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In-line quantification of peroxidase-catalyzed cross-linking of α-lactalbumin in a microreactor

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ABSTRACT

Horseradish peroxidase can induce the oxidative cross-linking of proteins through the radicalization of tyrosine residues and subsequent formation of dityrosine bonds. The dityrosine bond absorbs light at 318 nm which can be used to monitor in-line the peroxidase-catalyzed cross-linking of proteins in a microfluidic system. In this study calcium-depleted α-lactalbumin is used as model protein. To quantify the progress of the reaction, the absorbance increase at 318 nm was monitored in-line and compared with the amount of reacted monomeric α-lactalbumin as determined with size-exclusion chromatography (SEC) at various residence times. The increase in absorbance at 318 nm shows a logarithmic relation with the extent of reacted monomer. The logarithmic relation can be explained using a reaction model describing minimum and maximum formation of dityrosine cross-links to reacted monomer. Since the size distribution of reaction products was found to be reproducible, the absorbance increase at 318 nm can be used as a fast in-line screening method for the peroxidase-mediated cross-linking of proteins.

1. Introduction

The cross-linking of food proteins is a tool to form potential new food ingredients. A quite novel approach in food protein cross-linking is enzymatic oxidation. The use of laccase and peroxidase to cross-link proteins has been well described [1–3]. By catalyzing the conversion of tyrosine residues to phenolic radicals, peroxidase (EC 1.11.1.7) can initiate the formation of dityrosines [4] and trityrosines (*in vivo*) [5]. This can be used to cross-link food proteins as was shown for α-lactalbumin and β-lactoglobulin [3,6].

Oudgenoeg [6] studied the importance of the protein conformation of α-lactalbumin for the availability of tyrosine residues and the formation of dityrosine protein cross-links. Using calcium-depleted α-lactalbumin (apo-form), the tyrosine residues became more exposed, resulting in a range of cross-linked protein oligomers and polymers. Within food chemistry increasingly, attention shifts towards understanding the functionality of individual molecules instead of that of mixtures of related components. For this a functionality profile of more specific products, on the molecular level, is required. To direct the formation of specific products with certain functionality, more control is required over the peroxidase-mediated protein cross-linking reaction than is currently achieved at lab scale reactions.

In recent years, the use of microfluidic systems in enzymatic reactions has increased considerably. Due to their small dimensions, localized control over concentration gradients, temperatures and separations can be obtained [7,8]. This increases the precise control of the system and the formation of unwanted side products can be prevented. The small dimensions and sample size enable control of enzymatic reactions in microfluidic devices and make them suitable for kinetic parameter determination [9,10]. Enzyme kinetics on microreactor scale do not significantly differ from lab scale reactions giving rise to the possible use for reaction optimization [7]. Microreactors are ideal for directing complex enzymatic synthesis, like multienzyme catalysis [11,12] and cascade reactions [13]. With the possibility to place microreactors in parallel [14] enzymatic reactions in microreactors are easy to scale-up and show great potential in catalysis.

Here, we studied the peroxidase-catalyzed cross-linking of calcium-depleted α-lactalbumin in a microreactor. To make better use of the possibilities of a microreactor, an in-line detection of the extent of formation of reaction products is needed. This would enable a direct detection of product formation, instead of the time-consuming subsequent off-line analysis. To our knowledge, there is, however, no in-line detection method to quantify the extent of oxidative cross-linking of proteins. As the dityrosine bonds formed

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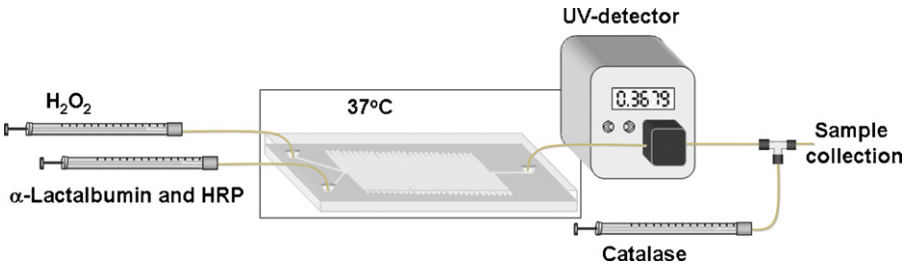

Fig. 1. Schematic representation of the microreactor set-up.

can be monitored at 318 nm [15], an UV-detector was connected in-line with a microreactor. Since the reaction products are heterogeneous [6], it is not clear whether UV-detection can be used to quantify the extent of cross-linking of proteins. Therefore, the aim of the present study is to investigate a possible correlation of absorbance at 318 nm and extent of cross-linking. Modifications of α-lactalbumin were carried out in a microreactor and in lab scale, to obtain a wide range of extents of cross-linking. The UV-signals were compared with the conversion of monomeric α-lactalbumin determined by size-exclusion chromatography (SEC).

2. Materials and methods

2.1. Materials

A commercial α-lactalbumin powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the cross-linking experiments. According to the manufacturer, the powder's protein content was 95% (w/w) (90% (w/w) α-lactalbumin) and 0.55‰ (w/w) calcium. Horseradish peroxidase (HRP) type VI-a (P6782), catalase (C30) and β-lactoglobulin (L6879) were obtained from Sigma (Sigma Chemical CO, St. Louis, MO, USA). All other (bio)chemicals were analytical grade and purchased from Sigma or Merck (Darmstadt, Germany)

2.2. Microreactor experiments

Cross-linking of α-lactalbumin was performed in a Y-shaped microfluidic chip (R150.676.1) of Micronit (Enschede, The Netherlands). The volume of the microreactor was 10.1 μl, the channel is 150 μm wide and deep, and 676 mm long. The microreactor was placed in a water bath at 37 °C and connected to an Ultimate UV-detector with an ULT-UZ-M10 flow cell (LC Packings, Sunnyvale, CA, USA), monitoring at 318 nm. The total reaction volume until the detector was 15.3 μl. Reagents were inserted by Hamilton 250 μl luer lock syringes (Reno, NV, USA), which were loaded onto a Harvard Apparatus Pico Plus 11 syringe pump (Holliston, MA, USA). All connections were made with 150 μm ID fused silica capillaries and MicroTight connectors (P-772 and P-888, Upchurch Scientific, Oak Harbor, WA, USA) (Fig. 1).

Syringes were loaded with 2 mM H_2O_2, 9 μg/ml catalase, and a mixture of 2% (w/v) α-lactalbumin and 1 mg/ml HRP, respectively. All solutions contained 0.1 M ammonium acetate. The oxidizing substrate was loaded separately from the enzyme to prevent the inactivation of HRP [16]. The H_2O_2 and enzyme–substrate mixture were guided through the microreactor. To quench the reaction, catalase was introduced in the capillary. This was done after the UV-detector to prevent disturbance of UV-signal by the formed oxygen. For the blank, the syringe with H_2O_2 was replaced with a syringe containing only 0.1 M ammonium acetate. Reaction time was changed by changing flow rate (0.5–10 μl/min per syringe) equal for all three syringes. After each change in flow rate, the system was left to equilibrate for 150 μl. After equilibration, the UV absorbance was quantified and samples (60 μl) were collected at room temperature.

2.3. Lab scale experiments

A 1% (w/v) protein solution of 1 ml was incubated at 37 °C with 1 mM H_2O_2 and 0.5 mg/ml HRP. Catalase (3 μg/ml, final concentration) was added to quench the reaction at the desired incubation time. The absorption increase at 318 nm was measured after incubation using the Ultimate UV-detector.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The formation of covalently linked protein oligomers was analyzed with SDS-PAGE, using a Phast-system (Amersham Biosciences, Uppsala, Sweden) according to the supplier's instructions. PhastGels Gradient 8-25 (Amersham) was used for protein separation. Before application, protein samples were treated with β-mercaptoethanol and heated for 5 min at 100 °C in the presence of SDS. Proteins were stained with Coommassie Brilliant Blue and a protein molecular weight marker (article 17-0446-01; GE-Healthcare) was used for calibration.

2.5. Size-exclusion chromatography (SEC)

The conversion of monomeric α-lactalbumin to oligomers was determined by SEC. Diluted protein samples (20 μl, 5 mg/ml) in 0.1 M ammonium acetate were applied to a Superdex 75 10/300 GL column (Amersham Biosciences, Uppsala, Sweden) with a Äkta Purifier system at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate solution at a flow rate of 0.9 ml/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low molecular weight gel filtration calibration kit (Amersham Biosciences). The kit contained blue dextran (2000k), albumin (67k), ovalbumin (43k), chymotrypsinogen A (25k) and ribonuclease A (13.7k).

The areas (at 280 nm) of the peaks were calculated with Unicorn software (GE-Healthcare, Chalfont St. Giles, UK) divided over four sections. This calculation gave the proportions of decrease in monomeric α-lactalbumin and the increase in oligomers.

3. Results and discussion

3.1. Cross-linking in a microreactor

To quantify, in-line, the cross-linking of α-lactalbumin in a microreactor one needs a reliable and simple monitoring technique. As dityrosine formation results in an increase in absorbance at 318 nm [15], direct connection of a UV-detector to the microreactor provides a convenient method to measure the cross-linking. Different flow rates, and consequently residence times, were applied to monitor the extent of cross-linking in time.

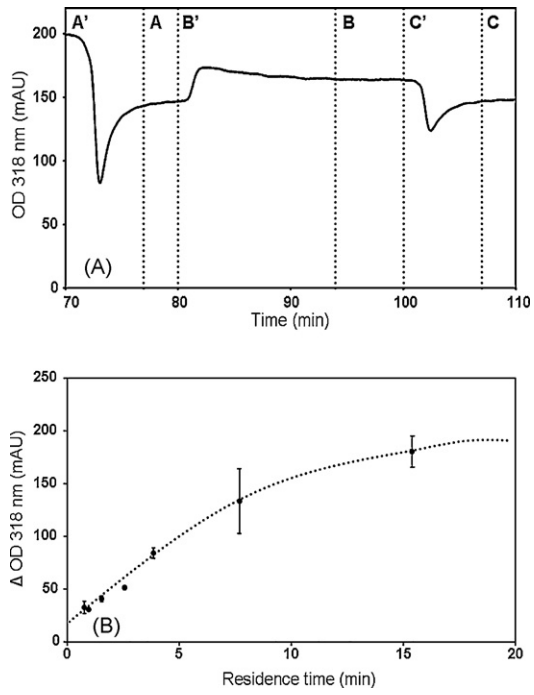

Fig. 2. In-line UV-detection of the peroxidase-mediated cross-linking of α-lactalbumin in a microreactor. (a) Fragment of a typical in-line absorption trace recorded at 318 nm. At 70, 80 and 100 min flow rate was changed. Sections A′, B′ and C′ represent equilibration time. Sections A, B and C correspond to plateau values with residence times of 1.03, 2.20 and 1.10 min, respectively. (b) Values of the absorbance at 318 nm at different residence times.

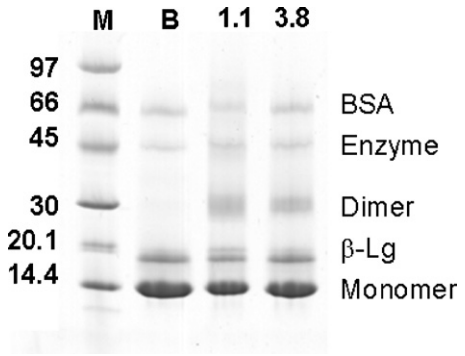

Fig. 3. SDS-PAGE of the peroxidase-mediated cross-linking of commercial α-lactalbumin in a microreactor. M: marker proteins with their molecular weights indicated at the left; B: blank reaction without H_2O_2; 1.1 and 3.8: residence times of the corresponding sample in the microreactor; enzyme: HRP; monomer and dimer: α-lactalbumin; β-Lg: β-lactoglobulin.

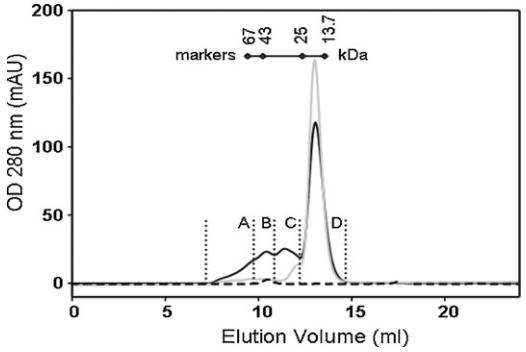

Fig. 4. Size-exclusion chromatogram of untreated α-lactalbumin (grey line), α-lactalbumin after peroxidase-mediated cross-linking (black line) and HRP (dashed line). The residence time of the cross-linking reaction was 15.4 min. Elution volumes of standard proteins (albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa)) are included. Sections A–D represent the area of integration to determine cross-linking.

Fig. 2a shows a fragment of a typical in-line absorption trace recorded at 318 nm, consisting of three different residence times. The flow rate was increased at 70 and 100 min and decreased at 80 min. Each change in flow rate was followed by a two-step change in absorbance. Sections A′, B′ and C′ represent the equilibration phase needed to stabilize signal intensity and pump pressure [17]. Fractions were collected when the signal intensity was stable (Sections A, B and C) and the average signal intensities were calculated from the stable sections. In Fig. 2b, signal intensities at different flow rates (1–20 μl/min) are shown as a function of their corresponding residence time, which is inversely related to the flow rate. The absorbance relates linearly to the residence time up to 4 min. At longer residence times the amount of substrate becomes a limiting factor.

3.2. Detection of cross-linked product

Quenched samples were analyzed by SDS-PAGE and SEC. The SDS-PAGE separation under reducing conditions is shown in Fig. 3. The blank reaction (B), consists of HRP (45 kDa) and the commercial α-lactalbumin (a mixture of α-lactalbumin (15 kDa), β-lactoglobulin (18 kDa) and BSA (66 kDa)). The enzymatic oxidation reactions with residence times 1.1 and 3.8 min show an additional protein band at ~30 kDa, corresponding to dimeric α-lactalbumin. Detection of trimer (~45 kDa) and tetramer (~60 kDa) formation is hindered by the presence of HRP and BSA. These results indicate the formation of covalently cross-linked oligomers of α-lactalbumin.

Fig. 4 shows a typical size-exclusion chromatogram of HRP (dashed line), untreated α-lactalbumin and α-lactalbumin incubated with HRP and H_2O_2. Untreated α-lactalbumin shows one major peak eluting at 13.0 ml with a small shoulder at 12.3 ml, originating from β-lactoglobulin as determined via separate analysis (no further data shown). The minor peak eluting at 9.6 ml is originating from BSA, based on the results of SDS-PAGE analysis and HRP elutes at 10.5 ml. Incubated α-lactalbumin shows di- and trimeric α-lactalbumin eluting at 11.4 and 10.4 ml, based on the results of SDS-PAGE analysis. The recorded size-exclusion chromatograms were used to determine the extent of cross-linking by integration of the separate peaks of the chromatogram (Table 1). Besides oligomers, formed at short residence times, polymeric α-lactalbumin (excluded volume 7.5 ml) is formed at longer residence

Table 1
Size distribution of cross-linked α-lactalbumin products formed at different residence times. The relative concentration of product in each size range is given as percentage of the total (with standard deviations), measured by SEC (Fig. 4).

Integration section	Residence time (min)						
	Blank	0.77	1.54	2.57	3.85	7.70	15.39
A	3.2 (0.6)	6.4 (0.5)	7.3 (1.1)	7.4 (2.5)	11.8 (2.3)	17.3 (4.1)	18.1 (2.9)
B	3.4 (0.2)	6.6 (0.4)	7.5 (0.6)	7.7 (1.3)	10.3 (0.9)	12.7 (1.3)	14.9 (0.6)
C	6.5 (0.4)	10.4 (0.6)	11.3 (0.9)	12.1 (1.6)	13.5 (0.1)	13.8 (0.6)	16.1 (1.8)
D	86.8 (0.9)	76.6 (1.5)	73.9 (2.6)	72.8 (5.4)	64.4 (3.3)	56.2 (6.0)	50.9 (1.8)

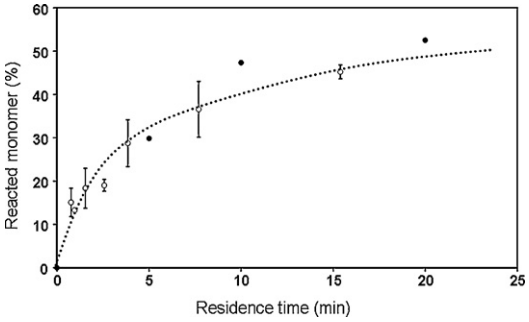

Fig. 5. Average decrease in monomeric α-lactalbumin upon peroxidase-mediated cross-linking at different residence times in a microreactor (○) and in a lab scale reaction (●).

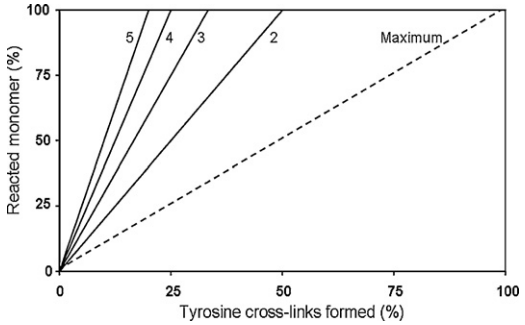

Fig. 6. Tentative model for α-lactalbumin cross-linking. Expected correlation for the reacted monomer and the formation of tyrosine cross-links. The minimal formation (solid lines) is indicated for α-lactalbumin when two to five dityrosine bonds can be formed. The dashed line indicates the maximum formation of tyrosine cross-links.

times. This results in a broad range of molecules, as previously shown by Oudgenoeg [6]. Similar size distribution in cross-linking patterns was observed with similar residence times (Table 1), indicating a reproducible cross-linking.

Besides α-lactalbumin, traces of β-lactoglobulin are present in the commercial α-lactalbumin preparation. As reported in previous research, peroxidase catalyzes the cross-linking of β-lactoglobulin in whey protein isolate solutions [3]. In this research, however, purified commercial β-lactoglobulin did not show any tendency to cross-link, when treated with HRP and H_2O_2 in lab scale reactions (SDS-PAGE and SEC results not shown). This indicates that only the cross-linking of α-lactalbumin is causing the increase in absorbance at 318 nm.

3.3. Correlation between reacted α-lactalbumin and increase in absorbance at 318 nm

Because molar absorption coefficients of oligomeric α-lactalbumin molecules are not known, the increase in absorbance at 318 nm due to dityrosine bond formation cannot directly be used for determining the extent of α-lactalbumin cross-linking. Thus, the amount of reacted monomeric α-lactalbumin was determined from the decrease in absorbance at 280 nm in SEC experiments. Fig. 5 shows the average extent of reacted monomer at their respective residence times. In agreement with the absorbance measurements (Fig. 2b), the extent of reacted monomer levels off with increasing residence time. The extent of reacted monomer is, as expected, quantitatively related to the absorbance at 318 nm. The cross-linking in a lab scale reaction (filled dots, Fig. 5) shows comparable results as in microfluidic cross-linking. Both systems show a conversion of monomeric α-lactalbumin up to a maximum of 50%, upon a single addition of 1 mM H_2O_2. Similar observations for microreactor and lab scale reactions were made in lipase-catalyzed esterification [7]. Because of good reproducibility and comparability the results obtained in both systems can be combined.

The peroxidase-catalyzed cross-linking of α-lactalbumin can most simply be described by the following reaction sequences:

$$2\text{N}\alpha\text{-lac} \rightarrow \text{N}(\alpha\text{-lac})_2 \quad (1)$$

$$\alpha\text{-lac} + (\alpha\text{-lac})_n \rightarrow (\alpha\text{-lac})_{n+1} \quad (2)$$

Minimum formation of tyrosine cross-links in relation to reacted monomer is achieved when only dimeric α-lactalbumin is formed (reaction (1)). The conversion of monomer to dimer and subsequent to trimer, etc. describes maximum formation of cross-links in relation to reacted monomer (reaction (2)).

α-Lactalbumin contains four tyrosine residues and with the theoretical ability to form trityrosines [5] a maximum of eight cross-links per molecule can be formed. Fig. 6 shows the correlation of reacted monomer to the proportion (%) of tyrosine cross-links

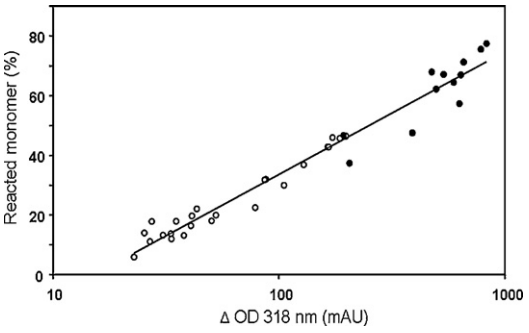

Fig. 7. Correlation of reacted monomeric α-lactalbumin (measured with size-exclusion chromatography) and absorbance increase at 318 nm in a microreactor (○) with in-line UV-detection and in a lab scale reaction (●).

formed. In a system with only two possibilities to form a dityrosine bond, a 100% conversion of monomeric α-lactalbumin will result, according to reaction (1), in a 50% formation of the total amount of possible dityrosine bonds (line 2 in Fig. 6). Reaction (2), in which maximum formation of cross-links is described, will result in a 100% formation of tyrosine cross-links (dashed line in Fig. 6). Minimum formation of tyrosine cross-links is also shown in Fig. 6 for systems in which, arbitrarily chosen, 3–5 tyrosine cross-links per molecule can be formed. When the absorption increase at 318 nm is equal for all different tyrosine bonds the *x*-axis legend 'tyrosine cross-links formed' can be replaced by 'ΔOD 318 nm'.

The correlation between the extent of reacted α-lactalbumin and absorbance increase at 318 nm can be described well by a logarithmic function ($R^2 > 0.96$, Fig. 7). This correlation is independent of the cross-linking system: lab scale reaction (filled dots) and microreaction (open dots) show the same trend. Cross-linking percentages up to 80%, in batch reaction, were obtained by adding aliquots of 1 mM H_2O_2. The logarithmic correlation is explained by the fact that at first only dimeric α-lactalbumin is formed (reaction (1); solid lines in Fig. 6). When the reaction continues dimers will react further and an increase in absorption at 318 nm is seen without a decrease in monomeric α-lactalbumin. This results in a shift towards the maximum formation of tyrosine cross-links, following a logarithmic function, until the maximum has been reached. With the relation described, an in-line determination of extent of reacted monomer is obtained.

4. Conclusion

The increase in absorption at 318 nm is a good tool to quantify in-line the peroxidase-catalyzed cross-linking of monomeric (calcium-depleted) α-lactalbumin in a microreactor. Since cross-

linking was shown to be reproducible, with respect to both the amount of reacted monomer and to the size distribution of formed products, a fast validation of the peroxidase-catalyzed cross-linking of α-lactalbumin in a microreactor is obtained. This is a first step in precisely controlling the oxidative cross-linking of food proteins.

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