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Thermal treatment of lupin-based milk alternatives – Impact on lupin proteins and the network of respective lupin-based yogurt alternatives

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ABSTRACT

In this study lupin-based milk alternative (LBMA), obtained from protein isolate of *Lupinus angustifolius* cv. Boregine, was homogenized and was subjected to different heat treatments (unheated; pasteurized: 80 °C, 60 s; ultra-high temperature heating (UHT): 140 °C, 10 s). Upon thermal treatment, lupin proteins, namely β-conglutin and α-conglutin, were stepwise denatured, which was accompanied by the exposure of masked sulfhydryl groups, the decrease of free sulfhydryl groups and reoxidation to disulfide bridges. Moreover, quaternary structure of lupin proteins was influenced upon heat treatment displayed with molecular weight distribution (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Further, particle size distribution showed that heat treatment promoted the formation of smaller particles. Differently heated LBMA were additionally fermented to lupin-based yogurt alternatives (LBYA) with different lactic acid bacteria (*Lactobacillus plantarum* TMW 1.460 and 1.1468, *Pediococcus pentosaceus* BGT B34 and *Lactobacillus brevis* BGT L150) and microstructural observations (Confocal Laser Scanning Microscopy, cryo-Scanning Electron Microscopy) were performed. Micrographs showed that harsher thermal treatments of LBMA (UHT heating) led to more close-meshed microstructures of respective LBYA. Further, the choice of microorganisms seemed to play a decisive role in microstructural appearance of respective LBYA as the composition of the networks were fundamental different.
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1. Introduction

Interest in plant-based dairy alternatives has been rising in recent years due to ethical and sustainability issues. Nowadays, soy-based products like milk and yogurt alternatives or tofu are widely used. However, other plant raw materials should be considered to broaden the variety of plant derived dairy alternatives. Lupins, another protein-rich legume belonging to the genus *Lupinus* (family of *Fabaceae*) are promising candidates. Besides to the high protein content, sweet lupin seeds are characterized by a high content of fiber, as well as a low fat and starch content (Lampart-Szczapa et al., 2003). They are in contrast to soybeans free from trypsin inhibitors (van Barneveld, 1999). Moreover, beneficial cardiovascular effects can be attributed to lupin, like lowering serum cholesterol levels (Pilvi et al., 2006). Further, lupin tolerates nutritionally poor soils (Trinick, 1977) and no genetically modified varieties are commercially available (Eapen, 2008).

Development of leguminous dairy substitutes like yogurt alternatives is a difficult task as most of these plant materials comprise disadvantages like beany off-flavors (Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998), contents of antinutritives causing flatulence (Gibson & Roberfroid, 1995) and textural properties that differ to conventional products based on cow milk (Hickisch, Beer, Vogel, & Toelstede, 2016; Jimenez-Martinez, Hernandez-Sanchez, & Davila-Ortiz, 2003; Yang, Fu, & Li, 2012). To overcome beany off-flavors and to reduce antinutritives, fermentation can be applied (Camacho et al., 1991; Fritsch, Vogel, & Toelstede, 2015; Schindler et al., 2011). However, textural properties of yogurt-like products like gel firmness or water-holding capacity are based on the behavior of involved proteins: whereas the interaction of caseins and whey proteins leads to the formation of a stable yogurt network (Lucey, 2002), conglutins of lupin protein behave fundamental different.

The major lupin protein fractions are globular proteins, namely conglutin α (legumin fraction, 11-S globulin) and conglutin β (vicilin fraction, 7-S globulin) (Wäsche, Müller, & Knauf, 2001). Denaturation of conglutin β begins at ~81 °C, whereas conglutin α is thermally more stable with ~96 °C (Bader, Oviedo, Pickardt, & Eisner, 2011). In general, globular proteins undergo several reactions during gel formation such as molecular unfolding (denaturation), dissociation–association and aggregation (Batista, Portugal, Sousa, Crespo, & Raymundo, 2005; Hermansson, 1986). Denaturation is any modification in conformation involving changes in the secondary, tertiary or quaternary structure and thereby the conversion from a native into a more unfolded

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state (Tanford, 1968). Along with denaturation rupture of various inter- and intramolecular bonds that stabilize the native conformation can occur leading to exposure and activation of functional groups e.g. masked sulfhydryl groups (SH) or hydrophobic groups (Boye, Harwalkar, & Ma, 1996). These reactive groups interact with each other and disulfide bondings (SS) and hydrophobic reactions occur, which facilitate protein aggregation (Wang & Damodaran, 1991). Finally aggregated proteins are arranged to a network. For cow milk yogurt manufacture gel formation is acid-induced, but when combined with thermal denaturation of proteins prior to fermentation physical properties and network density of respective yogurts were enhanced as degree of denaturation and thus the amount of crosslinkings were increased (Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Lucey, Munro, & Singh, 1999; Lucey, Teo, Munro, & Singh, 1997; Parnell-Clunies, Kakuda, Mullen, Arnott, & deMan, 1986; Remeuf, Mohammed, Sodini, & Tissier, 2003). A strong relation between heat treatment of lupin-based milk alternatives (LBMA) and physical properties of respective lupin-based yogurt alternatives (LBYA) was also seen in our previous works favoring harsher heat treatments (Hickisch et al., 2016). However, the extent of unfolding and related SH-SS-transitions as well as the impact on texture and network density has not been studied yet for lupin. With regard to textural deficits of LBYA (Hickisch et al., 2016), a better understanding of heat-induced alterations of lupin proteins is needed.

Thus, the objective of this study was to elucidate the effect of protein denaturation and exposure of reactive groups upon LBMA production on the network of respective LBYA. Therefore LBMA was differently heat-treated and degree of denaturation of lupin protein fractions was analyzed. Further, SH-SS-transitions were recorded, as well as particle size and molecular weight distribution. To relate the effect of thermal processing of LBMA on the network of the respective LBYA microstructural observations were performed.

2. Material and methods

2.1. Manufacture of LBMA

Protein isolate manufacture from lupin species *Lupinus angustifolius* cv. Boregine was performed at pilot-scale according to the process by Wäsche et al. (2001), followed by LBMA production as outlined by Jacobs, Stephany, Eisner, and Toelstede (2016). Therefore, a lupin protein isolate solution (LPI, 2.0% (w/v)) was prepared, dextrose (4.0% (w/v)) was added and coconut fat (4.0% (w/v)) was emulsified with water applying Ultraturrax (Janke & Kunkel IKA Labortechnik, Staufen, Germany) with 4450 rpm for 10 min at 50 °C and a high pressure homogenizer at 250 bar/50 bar (APV-2000, SPX FLOW Inc., Charlotte, USA) as well at 50 °C. Thereby the pH was maintained at pH 7.0 ± 0.1 the entire time to receive a stable LBMA. Then LBMA was subjected to different heat treatments, namely pasteurization (80 °C, 60 s) and ultrahigh temperature (UHT) treatment at 140 °C for 10 s with a tubular heat exchanger (HT 220 HTST/UHT System, Omwe, Schalkwijk, Netherlands) followed by storage at 1 °C. To monitor the samples throughout the production process, five different samples were drawn: LPI solution 2.0% (w/v); LBMA prior to homogenization; LBMA after homogenization: unheated, pasteurized and UHT-heated. These aliquots were analyzed with differential scanning calorimetry (DSC), content of free SH, total SH and SS was determined, as well as particle size distribution and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two independent measurements were performed with triplicate repetition. All chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, Missouri, USA) unless stated otherwise.

2.1.1. DSC-analysis

The thermal denaturation of lupin proteins in LPI and LBMA was examined using DSC Q 2000 system (TA Instruments, New Castle, USA). Samples were gently evaporated under vacuum to one fifth by a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik GmbH, Essen, Germany) to obtain a higher protein concentration. About 15 mg of concentrated samples were weighed into aluminum pans and were sealed hermetically. An empty DSC pan was taken as reference. Samples were heated with a heating rate of 2 °C/min from 30 °C to 130 °C. Peak denaturation temperature (T_d), onset temperature (T_{onset}) and enthalpy of denaturation (ΔH) for respective endothermic peaks were calculated by TA Universal Analysis software.

2.1.2. Free SH, total SH and SS content

The content of free SH groups (Koka, Mikolajcik, & Gould, 1968), of total SH groups (free and masked groups) (Beveridge, Toma, & Nakai, 1974) and total SS bonds (Beveridge et al., 1974; Huang, Hua, & Qiu, 2006) was determined with Ellmans reagent (5,5′-dithiobis(2-nitrobenzoic acid)), DTNB) based on the general procedure of Ellman (1959).

For free SH-group determination, 5 ml of each sample was purged with nitrogen for 10 s in the test tubes. Then, 0.1 ml DTNB solution (39.37 mg DTNB in 10 ml 0.1 M phosphate buffer pH 7.0) was added. After the yellow color was developed for 3 min at room temperature, 1 ml 0.1 M phosphate buffer (pH 8.0) and 4 ml distilled water was added and mixed carefully. For clarification, the mixture was treated with 2 g ammonium sulfate and was centrifuged at 9000 rpm for 10 min. The supernatant was sterile-filtered with a 0.45 μm filter (Chromafil® O-45/15 MS Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the absorbance of the filtrate was read at a wavelength of 412 nm with a spectrophotometer (Specord® 210 Plus, Analytik Jena AG, Jena, Germany) versus reagent blanks, treated the same way. The concentration of free SH-groups was calculated based on the following equation with a dilution factor of 2.02:

$$c\ SH\left[\frac{\mu mol}{g}\right] = \frac{A_{412}}{\varepsilon * z} \times \frac{D}{C} \times 1000 \quad (1)$$

with ΔA_{412} = absorbance at 412 nm; ε = extinction coefficient of 9500 l mol^{-1} cm^{-1} (Koka et al., 1968); z = path length of 1 cm; D = the dilution factor; C = protein concentration in g/ml.

For total SH-group determination 1 ml of each sample was mixed with 5 ml 8 M urea in Tris-glycine buffer (10.4 g Tris, 6.9 g glycine, 1.2 g ethylenediaminetetraacetic acid (EDTA) per l) and 0.02 ml DTNB-solution (2 ml DTNB in Tris-glycine buffer). After 5 min of color development, samples were centrifuged at 9000 rpm for 10 min. Again, the supernatant was sterile-filtrated with a 0.45 μm filter and the absorbance of the filtrate was read at a wavelength of 412 nm versus reagent blank. The concentration of total SH-groups was calculated based on Eq. (1) with ε = 13,600 l mol^{-1} cm^{-1} (Ellman, 1959) and D = 6.04.

For the determination of SS-content 1 ml of each sample was mixed with 0.05 ml of 2-mercaptoethanol and 4 ml urea-guanidinhydrochlorid (8 M urea and 5 M guanidinhydrochlorid) in 0.1 M phosphate buffer pH 8.0, 1 mM EDTA and 1% (w/v) SDS and were incubated for 1 h at 25 °C. Then, 10 ml of 12% (w/v) trichloroacetic acid (TCA) were added and were incubated for an additional hour at 25 °C. After incubation samples were centrifuged at 9000 rpm for 10 min. The precipitate was resuspended twice in 5 ml of 12% (w/v) TCA and was centrifuged to remove 2-mercaptoethanol. The washed precipitate was dissolved thoroughly in 10 ml of 0.1 M phosphate buffer (pH 8.0), 1 mM EDTA and 1% (w/v) SDS. For color development 0.08 ml DTNB solution was added after 5 min, absorbance was measured at 412 nm versus reagent blank. Before measurement the samples were sterile filtered as described above. The concentration of total SH and SS-groups was calculated based on Eq. (1) with ε = 13,600 l mol^{-1}

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