h</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 7.2.

<sup>i</sup> 0.05 U/mg milk protein, 4 h, 40 °C, pH 7.0.

<sup>j</sup> 4.8 U/mg milk protein and 1.8 μmol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.0.

<sup>k</sup> 0.011 U/mg milk protein and 0.08 μmol chlorogenic acid, 1 h, 40 °C, pH 7.0.

<sup>l</sup> 0.01 U/mg milk protein, 16 h, 20 °C, pH 7.0.

milk proteins are supposedly due to an oxidative, enzyme-induced unfolding of tertiary protein structures as well as limited fragmentation reactions which could also occur upon enzyme-induced oxidation reactions (Davies & Delsignore, 1987; Dean et al., 1997).

### 3.2. Techno-functional properties of enzymatically treated milk proteins

Milk proteins pre and post enzymatic modification were analysed for changes of heat stability, surface/interfacial properties, viscosity and antioxidative capacity. Summarising the results from Figs. 2–5, it can be observed that – although oligomeric and polymeric reaction products were enzymatically synthesised from milk protein monomers in all cases – the reaction products exhibited different techno-functional properties.

Figs. 3–5 show that heat stability markedly decreased during incubation of milk proteins with oxidoreductases, whereas increased heat stability was observed for transglutaminase-treated whey protein isolate (Fig. 2). According to Flanagan et al. (2003), Lorenzen (2000a, 2000b) and Tang et al. (2005), the formation of intra- and inter-molecularly isopeptide-bonded protein polymers of mainly compact molecule structure delays a heat-induced unfolding and denaturation of transglutaminase-treated proteins. Less compact, extended secondary and tertiary molecule structures of lactoperoxidase-, laccase- and glucose oxidase-treated milk proteins (Davies & Delsignore, 1987; Dean et al., 1997) seem to impair their heat stability (Figs. 3–5). Analogies between the mechanisms of an enzymatic oxidation (Matheis & Whitaker, 1984; Özer et al., 2003) and a heat-induced denaturation (Sava, van der Plancken, Claeys, & Hendrickx, 2005) – like an unfolding of protein structures or an oxidation of sulfhydryl groups to disulfide bonds – may also be relevant in this respect. In the literature, little has as yet been reported about the heat stability of oxidoreductase-treated proteins. Actin and myosin were demonstrated to be more heat stable post laccase treatment (Lantto, Puolanne, Kalkkinen, Buchert, & Autio 2005), whereas physicochemically oxidised myofibrillar proteins (Liu, Xiong, & Butterfield 2000) and lysozyme/chlorogenic acid as well as serum albumin/chlorogenic acid products (Prigent et al., 2003; Rawel, Rohm, Kruse, & Kroll, 2002) performed minor heat stability than their untreated forms.

Figs. 2–5 display that milk proteins performed increased surface hydrophobicity post enzymatic modification. This increase in

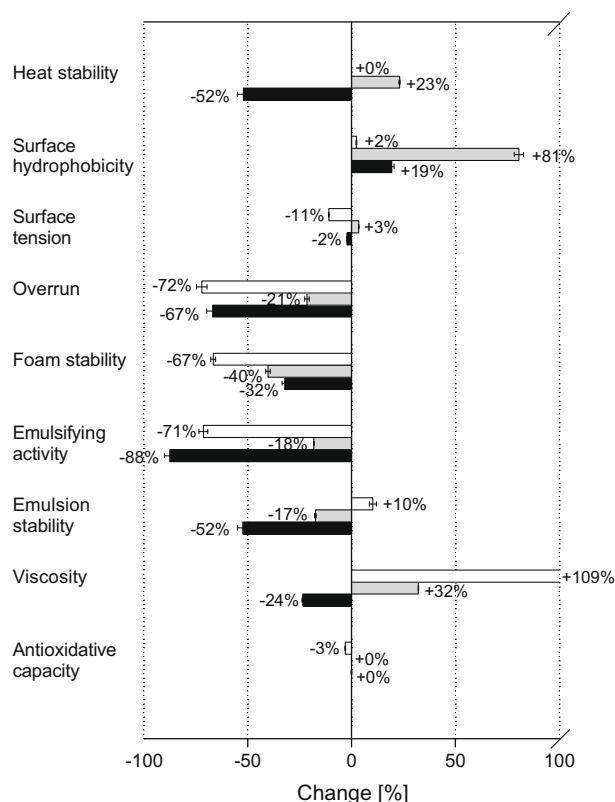


**Fig. 2.** Changes in techno-functional properties of sodium caseinate (0.05 U/mg milk protein, 16 h, 6 °C, pH 6.7) (□), whey protein isolate (0.05 U/mg milk protein, 16 h, 6 °C, pH 7.0) (▒) and total milk protein (0.05 U/mg milk protein, 4 h, 40 °C, pH 7.0) (■) upon incubation with transglutaminase.

surface hydrophobicity corresponds to an unfolding of protein structure upon enzymatic oxidation (Davies & Delsignore, 1987; Dean et al., 1997) and thus an exposure of hydrophobic amino acid residues which were buried within the tertiary structure of untreated protein molecules. Hirano, Hirano, Oooka, and Hatanaka (1998) and Khalid and Masud (2004) hypothesised that an increase in protein hydrophobicity was related to the formation of soft,

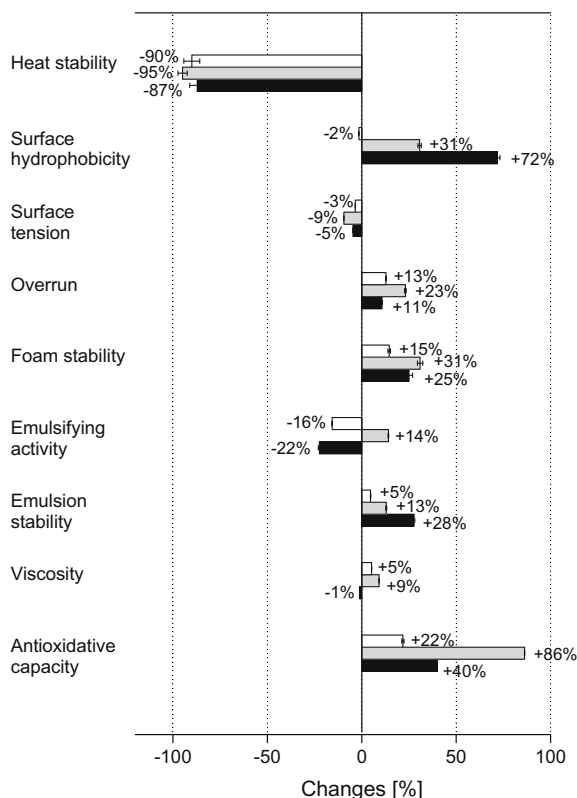


**Fig. 3.** Changes in techno-functional properties of sodium caseinate (47.5 U/mg milk protein and 1.8  $\mu\text{mol H}_2\text{O}_2$ , 16 h, 40 °C, pH 6.8) (□), whey protein isolate (47.5 U/mg milk protein and 18.0  $\mu\text{mol H}_2\text{O}_2$ , 1 h, 50 °C, pH 7.2) (■) and total milk protein (4.8 U/mg milk protein and 1.8  $\mu\text{mol H}_2\text{O}_2$ , 1 h, 50 °C, pH 7.0) (■) upon incubation with lactoperoxidase.

weak yoghurt gels made from lactoperoxidase-treated milk. Rawel et al. (2002) also obtained increased surface hydrophobicity of serum albumin/chlorogenic acid adducts.

Surface tension of milk protein solutions was shown to be slightly decreased by enzymatic protein cross-linking (Figs. 2–5). Decreasing surface tension – or a stronger affinity of milk protein molecules towards interfaces – theoretically (Borchering, Lorenzen, Hoffmann, & Schrader, 2008) corresponds to increasing surface hydrophobicity values as diagrammed in Figs. 2–5. Kato and Nakai (1980) and Kristinsson and Hultin (2003) supported a negative correlation between the surface tension of protein solutions and the surface hydrophobicity of milk protein molecules. It may also be discussed that protein unfolding reactions (Davies & Delsignore, 1987), changing amounts of cationic, hydrophobic amino acid residues on the protein surface (Cardamone & Puri, 1992; Patel & Kilara, 1990) and increasing protein net charges (Rawel et al., 2002; Stahmann et al., 1977) – which result from lactoperoxidase-, laccase- or glucose oxidase-induced protein oxidation – also relate the diminished surface tension of proteins post enzymatic modification.

Analyses of foam forming properties revealed that lactoperoxidase-treated milk proteins (Fig. 3) and laccase-treated whey protein isolate (Fig. 4) formed higher overrun (up to +13%) and performed increased foam stability (up to +58%) in relation to untreated proteins. In opposite, transglutaminase (Fig. 2) and glucose oxidase treatment (Fig. 5) generally impaired foaming forming properties of milk proteins.

Flanagan et al. (2003) and Lorenzen (2000a, 2000b) found out that impaired foam forming properties of cross-linked milk proteins (esp. transglutaminase-treated milk proteins) relate to their highly increased molar masses as well as their low activity towards

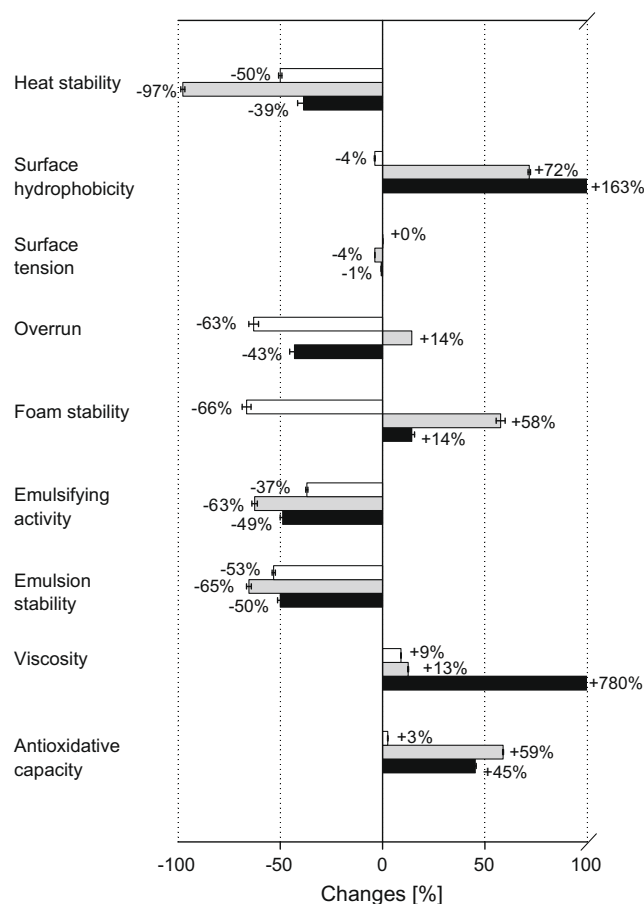


**Fig. 4.** Changes in techno-functional properties of sodium caseinate (0.011 U/mg milk protein and 0.02  $\mu\text{mol}$  chlorogenic acid, 16 h, 40 °C, pH 6.8) (□), whey protein isolate (0.011 U/mg milk protein and 0.08  $\mu\text{mol}$  chlorogenic acid, 1 h, 40 °C, pH 7.2) (■) and total milk protein (0.011 U/mg milk protein and 0.08  $\mu\text{mol}$  chlorogenic acid, 1 h, 40 °C, pH 7.0) (■) upon incubation with laccase.

interfaces. In the literature, little has as yet been reported about the foam forming properties of oxidoreductase-treated proteins; only Thalmann (2003) described that whey proteins formed higher overrun at pH 5.0 and decreased overrun at pH 7.0 post tyrosinase treatment. Regarding the outstanding foam forming properties of lactoperoxidase-treated proteins in this study (Fig. 3), it remains to be further analysed, how the complex interaction of their enzymatically modified molar mass, particle size, viscosity, surface tension and surface hydrophobicity affects their surface properties.

Analyses of the emulsifying properties of milk proteins revealed that enzymatic cross-linking generally impaired emulsifying activity as well as emulsion stability of milk proteins (Figs. 2–5). Increased emulsifying activity (+14%) was only detected for lactoperoxidase-treated whey protein isolate (Fig. 3), increased emulsion stability (up to +28%) for transglutaminase-treated sodium caseinate (Fig. 2) as well as lactoperoxidase-treated milk proteins (Fig. 3).

Liu and Damodaran (1999), Tang et al. (2005), Færgemand et al. (1998b), Lorenzen (2000a, 2000b) and Sharma et al. (2002) established that cross-linked proteins perform lower emulsifying activity because their high molar masses and extended protein structures retard interfacial adsorption and surface denaturation. Concerning their emulsion stabilisation behaviour, some studies report that cross-linked proteins form thick interfacial protein layers, stabilising oil droplets in o/w-emulsions against aggregation and coalescence (Liu & Damodaran, 1999; Lorenzen, 2000a, 2000b), whereas other studies suggest that the huge, extended

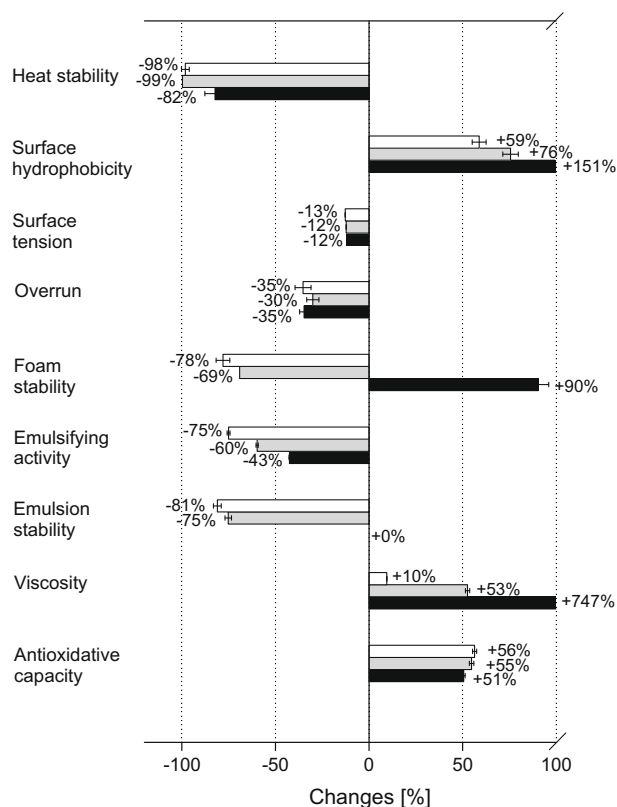


**Fig. 5.** Changes in techno-functional properties of sodium caseinate (0.5 U/mg milk protein, 16 h, 40 °C, pH 6.8) (□), whey protein isolate (0.5 U/mg milk protein, 16 h, 40 °C, pH 7.2) (■) and total milk protein (0.01 U/mg milk protein, 16 h, 20 °C, pH 7.0) (■) upon incubation with glucose oxidase.

protein oligomers/polymers facilitate aggregation and coalescence by simultaneously participating in the interfacial areas of different oil droplets (Flanagan et al., 2003).

Figs. 2–5 suggest that the viscosity of protein solutions mainly increased during enzymatic modification of milk proteins. Decreased viscosity was only detected for solutions of transglutaminase- and lactoperoxidase-treated total milk protein (Figs. 2 and 3), whereas markedly increased viscosity (up to +780%) was obtained for solutions of laccase- as well as glucose oxidase-treated total milk protein (Figs. 4 and 5). Further experiments under different incubation conditions revealed that solutions of total milk protein even gelled upon incubation with laccase (0.011 U/mg milk protein and 0.02 μmol chlorogenic acid, 1 h, 40 °C, pH 7.0) or glucose oxidase (0.01 U/mg milk protein, 16 h, 40 °C, pH 7.0) (results not shown).

In the literature, higher viscosity was also obtained for milk protein solutions post transglutaminase (Lorenzen, 2000a, 2000b), peroxidase (Færgemand et al., 1998a) or laccase treatment (Færgemand et al., 1998a; Shotaro, 2002) as well as for cereal protein dispersions post peroxidase (Peyron, Abecassis, Autran, & Ronan, 2001) or glucose oxidase treatment (Schooneveld-Bergmans, Dignum, Grabber, Beldman, & Voragen, 1999; Vernullapalli & Hoseneey, 1998). Shotaro (2002) observed a correlation between protein concentration and laccase-induced viscosity increase of casein and serum albumin solutions. Enzymatic gelation of milk protein solutions by laccase (Matheis & Whitaker, 1984) was obtained for protein solutions (w/w >1% protein) (Shotaro, 2002). Norsker, Jensen, and Adler-Nissen (2000) reported about an enzymatic gelation of pectin enriched milk (w/w = 2% pectin) by peroxidase or laccase. Lantto, Puolanne, Katina, et al. (2007) and Lantto et al. (2005) produced elastic protein gels by the incubation of myofibrillar proteins with laccase.

Figs. 3–5 diagram that markedly increased (up to +86%) antioxidative capacity of milk proteins was assessed post lactoperoxidase, laccase or glucose oxidase treatment. In opposite, antioxidative capacity of proteins remained mainly unaffected by transglutaminase treatment (Fig. 2). Though it might seem paradox that the antioxidative capacity of proteins increased upon enzymatically induced oxidation (Figs. 3–5), this increase may relate to an enzymatically induced, spontaneous unfolding of tertiary protein structures (Davies & Delsignore, 1987; Dean et al., 1997). Thus, amino acid side chains with antioxidative functional groups – which were originally buried within the protein structure – might have been exposed. Antioxidative capacity of laccase-treated milk proteins probably also increased upon the enzyme-induced cross-linking of protein and phenolic acid molecules (Barbeau & Kinsella, 1985). In the literature, superior antioxidative properties of protein cross-linking products have as yet only been reported for tyrosinase-treated casein (Halaoui et al., 2005).

### 3.3. Tropho-functional properties of enzymatically treated milk proteins

Milk proteins pre and post enzymatic modification were analysed for changes in their essential amino acid profile and pancreatic *in vitro* digestibility (Table 4).

Analyses of the essential amino acid profiles revealed that mainly leucine, lysine, methionine and isoleucine were modified during enzymatic treatment (Table 4). A modification of lysyl residues – which corresponds to the formation of ε-(γ-glutamyl-)-lysine cross-linked protein reaction products – was already reported for transglutaminase-treated proteins (Flanagan et al., 2003; Jaros et al., 2006; Lorenzen, 2000a, 2000b; Lorenzen, 2007; Yokoyama et al., 2004). Concerning the less amino acid specific cross-linking of proteins by lactoperoxidase, laccase or glucose oxidase, it is probable that several amino acid residues may enzymatically be modified (Matheis & Whitaker, 1984). In the literature, it was reported that amino acid modification by lactoperoxidase (Aeschbach et al., 1976; Boeriu et al., 2004; Michon, Wang, Ferrasson, & Gueguen, 1999; Thomas & Aune, 1977; Østdal, Bjerrum, Pedersen, & Andersen, 2000), laccase (Færgemand et al., 1998a; Mattinen et al., 2005) or glucose oxidase (Matheis & Whitaker, 1984) was mainly determined by the susceptibility of amino acids residues towards enzymatic oxidation as well as the steric availability of amino acid side chains in the protein structure. Davies and Delsignore (1987) and Dean et al. (1997) found out that principally any amino residue of serum albumin could oxidatively be modified. It was reported that tyrosine, glutamine, leucine, lysine, methionine, cysteine, tryptophane and phenylalanine are the most susceptible amino acid residues towards oxidative modification (Davies & Delsignore, 1987; Dean et al., 1997; Matheis & Whitaker, 1984; Stahmann et al., 1977).

Concerning an incubation of proteins with peroxidases/hydrogen peroxide, modifications of especially tyrosine residues were reported (Aeschbach et al., 1976; Michon et al., 1999; Oudgenoeg et al., 2001; Peyron et al., 2001; Schooneveld-Bergmans et al., 1999). Isodityrosine and isotrytyrosine were demonstrated to be formed upon “phenolic coupling” of oxidised aromatic tyrosine rings (Aeschbach et al., 1976). Coupling positions between functional groups of the aromatic tyrosine rings depend on incubation conditions (Grabber, Ralph, & Hatfield, 2000) and amino acid residues neighbouring tyrosine residues in the protein structure (Oudgenoeg et al., 2001). Thus, Oudgenoeg et al. (2001) described heterogeneous reaction products of different conformation and functional properties. In the present study, tyrosine residues were modified by 26% in sodium caseinate, by 63% in whey protein isolate and by 75% in total milk protein during lactoperoxidase treatment (results not shown). In agreement, Aeschbach et al. (1976)

**Table 4**

Changes in tropho-functional properties of milk proteins upon enzymatic modification.

|                                        |     | Enzymatic modification |                |                |                |
|----------------------------------------|-----|------------------------|----------------|----------------|----------------|
|                                        |     | TG                     | LPO            | PPO            | GOX            |
| <i>Amount of essential amino acids</i> |     |                        |                |                |                |
| Isoleucine                             | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | — <sup>c</sup> | 0 <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | 0 <sup>f</sup> | — <sup>g</sup> | 0 <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | 0 <sup>j</sup> | — <sup>k</sup> | 0 <sup>l</sup> |
| Leucine                                | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | — <sup>c</sup> | — <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | — <sup>f</sup> | — <sup>g</sup> | — <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | — <sup>j</sup> | — <sup>k</sup> | — <sup>l</sup> |
| Lysine                                 | SC  | — <sup>a</sup>         | 0 <sup>b</sup> | 0 <sup>c</sup> | — <sup>d</sup> |
|                                        | WPI | — <sup>e</sup>         | — <sup>f</sup> | — <sup>g</sup> | — <sup>h</sup> |
|                                        | TMP | — <sup>i</sup>         | — <sup>j</sup> | — <sup>k</sup> | — <sup>l</sup> |
| Methionine                             | SC  | 0 <sup>a</sup>         | — <sup>b</sup> | — <sup>c</sup> | — <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | — <sup>f</sup> | 0 <sup>g</sup> | — <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | — <sup>j</sup> | 0 <sup>k</sup> | 0 <sup>l</sup> |
| Phenylalanine                          | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | 0 <sup>c</sup> | 0 <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | 0 <sup>f</sup> | 0 <sup>g</sup> | 0 <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | 0 <sup>j</sup> | 0 <sup>k</sup> | 0 <sup>l</sup> |
| Threonine                              | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | 0 <sup>c</sup> | 0 <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | 0 <sup>f</sup> | — <sup>g</sup> | 0 <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | 0 <sup>j</sup> | 0 <sup>k</sup> | 0 <sup>l</sup> |
| Tryptophane                            | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | 0              | 0 <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | 0 <sup>f</sup> | 0 <sup>g</sup> | 0 <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | 0 <sup>j</sup> | 0 <sup>k</sup> | 0 <sup>l</sup> |
| Valine                                 | SC  | 0 <sup>a</sup>         | 0 <sup>b</sup> | 0 <sup>c</sup> | 0 <sup>d</sup> |
|                                        | WPI | 0 <sup>e</sup>         | 0 <sup>f</sup> | 0 <sup>g</sup> | 0 <sup>h</sup> |
|                                        | TMP | 0 <sup>i</sup>         | 0 <sup>j</sup> | 0 <sup>k</sup> | 0 <sup>l</sup> |
| <i>In vitro digestibility</i>          |     |                        |                |                |                |
|                                        | SC  | — <sup>a</sup>         | 0 <sup>b</sup> | — <sup>c</sup> | + <sup>d</sup> |
|                                        | WPI | — <sup>i</sup>         | + <sup>f</sup> | — <sup>g</sup> | + <sup>h</sup> |

+, Increase; —, decrease; 0, no change; SC, sodium caseinate; WPI, whey protein isolate; TMP, total milk protein in skim milk powder; TG, transglutaminase; LPO, lactoperoxidase; PPO, laccase; GOX, glucose oxidase.

<sup>a</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 6.7.

<sup>b</sup> 47.5 U/mg milk protein and 1.8 μmol H<sub>2</sub>O<sub>2</sub>, 16 h, 40 °C, pH 6.8.

<sup>c</sup> 0.011 U/mg milk protein and 0.02 μmol chlorogenic acid, 16 h, 40 °C, pH 6.8.

<sup>d</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 6.8.

<sup>e</sup> 0.05 U/mg milk protein, 16 h, 6 °C, pH 7.0.

<sup>f</sup> 47.5 U/mg milk protein and 18.0 μmol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.2.

<sup>g</sup> 0.011 U/mg milk protein and 0.08 μmol chlorogenic acid, 1 h, 40 °C, pH 7.2.

<sup>h</sup> 0.5 U/mg milk protein, 16 h, 40 °C, pH 7.2.

<sup>i</sup> 0.05 U/mg milk protein, 4 h, 40 °C, pH 7.0.

<sup>j</sup> 4.8 U/mg milk protein and 1.8 μmol H<sub>2</sub>O<sub>2</sub>, 1 h, 50 °C, pH 7.0.

<sup>k</sup> 0.011 U/mg milk protein and 0.08 μmol chlorogenic acid, 1 h, 40 °C, pH 7.0.

<sup>l</sup> 0.01 U/mg milk protein, 16 h, 20 °C, pH 7.0.

determined up to 30% modified tyrosine residues in peroxidase-treated milk proteins and Michon et al. (1999) up to 12% modified tyrosine residues in peroxidase-treated cereal proteins. About 1.4–2.0% dityrosine were reported to be formed (Aeschbach et al., 1976; Østdal et al., 2000). Davies and Delsignore (1987) observed that besides tyrosine also tryptophane was oxidatively modified by 70% in serum albumin and by 30% in α<sub>s</sub>-casein.

In agreement with Tables 3 and 4, modifications of sulfur-containing amino acids by peroxidase/hydrogen peroxide were reported by Marin, Sanchez, Perez, Pocovi, and Calvo (2005), Dean et al. (1997), Østdal et al. (2000), Özer et al. (2003) and Matheis and Whitaker (1984). Thomas and Aune (1977) found out that 1 mol hydrogen peroxide oxidised 1 mol sulfhydryl groups in serum albumin or β-lactoglobulin. Marin et al. (2005) detected 80% less methionine as well as a 50% less cysteine in peroxidase/hydrogen peroxide sterilised milk.

Concerning an incubation of proteins with phenol oxidases, modifications of lysine (Hurrel, Finot, & Cuq, 1982; Lantto, Puolanne, Katina, et al., 2007; Lantto, Puolanne, Kruus, et al., 2007;

Matheis & Whitaker, 1984; Petzke, Schuppe, Rohn, Rawel, & Kroll, 2005; Rawel et al., 2002), histidine (Hurrel et al., 1982; Matheis & Whitaker, 1984; Shotaro, 2002), sulfur-containing amino acids (Hurrel et al., 1982; Lantto, Puolanne, Katina, et al., 2007; Lantto, Puolanne, Kruus, et al., 2007; Matheis & Whitaker, 1984; Mattinen et al., 2005; Petzke et al., 2005; Rawel et al., 2002; Shotaro, 2002), tryptophane (Boeriu et al., 2004; Hurrel et al., 1982; Matheis & Whitaker, 1984; Rawel et al., 2002) and phenylalanine (Hurrel et al., 1982) were reported. In agreement with Table 4, Hurrel et al. (1982) detected leucine and isoleucine modifications in casein by tyrosinase. Anyhow, no changes in the limiting amino acid profiles occurred upon casein/cafeic acid (Hurrel et al., 1982) or β-lactoglobulin/chlorogenic acid cross-linking (Petzke et al., 2005).

Lantto, Puolanne, Katina, et al. (2007), Lantto, Puolanne, Kruus, et al. (2007) and Mattinen et al. (2005) detected heterogeneous, dityrosine cross-linked reaction products post laccase treatment of proteins and Prigent et al. (2003) upon non-enzymatic protein/polyphenol interactions. In contrast, no major modification of tyrosine residues by laccase was detected in this study (results not shown). It is probable that laccase without tyrosinase side activity (with was applied in this study) favours diphenols (like e.g. caffeic or chlorogenic acid) rather than monophenolic tyrosine side chains of proteins (Færgemand et al., 1998a).

In agreement with Table 4, modifications of sulfur-containing amino acids and disulfide bonding by glucose oxidase were also reported by Primo-Martin et al. (2003), Dean et al. (1997), Schooneveld-Bergmans et al. (1999), Vernullapalli and Hoseney (1998), Bonet et al. (2006) and Poulsen and Bak Høstrup (1998). In this study, changes in the amount of dityrosine were not detected (results not shown), whereas Tilley et al. (2001) and Hanft and Köhler (2006) detected dityrosine in glucose oxidase-treated cereal proteins.

According to Table 4, pancreatic *in vitro* digestibility of milk proteins was impaired by transglutaminase and laccase treatment, but enhanced by incubation with glucose oxidase. Enhanced *in vitro* digestibility was also detected for lactoperoxidase-treated whey protein isolate, whereas digestibility of lactoperoxidase-treated sodium caseinate remained unaffected (Table 4). Fig. 6 illustrates the pancreatic *in vitro* digestibility of sodium caseinate pre and post enzymatic cross-linking, expressed as the amount of soluble amino nitrogen released during a pancreatic hydrolysis of peptide bonds. Fig. 6 displays that the amount of soluble amino nitrogen increases during pancreatic proteolysis for untreated as well as enzymatically treated protein samples; highest increase – in relation to untreated sodium caseinate – is diagrammed for glucose oxidase-treated sodium caseinate, equal increase for lactoper-

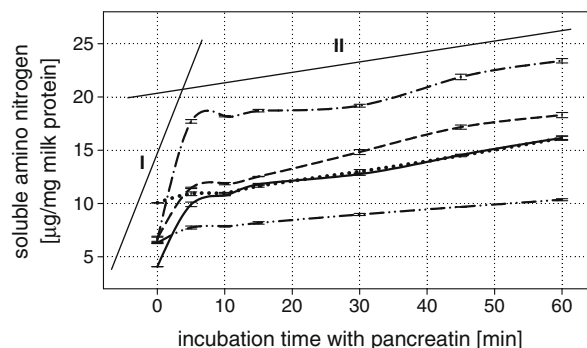


**Fig. 6.** Changes in soluble amino nitrogen of enzymatically treated sodium caseinate (untreated milk protein (—); transglutaminase-treated milk protein (---); lactoperoxidase-treated milk protein (- · -); laccase-treated milk protein (···); glucose oxidase-treated milk protein (— · —)) during pancreatic *in vitro* proteolysis (enzyme/substrate ratio = 1/500, 60 min, 37 °C, pH = 7.8).

oxidase-treated sodium caseinate and lowest increase for transglutaminase- as well as glucose oxidase-treated sodium caseinate (Fig. 6). Differences were especially assessed during the first 5–10 min of the incubation (reaction kinetic I), whereas during further incubation no major differences were apparent (reaction kinetic II) (Fig. 6).

Highly cross-linked proteins – sterically shielding peptide bonds against proteolysis (Roos, Lorenzen, Sick, Schrezenmeir, & Schlimme, 2003) – may be the cause of impaired *in vitro* digestibility of laccase- and transglutaminase-treated milk proteins. On the other hand, an unfolding of the secondary and tertiary structure of protein molecules during enzymatic oxidation (Davies & Delsignore, 1987; Dean et al., 1997; Kamin-Belsky, Brillon, Arav, & Shaklai, 1996) or protein/phenolic acid cross-linking (Barbeau & Kinsella, 1985) may facilitate proteolysis of previously buried peptide bonds (Dean et al., 1997; Matheis & Whitaker, 1984) and explain the improved *in vitro* digestibility of lactoperoxidase-treated whey protein isolate and glucose oxidase-treated milk proteins. In contrast, “strong, progressed protein oxidation” and “excessive protein cross-linking” were described to diminish protein digestibility (Dean et al., 1997; Matheis & Whitaker, 1984).

Rawel et al. (2002) as well as Kroll, Rawel, and Seidelmann (2000) reported about a lower susceptibility of serum albumin/chlorogenic acid as well as myoglobin/caffeic acid cross-linked products towards peptic hydrolysis, whereas tryptic or chymotryptic digestibility were less affected. Retarded peptic proteolysis was especially assessed during the first minutes of protein/proteinase incubation (Kroll et al., 2000). Similar results were obtained for physicochemically oxidised and cross-linked myoglobin (Kamin-Belsky et al., 1996). Anyhow, biological value of tyrosinase-treated casein *in vivo* was shown not to significantly differ from untreated casein (Hurrel et al., 1982). According to Matheis and Whitaker (1984) and Marin et al. (2005) amino acid oxidation products like methionine sulfoxide are still partly available *in vivo*.

#### 4. Conclusions

The results suggest that high degrees of milk protein cross-linking are not only detected upon enzymatic modification by transglutaminase, but also upon incubation with oxidoreductases like lactoperoxidase, laccase as well as glucose oxidase. Due to different reaction mechanisms, oligomeric and polymeric reaction products with different functional property profiles were synthesised. In contrast to transglutaminase-treated milk protein, e.g., lactoperoxidase-treated milk proteins performed outstanding interfacial properties and glucose oxidase-treated milk proteins higher *in vitro* digestibility. Oxidoreductase-treated protein was shown to exhibit increased antioxidative capacity. Laccase was demonstrated to generate oligomeric as well as polymeric protein/protein and protein/phenolic acid reaction products.

The qualitative and quantitative characterisation of enzymatically cross-linked proteins in this study makes it possible to specifically select modified proteins for industrial applications according to the requirements towards food ingredients, weighing changes in techno- and tropho-functional protein properties.

#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodres.2009.04.022.

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