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ARTICLE

Directing the Oligomer Size Distribution of Peroxidase-Mediated Cross-Linked Bovine α-Lactalbumin

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Enzymatic protein cross-linking is a powerful tool to change protein functionality. For optimal functionality in gel formation, the size of the cross-linked proteins needs to be controlled, prior to heating. In the current study, we addressed the optimization of the horseradish peroxidase-mediated cross-linking of calcium-depleted bovine α-lactalbumin. To characterize the formed products, the molecular weight distribution of the cross-linked protein was determined by size exclusion chromatography. At low ionic strength, more dimers of α-lactalbumin are formed than at high ionic strength, while the same conversion of monomers is observed. Similarly, at pH 5.9 more higher oligomers are formed than at pH 6.8. This is proposed to be caused by local changes in apo α-lactalbumin conformation as indicated by circular dichroism spectroscopy. A gradual supply of hydrogen peroxide improves the yield of cross-linked products and increases the proportion of higher oligomers. In conclusion, this study shows that the size distribution of peroxidase-mediated cross-linked α-lactalbumin can be directed toward the protein oligomers desired.

KEYWORDS: α-Lactalbumin; horseradish peroxidase; protein cross-linking; ionic strength

INTRODUCTION

Enzymatic cross-linking of whey proteins, prior to heating, has been used to decrease gel point temperature and to improve gel strength upon gelation. Because only limited cross-linking showed these beneficial effects (*1*, *2*), there is a need to direct the enzymatic cross-linking process from polymer formation into oligomer formation to produce cross-linked proteins of desired functionality.

Enzymes such as transglutaminase, laccase, and peroxidase have been applied to form cross-linked whey proteins (*2*−*6*). Transglutaminase (EC 2.3.2.13) induces isopeptide bond formation between glutamyl and lysine residues in proteins. Laccase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) catalyze the one-electron oxidation of tyrosine residues (*7*−*9*). The tyrosyl radicals generated induce (iso)dityrosine conjugation, and as a consequence, covalent protein cross-links are formed. Which tyrosines are radicalized depends on the accessibility of the tyrosine groups (*3*).

Bovine α-lactalbumin has been thoroughly investigated for its structural and folding properties (*10*−*13*). Removal of the calcium cofactor generates apo α-lactalbumin, which has a native-like, but more dynamic structure than holo α-lactalbumin (*10*, *12*−*14*). Apo α-lactalbumin is less thermostable than holo α-lactalbumin and more susceptible to pH- and ionic strength-induced structural changes (*14*). The plasticity of apo α-lactalbumin allows peroxidase-catalyzed intermolecular cross-linking (*8*), resulting in a range of protein oligomers (*6*, *8*), while the holo form is not reactive. The more flexible tertiary structure of the apo form leads to increased exposure of the tyrosine residues. However, as discussed elsewhere (*8*), no single tyrosine was identified as becoming more reactive. Thus, changes in temperature, pH and ionic strength are expected to cause conformational perturbations of tyrosine residues in apo α-lactalbumin. This suggests that changes in apo α-lactalbumin conformation will influence the cross-linking reactivity and hence the type of reaction products formed. Due to its flexibility, apo α-lactalbumin has the potential to direct the oligomer formation.

The catalytic mechanism of horseradish peroxidase (*15*) and the biological implications of peroxidase-mediated protein aggregation (*16*) have been addressed in detail. However, little attention has been paid to how the size of the cross-linked products can be specifically altered. In this research, we have addressed the product specificity of the peroxidase-mediated cross-linking of apo α-lactalbumin. Using different reaction conditions (pH, ionic strength and hydrogen peroxide (H_2O_2) concentration), the extent of cross-linking and type of products formed were monitored by size exclusion chromatography (SEC). Fluorescence spectroscopy, circular dichroism (CD), and differential scanning calorimetry (DSC) were used to investigate the protein conformation of the α-lactalbumin substrate. Future research will aim at the study of the structural and technofunctional food properties of the generated α-lactalbumin oligomers and their mode of cross-linking.

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MATERIALS AND METHODS

Materials. A commercial α-lactalbumin powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN) was used. According to the manufacturer, the protein content was 95% (w/w) of which 90% (w/w) α-lactalbumin. As α-lactalbumin is the only present protein, which can cross-link, we refer to it as pure α-lactalbumin (*6*). The calcium content of the α-lactalbumin powder was 0.55 ‰ (w/w). When indicated, excess calcium chloride (20 mM) or EDTA (20 mM) was added to create 100% holo or 100% apo α-lactalbumin, respectively. Horseradish peroxidase (HRP) type VI-a (P6782), and catalase (C30) were obtained from Sigma (Sigma Chemical CO, St. Louis, MO). All other (bio)chemicals were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

Enzymatic Cross-Linking. α-Lactalbumin solutions of 1% (w/v) with a volume of 1 mL were incubated at 20 and 37 °C for one hour in different ammonium acetate buffers (0.1 mM to 100 mM, pH 5.9 and 6.8). HRP (50 μL, 10 mg/mL) and hydrogen peroxide (2−96 μL, 0.5 M H_2O_2) were added to induce cross-linking. A catalytic amount of catalase (20 μL, 150 μg/mL) was added to quench the reaction after the desired incubation time. Incubation conditions were varied by changing the pH (5.9−6.8), the salt concentration (0.1 mM to 100 mM ammonium acetate), or H_2O_2 concentration (1−48 mM). The H_2O_2 cosubstrate was added either all at once or in aliquots of 2 μL at 30 min intervals.

Peroxidase Activity. ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 9.1 mM) in 0.1−100 mM ammonium acetate buffer pH 5.9 and 6.8, was incubated in a 1 mL cuvette at 37 °C in a UV-1601 spectrophotometer equipped with a CPS-240A cell positioner (Shimadzu SI, Columbia, MD). After addition of 16 μL of HRP (0.5 μg/mL) to 1 mL of ABTS solution, the mixture was left to equilibrate for 5 min. H_2O_2 (33 μL, 0.3% (w/w)) was added to start the reaction, and the increase in absorption at 405 nm was monitored for 10 min. The initial slope of $\Delta A_{405\ nm}$/minute was used to calculate the enzymatic activity via the UV-Probe software (Shimadzu).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Covalent protein cross-linking was analyzed by SDS−PAGE using a Phast-system (GE Healthcare, Uppsala, Sweden) according to the supplier's instructions. PhastGels Gradient 8-25 gels (Amersham) were used for protein separation. Before application, protein samples (5 mg/mL) were heated for 5 min at 100 °C in the presence of 1.25% (w/v) SDS and 1.25% (v/v) β-mercapthoethanol. Proteins were stained with Coomassie Brilliant Blue, and a protein molecular weight marker (GE Healthcare) was used for calibration.

Size-Exclusion Chromatography (SEC). Peroxidase-mediated cross-linking of α-lactalbumin was also analyzed by SEC. Diluted protein samples (20 μL, 5 mg/mL) in 0.1 M ammonium acetate buffer pH 6.8 were applied to a Superdex 75 10/300 GL column (GE Healthcare, Uppsala, Sweden) connected to an Äkta Purifier system at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate buffer pH 6.8 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 SEC calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

To describe the size distribution of the products formed, the SEC chromatogram was divided in four regions (60 kDa−V_0, 40−60 kDa, 25−40 kDa, and 10−25 kDa). For each region the amount of material under the peaks eluted was calculated with Unicorn software (GE Healthcare). From this information the decrease in monomeric α-lactalbumin and the increase in oligomers was estimated, arbitrarily assuming similar weight absorption coefficients for monomeric and oligomeric forms. The increase in oligomer fractions was calculated to obtain the size distribution of reaction products.

Circular Dichroism (CD). CD spectra were recorded at 37 °C on a J-715 (Jasco, Tokyo, Japan) CD spectrometer. Near UV CD spectra (250−350 nm, slit width 2 nm) were determined as an average over 20 repetitive scans in 10 mm quartz cuvettes with protein concentrations of 1 mg/mL. All spectra were corrected for reference spectra recorded in the absence of protein.

Tryptophan Fluorescence. Protein samples were diluted in 0.1−100 mM ammonium acetate buffer pH 6.8 to a final concentration of 0.05 mg/mL and equilibrated for 1 h at 20 or 37 °C. After this incubation, the sample was transferred to a thermostated 1 mL quartz cell. Tryptophan emission was recorded between 300 and 400 nm upon excitation at 295 nm with slit widths of 5 nm.

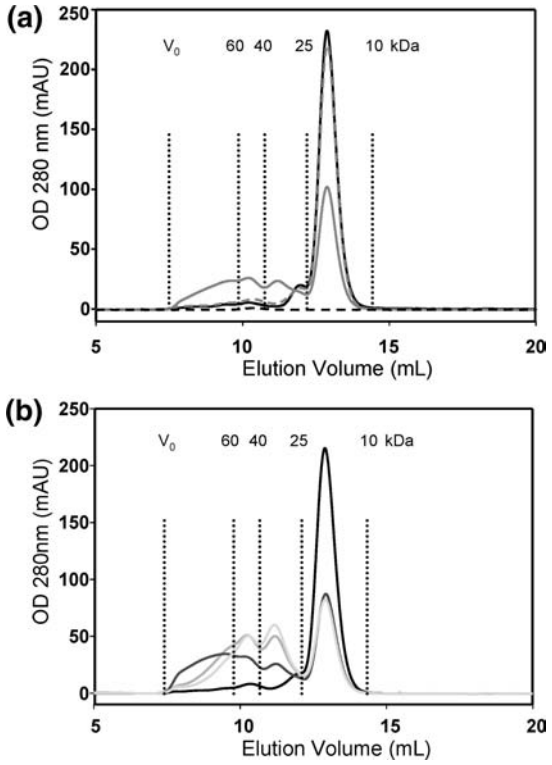

Figure 1. (**a**) Size exclusion chromatogram of untreated apo α-lactalbumin (black line), HRP (dashed black line), and apo α-lactalbumin after peroxidase incubation (gray lines). Apo α-lactalbumin in 100 mM ammonium acetate pH 6.8 was incubated at 37 °C with 1 mM H_2O_2 in the absence (solid line) and presence (dashed line) of 20 mM $CaCl_2$. (**b**) Size exclusion chromatogram of apo α-lactalbumin cross-linked by peroxidase treatment at different ionic strength (gray lines). Darker gray is indicating a higher ionic strength (respectively 0.1 mM, 10 mM and 100 mM ammonium acetate pH 6.8). Untreated α-lactalbumin (black line).

Differential Scanning Calorimetry (DSC). DSC measurements were performed on a VP-DSC MicroCalorimeter (Microcal Inc., Northhampton, MA). Protein samples (2.0 mg/mL, in 0.1−100 mM ammonium acetate buffer pH 6.8) were heated from 15 to 100 °C with a heating rate of 60 °C/h. All samples were thermostatted and degassed before analysis.

RESULTS AND DISCUSSION

Peroxidase-Catalyzed Cross-Linking of Apo α-Lactalbumin. Commercial Ca^{2+}-depleted α-lactalbumin exists for at least 80% in the Ca^{2+} -free apo form, according to the supplier's specification. The influence of pH and ionic strength on the peroxidase-mediated cross-linking of apo α-lactalbumin was monitored by size-exclusion chromatography (SEC). **Figure 1a** shows a typical size-exclusion chromatogram of untreated apo α-lactalbumin, and apo α-lactalbumin cross-linked with 1 mM hydrogen peroxide (H_2O_2) in 100 mM ammonium acetate buffer, pH 6.8 at 37 °C, either in the absence or presence of Ca^{2+}. Apo α-lactalbumin, cross-linked without added Ca^{2+}, shows a decrease in monomeric α-lactalbumin (10−25 kDa) and an increase in oligomers. As reported before (*8*), binding of Ca^{2+} ions inhibits the peroxidase-catalyzed cross-linking of α-lactalbumin and almost no cross-linking products are observed. The three major fractions of cross-linked α-lactalbumin in **Figure 1a** correspond to dimer (25−40 kDa), trimer (40−60 kDa), and higher oligomers (60 kDa−V_0) as determined by SDS−PAGE. The relative yield per fraction, for various reaction conditions at 37 °C, is summarized

Table 1. Quantitative Size Distribution of α-Lactalbumin Products Present in the Reaction Mixture at Different Ionic Strength and pH at 37 °C

	integration section (kDa)[a]			
incubation conditions	10−25	25−40	40−60	60−V_0
pH 6.8				
reaction blank	86	7	4	3
100 mM NH_4Ac + 20 mM $CaCl_2$	83	8 (32)[b]	4 (17)	5 (51)
100 mM NH_4Ac	38	14 (15)	16 (25)	32 (60)
10 mM NH_4Ac	35	22 (29)	22 (36)	22 (35)
0.1 mM NH_4Ac	34	26 (37)	23 (38)	16 (25)
pH 5.9				
100 mM NH_4Ac	39	13 (14)	13 (21)	34 (66)
10 mM NH_4Ac	33	14 (13)	15 (22)	38 (65)
0.1 mM NH_4Ac	31	15 (14)	17 (25)	37 (61)

[a]The relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. [b]Size distribution of oligomers.

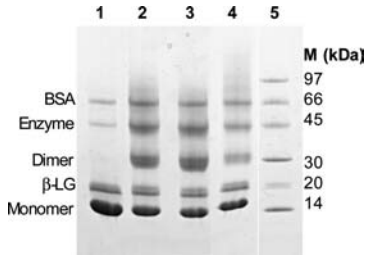

Figure 2. SDS−PAGE of apo α-lactalbumin cross-linked by peroxidase treatment at different ionic strength. 1: Blank reaction without H_2O_2. 2, 3 and 4: Reactions with 1 mM H_2O_2 in 10, 0.1, and 100 mM ammonium acetate buffer pH 6.8. 5: Marker proteins with their molecular masses indicated at the right. Enzyme = peroxidase. β-LG = β-lactoglobulin.

in **Table 1**. At low ionic strength and pH 6.8 relatively more dimers and trimers are formed, whereas at a high ionic strength at pH 6.8 more higher oligomers are formed (**Figure 1b**). At all ionic strengths and pH 5.9 the yield of cross-linked product is comparable to pH 6.8, but at pH 5.9 more higher oligomeric α-lactalbumin is formed (60 kDa−V_0, **Table 1**). The size distribution for each ionic strength does not show a clear difference at pH 5.9. The same reactions are performed at 20 °C as Griko and co-workers (*14*), and this research (Figure **3**) showed a decrease in disruption of tertiary structure at lower temperatures. At 20 °C the cross-linking shows a lower total conversion of monomeric α-lactalbumin (**Table 2**). A low ionic strength causes a small increase in monomer conversion as compared to the high ionic strength. At 20 and 37 °C, similar observations in size distribution correlated to ionic strength are observed at pH 6.8. To verify that the oligomers observed are formed by covalent bonds and not by non-covalent aggregation, the samples were studied by SDS−PAGE under dissociating and reducing conditions. **Figure 2** shows dimers and trimers of α-lactalbumin with a decreasing intensity under high ionic strength reaction conditions. This confirms that the aggregates observed in SEC are a result of covalent bonds. With SEC under reducing conditions the conversion of monomeric α-lactalbumin was comparable to the native SEC (not shown).

Changes in Enzyme Performance. The protein conformation of apo α-lactalbumin might be the main cause of different cross-linking behavior with various buffer conditions, but the peroxidase catalyst might also behave differently (*17*). The enzyme activity was tested using a spectrophotometric assay with ABTS, using the same reaction conditions. Horseradish peroxidase (HRP) shows a 4 to 5 times higher oxidizing activity with ABTS at pH 5.9 compared to pH 6.8 and a 1.0 to 1.1 times higher activity at high ionic strength compared to low ionic strength (no further data shown). Especially at pH 5.9, the higher activity might be an explanation for the preferred formation of α-lactalbumin higher oligomers (**Table 1**). However, with more higher oligomers formed an increased conversion of monomeric α-lactalbumin is also expected. Since at all conditions the consumption of α-lactalbumin was similar, the reactivity of monomeric α-lactalbumin is considered not to be a limiting factor. Most likely, protein conformational changes at the incubation conditions are causing different cross-linking products. Therefore, the conformational state of α-lactalbumin, at the cross-linking conditions, is investigated.

Conformational State of α-Lactalbumin. A change in the accessibility of tyrosine residues within the α-lactalbumin molecule likely causes a difference in cross-linking pattern. To understand this, we analyzed the near-UV circular dichroism (CD) and tryptophan fluorescence spectra of apo and holo α-lactalbumin at different salt concentrations.

Figure 3 shows the near-UV CD spectra for untreated α-lactalbumin at 20 and 37 °C at different ionic strengths at pH 6.8. In the absence of Ca^{2+} ions, the ellipticity around 270 nm is weaker at low ionic strength, indicating a conformational change around the tyrosine and phenylalanine residues (*18*). The decreasing magnitude of ellipticity resembles the gradual chemical denaturation of α-lactalbumin by guanidium hydrochloride (*19*), indicating α-lactalbumin to be slightly unfolded at low ionic strength. Furthermore, the ellipticity is lower at 37 °C and the effects of ionic strength on ellipticity are less profound compared to 20 °C. Similar temperature and ionic strength effects on the ellipticity of α-lactalbumin have been reported before (*14*).

Lowering the ionic strength (both at 20 and 37 °C) does not change the wavelength of the tryptophan fluorescence emission maximum of apo α-lactalbumin (not shown). Furthermore, no change is observed in the melting temperature of α-lactalbumin (**Figure 4**). A small change in enthalpy is observed, but this is negligible compared to the holo and apo α-lactalbumin enthalpy difference. Together with the unaltered far-UV CD properties of apo α-lactalbumin at different ionic strengths and temperatures (*20*, *21*), this indicates that apo α-lactalbumin retains much of its native structure at low ionic strength.

The tryptophans do not become exposed, but the near-UV CD data indicate some change in the orientation of tyrosines (*18*). When the orientation of the tyrosines shows minor ellipticity changes (100 mM at 20 °C; **Figure 3**), no cross-linking can occur. When major ellipticity changes occur (0.1 mM at 20 °C), cross-linking is observed. Local conformational changes of α-lactalbumin at low ionic strength might, therefore, increase the tyrosine availability for oxidation and cross-linking. With the increased availability, cross-links can be formed easier and multiple cross-links per molecule are formed. Quantitative analysis of dityrosine formation, via the absorption increase at 318 nm (*6*), supports this hypothesis: At high ionic strength more higher oligomers are formed than at low ionic strength, but no increase in the extent of dityrosine formation is observed. This suggests that the dimers and trimers formed at low ionic strength (cf. **Figure 1b**) contain a higher number of cross-links per molecule than the oligomers formed at high ionic strength. Interestingly, the same conversion speed of monomeric α-lactalbumin is observed in 10 and 100 mM ammonium acetate (results not shown).

Role of Cosubstrate. The concentration of the cosubstrate H_2O_2 is critical for the peroxidase-catalyzed protein cross-linking process. Besides from increasing the reaction rate, an increase in the amount of H_2O_2 can inhibit the mode of action of HRP (*22*). To study this, the peroxidase-mediated cross-linking of apo α-lactalbumin at 100 mM ammonium acetate buffer pH 6.8 with

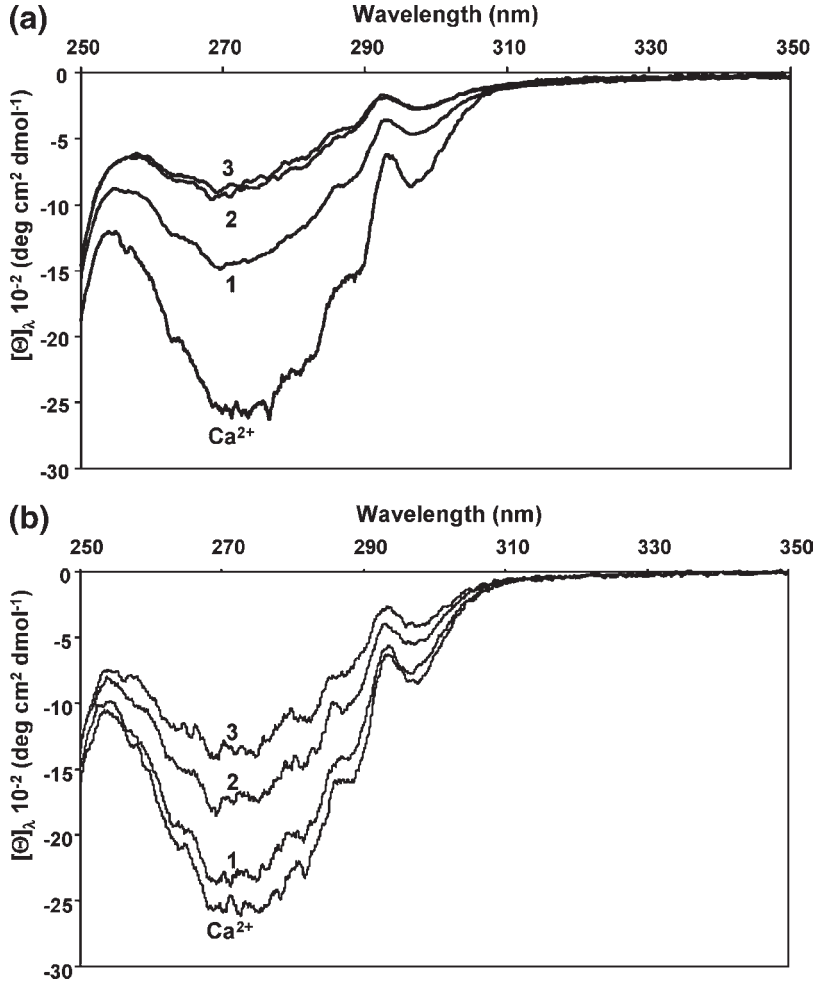

Figure 3. Near-UV CD spectra of apo α-lactalbumin in solutions of different ionic strength at 37 °C (**a**) and 20 °C (**b**). 1: 100 mM. 2: 10 mM. 3: 0.1 mM ammonium acetate buffer pH 6.8. Ca^{2+}: apo α-lactalbumin in 100 mM ammonium acetate buffer pH 6.8 with 20 mM $CaCl_2$.

Table 2. Quantitative Size Distribution of α-Lactalbumin Products Present in the Reaction Mixture at Different Ionic Strength and pH at 20 °C

	integration section (kDa)[a]			
incubation conditions	10−25	25−40	40−60	60−V_0
pH 6.8				
100 mM NH_4Ac	79	8 (19)[b]	5 (23)	8 (58)
10 mM NH_4Ac	72	14 (49)	8 (30)	7 (21)
0.1 mM NH_4Ac	70	17 (63)	8 (26)	5 (11)
pH 5.9				
100 mM NH_4Ac	72	12 (36)	6 (19)	10 (45)
10 mM NH_4Ac	56	20 (45)	20 (24)	13 (32)
0.1 mM NH_4Ac	52	14 (21)	13 (27)	21 (51)

[a] The relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. [b] Size distribution of oligomers.

different H_2O_2 concentrations was monitored by SEC. Since there are no changes in ionic strength and pH, the conversion of monomeric apo α-lactalbumin was taken as a measure of cross-linking. **Figure 5** shows the time dependence of reacted monomer with 0.33, 1, 3, and 6 mM H_2O_2 in 100 mM ammonium acetate pH 6.8. With 0.33 mM H_2O_2 the highest initial conversion rate is observed. The highest degree of conversion (50−60%) of monomer is observed at 1 and 3 mM H_2O_2. All the conditions result in the same size distribution of oligomers (**Table 3**). At higher (≥12 mM) H_2O_2 concentrations, a lower conversion was obtained. This is likely due to (ir)reversible enzyme inhibition by excess H_2O_2 (*22*) as was also observed with the ABTS assay (not shown).

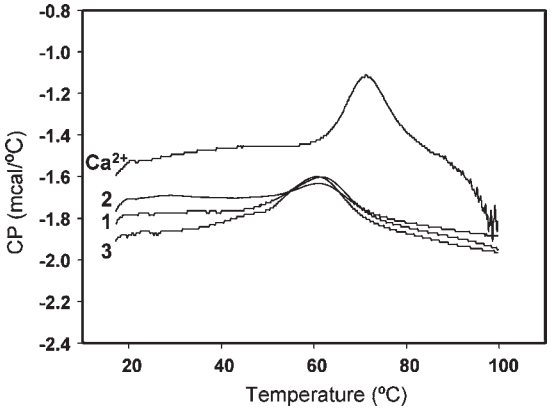

Figure 4. DSC-profiles of the temperature-dependent unfolding of α-lactalbumin at different ionic strength in the absence and presence of $CaCl_2$. Apo α-lactalbum in (1) 100 mM; (2) 10 mM and (3) 0.1 mM ammonium acetate buffer pH 6.8; Ca^{2+}: apo α-lactalbumin in 100 mM ammonium acetate buffer pH 6.8 with 20 mM $CaCl_2$.

When the amount of H_2O_2, equivalent to 6 mM, was added in aliquots of 2 μL (1 mM) at 30 min intervals, apo α-lactalbumin was almost completely (80%) converted into higher oligomers (**Figure 6** and **Table 3**). The high conversion of monomeric apo α-lactalbumin upon multiple additions of H_2O_2 is independent of ionic strength, and a total conversion of monomeric α-lactalbumin

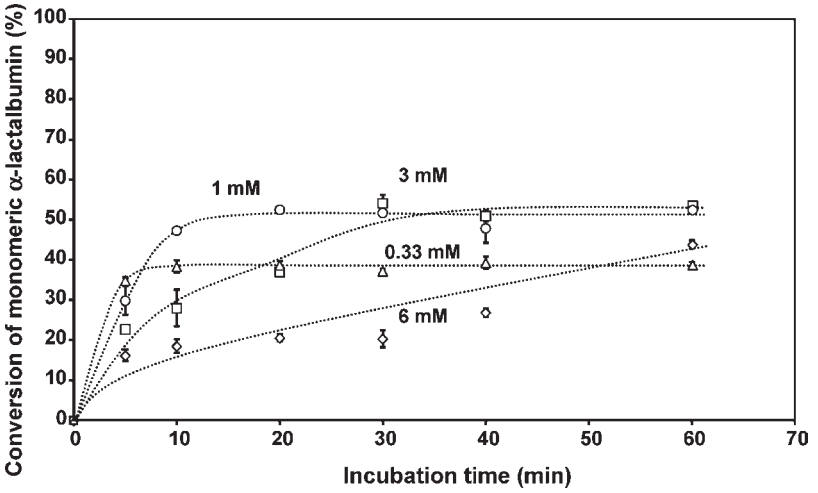

Figure 5. Time-dependent conversion of fraction monomeric α-lactalbumin upon treatment with peroxidase and various H_2O_2 concentrations in 100 mM ammonium acetate pH 6.8. Indicated is the molarity of H_2O_2 after addition.

Table 3. Quantitative Size Distribution of α-Lactalbumin Products Present in the Reaction Mixture at Different Ionic Strength and H_2O_2 Concentrations at 37 °C

		integration section (kDa)[b]			
buffer properties[a]	H_2O_2 concn	10−25	25−40	40−60	60−V_0
	Single Addition; *n* mM H_2O_2				
100 mM	0.33 mM	45	20 (31)[c]	18 (35)	18 (34)
	1 mM	33	17 (20)	20 (30)	30 (50)
	3 mM	29	16 (16)	20 (29)	35 (55)
	6 mM	35	18 (22)	20 (31)	27 (46)
	12 mM	66	16 (46)	10 (32)	8 (23)
	Multiple Additions; 1 mM H_2O_2				
100 mM	1×	38	14 (15)	16 (25)	32 (60)
	3×	23	7 (0)	9 (8)	61 (92)
	6×	19	4 (0)	4 (1)	73 (99)
10 mM	1×	35	22 (29)	22 (36)	22 (35)
	3×	18	10 (5)	18 (22)	53 (73)
	6×	15	6 (0)	11 (10)	68 (91)
0.1 mM	1×	34	26 (37)	23 (38)	16 (25)
	3×	18	15 (12)	25 (31)	42 (57)
	6×	15	9 (3)	17 (19)	60 (79)
100 mM + 20 mM EDTA	3×	10	7 (1)	11 (9)	72 (90)
	6×	6	6 (0)	9 (6)	79 (95)

[a] Concentration ammonium acetate. [b] Relative protein absorbance in each size range, given as percentage of the total absorbance at 280 nm, as measured by SEC. [c] Size distribution of oligomers.

of 80% is reached (**Table 3**). However, at low ionic strength smaller oligomers are formed, compared to high ionic strength, even after multiple additions of H_2O_2. When EDTA is added to scavenge the residual Ca^{2+} ions, conversion of monomers is increased to at least 90%, and no changes in size distribution are observed. The addition of H_2O_2 in aliquots shows the great potential of modulating the H_2O_2 concentration to increase monomer conversion.

Conclusions. Peroxidase-mediated cross-linking of apo α-lactalbumin yields a wide range of oligomer products. The size distribution of the cross-linked products can be directed by changing the ionic strength and pH. With the obtained knowledge it is possible to direct the peroxidase-mediated cross-linking of α-lactalbumin to form biomacromolecules with a different number of cross-links and altered properties.

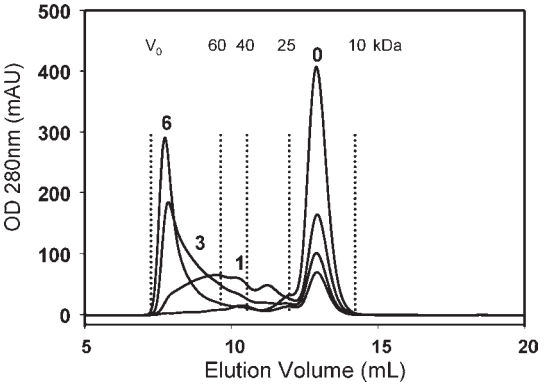

Figure 6. Peroxidase-mediated cross-linking of apo α-lactalbumin in 100 mM ammonium acetate pH 6.8 with multiple additions of 1 mM H_2O_2 (number of additions indicated) as measured by SEC.

ABBREVIATIONS USED

HRP, horseradish peroxidase; EDTA, ethylenediaminetetraacetic acid; H_2O_2, hydrogen peroxide; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; CD, circular dichroism; V_0, void volume; DSC, differential scanning calorimetry; β-LG, β-lactoglobulin; BSA, bovine serum albumin.

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