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Short communication

Optimization of enzymatic oligomerization reaction conditions for three milk protein products *via ceteris-paribus* approach

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ABSTRACT

Via a *ceteris-paribus* approach, optimum reaction conditions for an oxidoreductase- (lactoperoxidase; laccase; glucose oxidase) induced oligomerization of milk proteins were assessed for three different milk protein products (sodium caseinate; whey protein isolate; skim milk powder).

Optimum protein monomer modification conditions were enzyme- and substrate-specifically identified, yielding protein monomer modification levels of, e.g., 58% in case of lactoperoxidase-induced (w/w = 5% protein; 1.8 μmol hydrogen peroxide/mg protein; 4.8 U lactoperoxidase/mg protein; 50 °C; 1 h; pH 7.0), 92% in case of laccase-induced (w/w = 5% protein; 0.02 μmol chlorogenic acid/mg protein; 0.01 U laccase/mg protein; 40 °C; 1 h; pH 7.0) and 86% in case of glucose oxidase-induced (w/w = 1% protein; 0.5 U glucose oxidase/mg protein; 40 °C; 16 h; pH 7.0) modification of total milk proteins from skim milk powder.

The study for the first time provides a comprehensive overview over reaction conditions facilitating high degrees of milk protein monomer modification upon oxidoreductase-induced oligomerization in regard to food protein tailoring *via* application of less substrate- and reaction-specific enzymes than transglutaminase.

1. Introduction

Food protein tailoring has developed an essential tool to meet continuously rising requirements of consumers and producers towards techno-functional, tropho-functional and sensory attributes of proteins in food matrices (Chobert et al., 1996; Dube, Schäfer, Neidhart, & Reinhold, 2007; Ercili-Cura et al., 2009; Gerard, 2002; Lorenzen, 2007). Among the most innovative techniques, food protein modification *via* an enzymatic cross-linking of protein side chain groups to generate oligomeric (2×10^4–2×10^5 g/mol) and polymeric (>2×10^5 g/mol) protein reaction products has been intensively studied. Recent examinations elucidated that — besides the established transglutaminase-induced cross-linking of γ-glutaminyl and ε-lysyl residues of protein side chains (Jaros, Partschefeld, Henle, & Rohm, 2006; Lorenzen, 2007) — protein oligomerization can also be facilitated by an enzymatic oxidation of aromatic and sulfur-containing amino acid residues and spontaneous isopeptide-bonding of oxidized protein groups (Færgemand, Otte, & Qvist, 1998; Koppelmann & Wijngaards, 2000; Singh, 1991). Thus, applications of peroxidase/lactoperoxidase, laccase/tyrosinase or glucose oxidase to synthesize techno- and tropho-functionally improved proteins (Hiller & Lorenzen, 2009) as well as to cross-link milk (Ercili-Cura, Lille, Buchert, & Lantto, 2008; Hiller & Lorenzen, 2011), myofibrillar (Lantto et al., 2007) and cereal (Michon, Wang, Ferrasson, & Gueguen,1999) proteins in complex food matrices have been reported. Regarding applications on milk proteins (Hiller & Lorenzen, 2009), lactoperoxidase-treated proteins were demonstrated to perform outstanding interfacial properties, laccase-treated proteins to form highly viscous solutions and protein gels, and glucose oxidase-treated proteins to exert improved proteolytic digestibility. Furthermore, yoghurt from lactoperoxidase-, laccase- and glucose oxidase-treated milk was characterized by a minor acidity and whey drainage as well as by a more soft, homogeneous and creamy consistency than yoghurt form untreated milk (Hiller & Lorenzen, 2011).

In opposite to protein cross-linking by transferases, minor substrate- and reaction-specificity of oxidoreductases allows applications on various protein substrate sources, but necessitates strictly optimized reaction conditions to yield the desired reaction endpoints and protein oligomerization products.

In this context, the present study applied a *ceteris-paribus* approach to determine optimum reaction conditions for an enzymatic oligomerization of proteins of open-chain, random-coil tertiary structure (sodium caseinate), of compact, globular structure (whey protein isolate) and of a complex protein system (total milk proteins in skim milk powder) by lactoperoxidase, laccase and glucose oxidase. The *ceteris-paribus* approach for the first time creates a comprehensive overview over the impact of a single factor variation of protein concentrations, cofactor amounts, enzyme/substrate ratios, pH-values, incubation times and incubation temperatures on the extent of an oxidoreductase-induced protein oligomerization and thus expands

Abbreviations: SC, sodium caseinate; WPI, whey protein isolate; TMP, total milk proteins in skim milk powder.

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findings of preliminary, fixed factor investigations (Aeschbach, Amado, & Neukom, 1976; Boeriu et al., 2004; Özer, Grandison, Robinson, & Atamer, 2003).

2. Materials and methods

2.1. Materials

2.1.1. Milk proteins

Sodium caseinate (96.6 g protein, 0.1 g lactose, 3.6 g ash per 100 g dry matter) was from Fonterra New Zealand's Dairy Company (Hamburg, Germany), whey protein isolate (93.0 g protein, <1.0 g lactose, 2.0 g ash per 100 g dry matter) from MILEI GmbH (Stuttgart, Germany) and total milk proteins in low heat skim milk powder (35.9 g protein, 51.5 g lactose, 5.6 g ash per 100 g dry matter) from Nordmilch (Bremen, Germany).

2.1.2. Enzymes/cofactors

Lactoperoxidase [EC 1.11.1.7] from bovine milk (declared enzyme activity: 950 U/mg) was donated by DMV International (Veghel, Netherlands). Laccase [EC 1.10.3.2] from *Myceliophthera thermophila* (declared enzyme activity: 10,500 PCU/mL) was from Novozymes A/S (Bagsværd, Denmark). Glucoseoxidase [EC 1.1.3.4] from *Aspergillus niger* (declared enzyme activity: 10,000 U/g) was supplied by Novozymes A/S (Bagsværd, Denmark). Hydrogen peroxide was from Merck (Darmstadt, Germany), chlorogenic acid from Sigma-Aldrich (Steinheim, Germany).

2.2. Methods

2.2.1. Enzymatic protein oligomerization

Proteins in sodium caseinate, whey protein isolate and skim milk powder were enzymatically modified by lactoperoxidase (w/w = 1% milk protein; 47.5 U lactoperoxidase + 1.8 μmol H_2O_2/mg milk protein; 24 h; 40 °C; pH 7.0), laccase (w/w = 1% milk protein; 0.53 U laccase + 0.02 μmol chlorogenic acid/mg milk protein; 24 h; 40 °C; pH 7.0) or glucose oxidase (w/w = 1% milk protein; 0.5 U glucose oxidase/mg milk protein; 24 h; 40 °C; pH 7.0). To prevent microbial growth thymol (Merck KG, Darmstadt, Germany) was applied in amounts of w/w = 0.1%.

2.2.2. Protein oligomerization analysis

Protein oligomerization analysis was performed by SDS-PAGE under non-reducing conditions as previously described (Hiller & Lorenzen, 2010), using Rotimark® (Carl Roth GmbH, Karlsruhe, Germany) as well as α-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin (Sigma-Aldrich, Steinheim, Germany) as protein standard substances.

Amounts of protein monomers (integrated band intensities of α-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin) and protein oligomers (integrated band intensities between 2.9×10^4 and 3.0×10^5 g/mol) were densitometrically assessed. Shifts in molar mass distribution upon enzymatic modification were expressed as percentage decreases of protein monomer amounts. Densitometric band intensities of protein monomers in sodium caseinate, whey protein isolate and skim milk powder pre enzymatic modification were defined as 100%, whereas amounts of protein monomers post enzymatic oligomerisation were calculated as [monomer band intensity post enzymatic oligomerization]/[monomer band intensity pre enzymatic oligomerization] × 100%.

2.2.3. Enzymatic modification optimization trials

Enzymatic modification optimization trials were performed *via* a *ceteris-paribus* approach, analyzing the impact of shifted single incubation parameters on the extent of protein monomer modification assessed for the standard incubation system (section 2.2.1). The

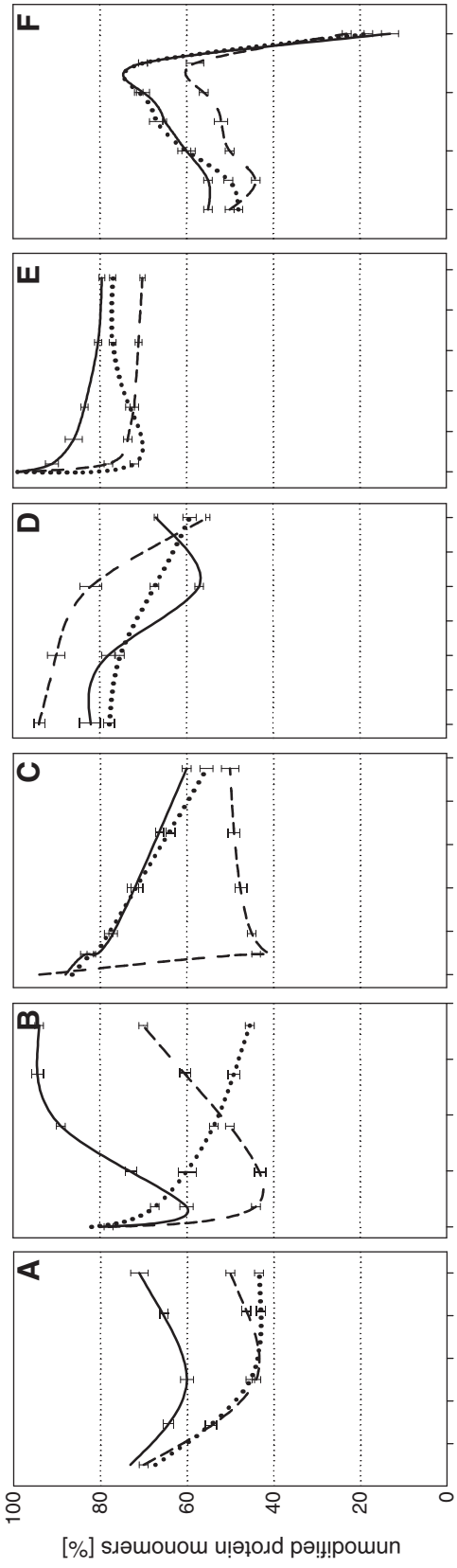

Fig. 1. Lactoperoxidase-induced modification of milk protein monomers (percentage diminution of unmodified protein monomers in sodium caseinate (—), whey protein isolate (· · ·) and skim milk powder (– –); error bars indicate standard deviations of three independent oligomerization trials) as affected by incubation conditions (*ceteris-paribus* approach to optimize standard incubation system (w/w = 1% protein; 47.5 U lactoperoxidase + 1.8 μmol H_2O_2/mg milk protein; 24 h; 40 °C; pH 7.0) by variations of (A) protein concentration (w/w = 1–10%), (B) hydrogen peroxide concentration (0.0–18.0 μmol/mg milk protein), (C) enzyme activity (0.0–47.5 U/mg milk protein), (D) incubation temperature (20°–50 °C), (E) incubation time (0–24 h) and (F) pH-value (pH 4–10)).