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Application of a recombinant laccase-chlorogenic acid system in protein crosslink and antioxidant properties of the curd

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ABSTRACT

Milk protein crosslink through the action of enzymes represents a feasible strategy to impart new functionalities to cheese. In this work we reported the effects of a laccase mediator system (LMS) on protein crosslink and antioxidant property of curd. The crosslinking activity of a purified recombinant laccase Ery4 and a commercial enzyme preparation (cLC), with three mediators was firstly evaluated in milk and then applied before curd manufacture. Only Ery4-LMS significantly increased curd weight compared to that of the control sample. SDS-PAGE revealed that similar high molecular weight bands produced by both LMSs in milk were also retained in curds. The antioxidant activity recorded in curds with Ery4-LMS was the highest among all samples both before and after gastro-pancreatic digestion. This is the first time that a CGA-based LMS is used in manufacture of curd with improved antioxidant properties. These results open new perspectives for dairy applications.

1. Introduction

Milk proteins modification is emerging as an innovative method to improve the technological, rheological, functional and sensory properties of dairy products, such as yield, heat stability, water and fat binding capacity and gel forming properties. In particular, protein incorporation in curd and cheese yield is a matter of great interest in the dairy industry, since considerable amounts of proteins are lost in whey during rennet clotting (Fox, Guinee, Cogan, & McSweeney, 2017).

Cheeses display a large difference in yield mostly in relation to milk quality, casein amount, k-casein types, fat content, cheese type and manufacturing methods (for a review see Fox et al., 2017). In particular, in the early stages of the cheese-making process, collapsing caseins incorporate fat globules and water; this latter is lately purged due to coalescence and applied pressure. The higher the amount of casein in the milk, the greater content of fat and water the cheese might incorporate.

Protein crosslink, obtained by means of chemical or enzymatic modifications, was shown to potentially increase cheese yield, texture properties, digestibility and to reduce the allergenicity of milk proteins (Zeeb, McClements, & Weiss, 2017). The enzymatic modification has the advantage of being highly specific and requiring mild reaction conditions. LCs (EC 1.10.3.2), tyrosinases (Tyr, EC 1.14.18.1) and transglutaminases (TGase, EC 2.3.2.13) were demonstrated to induce protein crosslink and to modulate some important functional properties of dairy and bakery products, including texture, gelling, foaming and emulsifying features as well as to increase whey protein recovery (Buchert et al., 2010).

LCs are copper-containing enzymes which catalyze the oxidation of substituted phenols, anilines and aromatic thiols, using oxygen as the electron acceptor and the release of water as byproduct. Substrates undergo a single electron oxidation, producing unstable and highly reactive radicals. LCs were also reported to directly oxidize tyrosine and cysteine residues of milk proteins, which could further interact with lysine, tyrosine and cysteine residues of other proteins (Selinheimo, Kruus, Buchert, Hopia, & Autio, 2006). Thus, the cross-linking activity is primarily due to non-enzymatic reactions, leading to polymerization. However, extensive protein crosslink could be also achieved by including several redox mediators in the so called Laccase Mediator System (LMS). By this way redox mediators, once oxidized, enhance protein crosslink acting as bridging agents between proteins (Steffensen, Andersen, Degn, & Nielsen, 2008).

It has been reported that the use of a cLC and polyphenols as mediators reinforced the viscoelastic properties of low-fat yoghurt

Abbreviations: ABTS, 2-azino-di-[3-ethylbenzo-thiazolin-sulphonate]; ALA, α-lactalbumin; ANOVA, analysis of variance; BLF, β-lactoferrin; BLG, β-lactoglobulin; CGA, chlorogenic acid; cLC, commercial laccase; CN, casein; FA, ferulic acid; LC, laccase; LMS, laccase mediator system; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEAC, trolox equivalent antioxidant capacity; TGase, transglutaminase; UHT, ultra high temperature; VAN, vanillin; WP, whey protein

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(Struch, Linke, Mokoonlall, Hinrichs, & Berger, 2015) and improved the protein emulsifying properties of sodium caseinate, allowing its supplementation in acidic products (Sato, Perrechil, Costa, Santana, & Cunha, 2015). This mode of action of LC was successfully exploited in several food research applications to improve the textural and rheological properties of bakery and dairy products (Pezzella, Guarino, & Piscitelli, 2015). New and already in use food enzymes need to be subjected to safety evaluation by the European Food Safety Authority (EFSA) and subsequently approved by the European Commission by means of a Union list. To date, an authorized food enzyme list does not exist, but a LC from *Trametes hirsuta* was included in the application list for the validity assessment (European Commission, 2015).

LC are nonspecific with regards to reducing substrates. They catalyze the oxidation of various organic substances, including *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, methoxy phenols, lignins, aryl diamines, and some inorganic ions with the simultaneous and direct reduction of dioxygen to water without the intermediate production of hydrogen peroxide (Giardina & Sannia, 2015).

LC mediators include organic compounds such as *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, methoxy phenols, lignins, aryl diamines. Polyphenolic compounds are exploited for their natural antioxidants and chemopreventive activity in production of nutraceutical foods. Indeed, functional foods enriched with polyphenols (dairy, bakery and meat products) have been recently developed (McDougall, 2017) and the evaluation of their antioxidant activity was also performed *in vitro* during gastro-pancreatic digestion (Helal, Tagliazucchi, Verzelloni, & Conte, 2015) and in *in vivo* assays (Bastide et al., 2017).

LMS application in milk is not limited to technological aspects but might be extended to safety issues. A recent study by Loi et al. (2017) reported the complete removal of aflatoxin M_1 *in vitro* as well as in artificially spiked milk obtained by means of LMS based on the recombinant Ery4 laccase, purified from *Pleurotus eryngii*, expressed in *Saccharomyces cerevisiae*. However, no details were given about the effect of this treatment in dairy applications.

This work aimed at investigating the effect of milk protein crosslink induced by a LMS on curd preparation and its antioxidant properties. Thus, Ery4 laccase was firstly assayed for milk protein crosslink in presence of three mediators, at two concentrations and at different times of incubation. Then, the selected mediator was added at the defined optimal concentration during curd making process in order to confer antioxidant properties of the final product and after *in vitro* gastro-pancreatic digestion.

2. Materials and methods

2.1. Chemicals and reagents

The following mediators: 2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), vanillin (VAN), ferulic acid (FA) and chlorogenic acid (CGA) were purchased from Sigma-Aldrich s.r.l. (Milan, Italy) and freshly diluted in working alcoholic solutions at 0.20 M before use. The enzymes pancreatin from porcine pancreas (8× USP specification activity), pepsin from porcine gastric mucosa (250 units mg/solid), cLC from *Trametes versicolor* (0.89 U/mg; cLC), α-, β-, κ-casein standards (α-CN; β-CN; *κ*-CN; cat. number C6780, C6905, C0406), *α*-lactoalbumin from bovine milk (*α*-LA; L6385-1VL), *β*-lactoglobulin (BLG; L3908) and bovine serum albumin (BSA, A2153) were obtained from Sigma-Aldrich. Marzyme15® was purchased from Danisco Italy S.p.A (Cernusco sul Naviglio, MI, Italy) and bovine lactoferrin (BLF) was obtained from Armor Proteines (Saint-Brice-en-Cogle, France).

The stained molecular weight markers Kaleidoscope (2.5–200.0 kDa) and HiMark™ High Molecular Weight Protein Standard were supplied by Bio-Rad Laboratories S.r.l, (Segrate, Italy) and Life Technologies Italia (Monza, Italy), respectively.

Commercial skimmed UHT milk (5% lactose, 3% protein, 0.1% fat) and whole fat pasteurized milk (5% lactose, 3.5% protein, 3.3% fat) used in this work were purchased in a local market.

2.2. Ery4 laccase production and purification

Ery4 laccase was obtained from *Saccharomyces cerevisiae* ITEM 17289 (Agri-Food Microbial Fungi Culture Collection of the Institute of Sciences of Food Production, www.ispa.cnr.it/Collection) engineered with pYES2 vector (Invitrogen, USA) bearing *ery4* gene sequence from *Pleurotus eryngii (*pY-*ery4*) as reported by Loi et al. (2017). Yeast culture was grown in minimal YNB medium [0.67% (w/v) yeast nitrogen base, supplemented with adequate quantities of auxotrophic requirements and glucose (2%, w/v)] in shaking conditions at 28 °C. Yeast cells were harvested by centrifugation at 0.8 OD_{600nm} and then suspended at 0.6 OD_{600nm} in induction medium (YNB medium supplemented with 4% (v/w) galactose and 1 mM $CuSO_4$. LC production was induced by incubating cells at 18 °C for 72 h. After induction, cultured medium was recovered by centrifugation (10,000*g* for 15 min), filtered and dialyzed at 4 °C against 50 mM Tris-HCl, pH 8.0 using a Vivaflow 200 apparatus (Vivascience AG, Hannover, Germany) equipped with a Hydrosart membrane module (nominal MW cut-off 10,000 Da) and a Masterflex L/S system pump (Cole-Parmer, Vernon Hills, IL, USA) up to a final volume of 50 mL. Ery4 was purified by using a High Q column (1.6 × 10 cm; Bio-Rad, USA) equilibrated with 50 mM Tris-HCl, pH 8. LC was eluted with the same buffer added with NaCl to a final concentration of 0.2 M at a flow rate of 1 mL/min. Active fractions were pooled, desalted by ultrafiltration with Vivaspin15 (Sartorius Stedim S.p.a., Milan, Italy) and concentrated in 1 mL of the same buffer. This enzyme solution was filter-sterilized and stored at −20 °C until use.

2.3. LC spectrophotometric activity assay

LC activity was photometrically measured (Ultraspec 3100pro, Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) according to Loi et al. (2016). Briefly, the reaction was performed in 100 mM sodium acetatepH 4.5, 5 mM ABTS and an appropriate amount of enzyme solution in a final volume of 1 mL. After 10 min of reaction the oxidized ABTS amounts were determined at 420 nm ($\varepsilon_{\lambda=420nm} = 36,000\ M^{-1}\ cm^{-1}$). One unit was defined as the amount of enzyme which oxidized 1 μmol of substrate per min.

2.4. Crosslinking activity of Ery4 in milk with different mediators

The ability of Ery4 laccase to crosslink milk proteins when combined with several mediators and at two different concentrations was evaluated in UHT skim milk. Crosslinked proteins were analyzed by SDS-PAGE as previously described (Loi et al., 2016).

Ery4 laccase (5 U/mL) was added alone or in combination with the selected mediator (VAN, FA or CGA) at 1 or 10 mM to 0.5 mL of milk previously equilibrated at 38 °C for 15 min. Milk control sample contained neither LC nor mediator. The reaction was performed in triplicate at 38 °C for 180 min. Then the samples were diluted for protein quantification by Bradford method (Bradford, 1976). In addition, the same determination was performed on the supernatants of each samples obtained by centrifugation at 13,000 rpm for 5 min. Aliquots of diluted samples and supernatants containing 7 μg of protein were diluted with sample buffer (1:1, v/v; 4% SDS, 3% b-mercaptoethanol, 10% glycerol, 50 mM Tris–HCl pH 6.8) and loaded on a 26-well 12% Criterion TM TGX precast gels (BioRad). Two different reference proteins mixtures (Casein, CN Mix1: *α*-CN, *β*-CN, and *κ*-CN and whey protein Mix2: β-lactoferrin (BLF), bovine serum albumin (BSA), α-lactoalbumin (ALA) and β-lactoglubulin (BLG), respectively) were also loaded on the gel. Electrophoresis was carried out on Criterion TM Cell at 150 V for 75 min. Staining protocol and gel acquisition was carried out as described by Quintieri et al. (2017). The appearance of high molecular weight bands combined with the reduction in intensity of any milk

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