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Nanostructure development during peroxidase catalysed cross-linking of α-lactalbumin

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ABSTRACT

Whereas extensive work has been done on the food functional and chemical aspects of enzymatic protein cross-linking, relatively little is known about the nanostructure and physical-chemical properties of enzymatically cross-linked protein. We investigate how nanostructure develops during enzymatic cross-linking of the 4 tyrosine residues of the globular protein apo α-lactalbumin. Protein cross-linking is catalysed by Horseradish Peroxidase, under the periodic addition of peroxide. We use on-line static and dynamic light scattering, combined with on-line UV-spectroscopy to simultaneously probe the development of nanostructure, the extent of dityrosine formation, and the catalytic state of the enzyme, as a function of the number of peroxide additions. It is found that initially, the rate of dityrosine formation is high, whereas the increase in the solution size of the cross-linked protein is limited. At later stages, the increase in solution size is significant whereas dityrosine formation slows down. Finally, the reaction stops due to enzyme inactivation. Off-line size exclusion chromatography shows that the initial phase corresponds to a fast cross-linking of monomers into small oligomers, followed by a slower joining of oligomers into large protein polymers. Consistent with this, Atomic Force Microscopy shows very heterogeneous polymers, apparently consisting of subunits that we identify with the oligomers formed in the first phase of the reaction. The dependence of the solution size on the molar mass of the cross-linked protein is determined using static and dynamic light scattering on fractionated reaction products. For sizes ranging from 30 nm to 80 nm, the protein polymers consist of 100–1000 α-lactalbumin subunits, and have molar masses of 10^6–10^7 g/mol. Apparent internal protein densities of the protein polymers calculated from these numbers are only a few weight percent, indicating a very dilute, open architecture of the cross-linked protein.

1. Introduction

Whereas enzymatic protein hydrolysis has a long history in food technology, the use of protein cross-linking enzymes is more recent, but very promising (Dickinson, 1997). For the commercially important whey proteins, functional effects that have been found upon cross-linking include enhanced foaming and foam stability, enhanced emulsion stability, enhanced thermal stability and improvements of gelation properties (Dickinson, Ritzoulis, Yamamoto, & Logan, 1999; Eissa, Bisram, & Khan, 2004; Faergemand, Otte, & Qvist, 1997; Truong, Clare, Catignani, & Swaisgood, 2004). In addition to food applications, various novel non-food applications of enzymatic protein cross-linking are also under investigation, for example in tissue engineering and for wool and leather (Zhu & Tramper, 2008). A lot of work has focused on the influence of microbial Transglutaminase (TGase) on the functionality and texture of dairy system (Ercili-Cura et al., 2013; Jaros, Heidig, & Rohm, 2007; Norziah, Al-Hassan, Khairulnizam, Mordi, & Norita, 2009; Wilcox & Swaisgood, 2002). For milk proteins, TGase is very efficient at cross-linking the caseins, but less so for the commercially important whey proteins β-lactoglobulin and α-lactalbumin due to their rigid globular structure (Traore & Meunier, 1992). Typically, reducing reagents such as DTT are used in order to increase substrate accessibility during TGase-mediated cross-linking for these proteins. For example, Eissa, Puhl, Kadla, and Khan (2006) report that the viscosity of denatured WPI solutions was decreased by several orders of magnitude after a TGase-treatment. This was attributed to a decrease of hydrophobic interactions after enzymatic cross-linking (Eissa & Khan, 2006). It has also been reported that the thermal stability of β-lactoglobulin in heat-shocked WPI is

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enhanced by a TGase-treatment, with an increase in denaturation temperature of 6–7 °C (Damodaran & Agyare, 2013). For food applications it would be better if reducing agents could be avoided, and some progress in this direction has been made for TGase-induced cross-linking of whey proteins (Tang & Ma, 2007).

An enzymatic cross-linking route that is complementary to cross-linking using TGase is oxidative cross-linking catalysed by enzymes such as laccase, tyrosinase and various peroxidases (Buchert et al., 2010). Whereas TGase introduces cross-links between γ-carboxyamide group and primary amines typically located on the outside and therefore typically reasonably accessible, the oxidative enzymes such as tyrosinase, laccase and peroxidase catalyse the formation of cross-links involving phenols. The phenols may be either located on tyrosine residues of proteins, or on side groups of polysaccharides such as arabinoxylan, containing reactive ferulic acid residue (Oudgenoeg, 2004). Research in this area has largely focused on determinants of chemical reactivity and accessibility of reactive groups (Hakulinen et al., 2002; Mattinen, Lantto, Selinheimo, Kruus, & Buchert, 2008; Thalmann & Lotzbeyer, 2002). But, in the mean time, promising effects on food functionality have also been reported for oxidative enzymatic cross-linking (Faergemand, Otte, & Qvist, 1998; Selinheimo, Kruus, Buchert, Hopia, & Autio, 2006; Selinheimo, Lampila, Mattinen, & Buchert, 2008). A major issue that so far has not been addressed in any detail, is architecture of clusters of cross-linked globular food proteins, and the relation between this architecture and changes in food functionality induced by enzymatic cross-linking.

Indeed, an understanding of the functional effects of enzymatic protein cross-linking first of all requires a thorough characterization of structural evolution induced by enzymatic cross-linking of proteins. Very little work has been performed however, on the structural characterization of enzymatically cross-linked proteins, even for the commercially important case of microbial transglutaminase. An exception is the work of Matsumura, Lee, and Mori (2000) who performed a detailed analysis of α-lactalbumin "protein-polymers" prepared by cross-linking α-lactalbumin using mammalian guinea pig liver (GTGase) and Microbial *Streptoverticillium mobaraense* MTGase in the presence of DTT. By using SEC with a multi-angle laser light scattering (SEC-MALLS) detector system, it was found that α-lactalbumin polymers were formed with solution molecular weights between 3.0×10^6 and 7.0×10^6. The relation between solution size (radius of gyration R_g) and solution molecular weight was found to be $R_g \sim M^v$ with an exponent $v \approx 0.33$ and 0.44 respectively for protein-polymers produced by MTGase and GTGase, indicating a compact architecture (Matsumura et al., 2000). A few other studies have also reported on the effect of enzymatic cross-linking on protein structures (Eissa et al., 2006; Hu, Zhao, Sun, Zhao, & Ren, 2011; Mattinen et al., 2006).

Previously, we have been interested in the chemistry of enzymatic cross-linking reactions that involve phenol groups, such as oxidative protein cross-linking catalysed by peroxidases, tyrosinase or laccase. Specifically, we have considered the case of the cross-linking of the globular whey protein α-lactalbumin via dityrosine bonds, catalysed by Horseradish Peroxidase (HRP) (Heijnis, Wierenga, Berkel, & Gruppen, 2010; Oudgenoeg, 2004), in the presence of peroxide. For each catalytic cycle that ends with the formation of two phenol radicals (that may combine to form a phenol–phenol crosslink), HRP requires one molecule of H_2O_2. Other oxidative cross-linking enzymes (tyrosinase, laccase) require dissolved oxygen rather than peroxide and an advantage of using peroxidase for studies of oxidative cross-linking is that the concentration of peroxide is more easily controlled than the concentration of dissolved oxygen. However, HRP is rather sensitive to excess peroxide, which leads to various side reactions and eventually to inactivation of the enzyme (Hernandez-Ruiz, Arnao, Hiner, Garcia-Canovas, & Acosta, 2001). Therefore the supply of peroxide during the reaction must be tuned carefully (van de Velde, van Rantwijk, & Sheldon, 2001). We also here use step-wise addition of peroxide, which enables us to control the reaction kinetics and particle size development during the reaction.

High conversion and high molecular weight products are found when performing the reactions at pH 7, in 0.1 M ammonium acetate (NH_4Ac) at 37 °C, using a simple scheme of multiple additions of small amounts of H_2O_2 (Heijnis et al., 2010). Also, the calcium depleted, and partly unfolded, apo-form of α-lactalbumin was found to be much more sensitive towards HRP-catalysed cross-linking than the calcium containing holo form (Oudgenoeg, 2004). Not all four tyrosines are equally reactive. Previous experimental data suggests that the first cross-link that is being formed is between Tyr18 and Tyr50 (Heijnis et al., 2011).

Whereas previous work (Heijnis et al., 2011) has mainly dealt with accessibility and reactivity in the early stages of the reaction, the aim of the present work is to perform a detailed nano-structural characterization of the formation of protein polymers if these reactions are ran to completion. Rather than varying reaction conditions (which has been done in previous work), (Heijnis et al., 2010), we here consider a single reaction condition that is known to lead to high conversion and high molecular weight products. For this condition, reactions run to completion in a number of hours, which allows for a detailed on-line probing of the reaction using dynamic and static light scattering, as well as UV-spectroscopy. Off-line analyses that are performed on both aliquots taken during the reaction and on the final reaction products include assays for enzyme activity, size-exclusion chromatography, and Atomic Force Microscope (AFM) imaging.

2. Materials and methods

2.1. Materials

Ca^{+2}-depleted-α-lactalbumin (L6010) was supplied by Sigma–Aldrich and was used without any purification. The calcium content reported is less than 0.3 mol Ca^{2+} per mol of α-lactalbumin. Horseradish peroxidase (HRP) type VI-A (P6782), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) was also supplied by Sigma–Aldrich. All other chemicals used were of analytical grade.

2.2. Sample preparation

Stock solutions of α-lactalbumin were prepared by dissolving 12 g/L in 0.1 M NH_4Ac at 25 °C. After centrifugation (3 h, 45,000× *g*, 25 °C), the pH of the supernatant was adjusted to pH 6.8 using 0.1 mM HCl, and was filtered using a 0.1 μm syringe filter. Finally, the protein concentration was determined spectrophotometrically assuming an absorbance $A_{280} = 20.1$ for a 1% (w/v) solution of α-lactalbumin (Kronman & Andreotti, 1964). HRP stock solutions were prepared by dissolving 15 g/L in 0.1 M NH_4Ac. Concentration of HRP solutions were checked using UV spectrophotometry assuming a molar extinction coefficient of $\varepsilon_{403} = 102.1\ mM^{-1}\ cm^{-1}$ (Ohlsson & Paul, 1976). For preparing hydrogen peroxide (H_2O_2) stock solutions, an aqueous solution of 30% (w/w) of peroxide was diluted with deionized water to a concentration of 0.05 M. Before use, concentrations of H_2O_2 stock solutions were checked spectrophotometrically assuming a molar extinction coefficient of $\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$ (Noble & Gibson, 1970).

2.3. Light scattering combined with titration and UV spectroscopy

For on-line probing of the enzymatic cross-linking reaction, a cell was developed that allows for simultaneous titration of H_2O_2,