Cross-Linking of Whey Proteins by Enzymatic Oxidation

Merete Færgemand,* Jeanette Otte, and Karsten Bruun Qvist

Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30[5], DK-1958 Frederiksberg C, Denmark

The applicability of enzymatic oxidation for polymerization of whey proteins [in whey protein isolate (WPI)] has been investigated, using three different oxidoreductases with different specificities (microbial peroxidase, fungal laccase, and bovine plasma monoamine oxidase) to induce oxidation. All three enzymes were able to induce formation of oligomers and polymers of whey proteins under various conditions, but their modes of action seemed to diverge, as they affected the two main whey proteins β-lactoglobulin and α-lactalbumin differently: for example, peroxidase (in the presence of hydrogen peroxide) mainly acted on β-lactoglobulin, laccase (in the presence of chlorogenic acid) mainly worked on α-lactalbumin, and monoamine oxidase acted somewhat on both proteins. None of the oxidoreductases induced full polymerization of WPI, as opposed to cross-linking with microbial transglutaminase with a reductant present, which polymerizes the whey proteins fully. None of the oxidoreductases could induce gelling of WPI solutions with 10−20% protein.

Keywords: *Enzymatic; oxidation; whey proteins; cross-linking; polymerization*

INTRODUCTION

Increasing interest is directed toward modifying proteins to enhance functionality and thereby add value to the proteins. Protein functionality can be changed by chemical, physical, and enzymatic methods. The advantage of using enzymatic modification instead of chemical modification is the higher specificity of enzymatic reactions, thus minimizing the risk of formation of possible toxic side products. Enzymatic cross-linking has been investigated in some detail using the enzyme transglutaminase (EC 2.3.2.13). Transglutaminase catalyzes acyl transfer reactions using peptide-bound glutamine residues as donors and various primary amines, for example, peptide-bound lysine, as acyl acceptors (Folk and Finlayson, 1977). The enzyme is thus able to form intra- or intermolecular ϵ-(γ-glutamyl)-lysine cross-links. Many food proteins are good substrates for transglutaminase-catalyzed cross-linking, and among the milk proteins the caseins are expecially excellent substrates for transglutaminase (Ikura et al., 1980; Sakamoto et al., 1994; Traoré and Meunier, 1991, 1992). It is possible to polymerize the other main part of the milk proteins, the whey proteins quite extensively using transglutaminase (Færgemand et al., 1997), but only when the whey proteins are partly unfolded. It is therefore of interest to investigate other possibilities for cross-linking of native whey proteins.

Matheis and Whitaker (1987) have reviewed various enzymatic reactions that may possibly induce cross-linking in food proteins and proposed that besides the transglutaminase-catalyzed reaction, especially oxidative cross-linking, using various oxidoreductases, should be considered. Several studies have demonstrated that oxidation of tyrosine as such or tyrosine residues in proteins with peroxidase and hydrogen peroxide results in an oxidation of tyrosine (residues), forming dityrosines or proteins cross-linked inter- or intramolecularly via dityrosine bonds (Gross and Sizer, 1959; Aeschbach et al., 1976). Furthermore, dityrosine cross-links have been isolated from some structural proteins, for example, resilin (Andersen, 1964), and the insolubility and elastic properties of these proteins have been ascribed to the presence of dityrosine cross-links.

Stahmann et al. (1977) showed that horseradish peroxidase could induce cross-linking of some proteins in the presence of hydrogen peroxide and a low molecular weight hydrogen donor. In their study the cross-linking of, for example, ovalbumin, β-lactoglobulin, and bovine serum albumin, yielding various oligomers and polymers, was detected using SDS gel electrophoresis and gel exclusion chromatography. Also, Matheis and Whitaker (1984a) cross-linked several proteins, for example, casein, lysozyme, and soybean protein, using peroxidase/hydrogen peroxide and detected oligomers and polymers using SDS−PAGE. Hurrell et al. (1982) was able to cross-link casein with polyphenol oxidase (EC 1.11.18.1) in the presence of a low molecular weight phenolic compound (caffeic acid).

Although it is established that oxidation can result in the formation of covalent cross-links in several food proteins, the effect on the functional properties has not been studied in any detail. Most work in this area has concentrated on improving wheat doughs by adding peroxidase (Kieffer et al., 1981) or polyphenol oxidase (Kuninori et al., 1976, 1978). As wheat dough contains proteins as well as other compounds containing phenol groups (for example, ferulic acid), many possible oxidative reactions exist, and the reactions resulting in the dough-improving effects of peroxidase or polyphenol oxidase are not fully understood. The film-forming properties of soy protein isolate (SPI) treated with horseradish peroxidase were investigated by Stuchell and Krochta (1994). This study showed that SPI treated with peroxidase formed films that were stiffer but more brittle than those formed without the enzyme treat-

* Author to whom correspondence should be addressed (telephone + 45 35 28 31 91; fax + 45 35 28 31 90; e-mail mef@kvl.dk).

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ment, whereas the treatment had no effect on water vapor permeability. The effects found were ascribed to polymerization of SPI.

The aim of the present study is to investigate if polymerization of whey proteins in whey protein isolate (WPI) can be induced by enzymatic oxidation with three different oxidoreductases: a peroxidase, a laccase, and a monoamine oxidase. Peroxidase has a wide specificity toward electron donors in the oxidation process but specifically uses hydrogen peroxide as electron acceptor, when phenolic compounds or amines are the hydrogen donors (Matheis and Whitaker, 1984b). In proteins, peroxidase mainly oxidizes the amino acids tyrosine, cysteine, and tryptophan (Sizer, 1953). Laccase and monoamine oxidase use oxygen as electron acceptor. Laccase primarily oxidizes diphenols as electron donors (Yaropolov et al., 1994), and monoamine oxidase oxidizes primary amines to yield aldehydes (Tipton, 1971), although the enzyme may also oxidize some secondary and tertiary amines (Yasunobu and Gomes, 1971). Thus, both of these enzymes have a narrower specificity regarding the electron donor than peroxidase. In addition to the study of the extent of polymerization, the effect of the enzymatic treatment on the viscosity of protein solutions was investigated, and the effect of the enzymatic oxidation was compared to the effect of cross-linking with transglutaminase.

MATERIALS AND METHODS

WPI (Bipro) containing 90% protein was from BioIsolates (Deeside, U.K.). A microbial peroxidase (EC 1.11.1.7, originally purified from *Coprinus cinereus*) and a laccase of fungal origin (EC 1.10.3.2, originally purified from *Polyporus pinsitus*) were supplied by Novo Nordisk A/S (Bagsværd, Denmark). The peroxidase preparation had a specific activity of 5000 PODU/mL, 1 PODU (peroxidase unit) catalyzing the conversion of 1 μmol of hydrogen peroxide/min at pH 7.0 and 30 °C and oxidizing 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS). The peroxidase (lactoperoxidase) content of WPI, as determined by a chromogenic assay performed according to the method of Schindler and Bardsley (1975) using ABTS as chromogen, was estimated to be 0.08 PODU/g, equal to 0.008 PODU/mL of 10% WPI solution. This concentration is approximately 5000 times lower than the lowest concentration of microbial peroxidase applied in the experiments, and we consider the presence of lactoperoxidase in this concentration insignificant for the results obtained. The specific activity of the laccase preparation was 100 LACU/mg, 1 LACU (laccase unit) converting 1 μM of syringaldazin to tetramethoxyazobis-(methylenequinone)/min at pH 5.5 and 30 °C under aerobic conditions in aqueous solution. Monoamine oxidase from bovine plasma (EC 1.4.3.4) was from Sigma Chemical Co. (St. Louis, MO) and had a specific activity of 2500 units/mg, 1 unit of monoamine oxidase oxidizing 1.0 μmol of benzylamine to benzaldehyde per minute at pH 7.4 at 25 °C under aerobic conditions in aqueous solution. A microbial transglutaminase from *Streptoverticillium mobaraense* was as described previously (Færgemand et al., 1997). All other chemicals were of AnalR grade from Sigma.

Preparation of Enzymatically Oxidized Protein Samples. Peroxidase-treated samples were made from WPI dissolved in 75 mM Tris-HCl buffers of pH 6.5, 7.0, or 8.0. The solutions were made up on protein basis, and the protein concentration is given in each case. Hydrogen peroxide in various known concentrations was added. Microbial peroxidase in various concentrations was then added, and the samples were incubated at 40 °C; aliquots were withdrawn at different reaction times during incubation. The withdrawn samples were diluted 10 times with 0.07% trifluoroacetic acid (TFA) to inhibit further enzymatic action. TFA-treated samples were frozen at −18 °C until analyzed. Before analysis, TFA-treated samples were dried by vacuum centrifugation using a Hetovac VR-1 (Heto Lab Equibment, Allerød, Denmark) and redissolved in run buffer. Laccase-treated samples were prepared with WPI dissolved in Tris-HCl buffers of pH 6.0 or 7.0. Chlorogenic acid in various known concentrations was added to some of the samples, the fungal laccase was then added, and the samples were incubated at 40 °C. Aliquots withdrawn at different reaction times were diluted with run buffer and frozen at −18 °C until analysis to stop further enzymatic action by low temperature. Prior experiments indicated that this procedure was sufficient to inhibit the enzyme during further analysis. Monoamine oxidase-treated samples were prepared with WPI dissolved in Tris-HCl buffers of pH 7.5 (the enzyme's optimum pH according to the manufacturer). Bovine plasma monoamine oxidase was then added, the sample was incubated at 40 °C, and aliquots were withdrawn at different reaction times. The aliquots were diluted with run buffer and either analyzed immediately or frozen at −18 °C until analysis to stop further enzymatic action.

Size Exclusion (SE) HPLC/FPLC. SE chromatography was performed using either an FPLC or HPLC system. SE-HPLC was performed at room temperature (21−22 °C) as described by Otte et al. (1996). A Waters HPLC system was used with a TSK Gel G-2000SWXL column (0.78 × 30 cm) and a SWXPRIOM guard column (0.6 × 4 cm), both from TosoHaas (Tokyo, Japan). Before analysis, the samples dissolved in run buffer were centrifuged (2 min; 12.000*g*), and 20 μL of supernatant was injected onto the column. The components were eluted with a nonreducing buffer consisting of 0.1 M disodium hydrogen phosphate and 0.3 M sodium chloride (pH 7.0), at 0.8 mL/min, and detected at 220 and 280 nm. The whey proteins were identified by analysis of standards. The protein standards were α-lactalbumin (14 200 Da; L6010), β-lactoglobulin (36 400 Da; L0190), bovine serum albumin (66 000 Da; A8531), β-amylase (200 000 Da; A8781), bovine thyroglobulin (669 000 Da; T9145), and blue dextran (2 000 000 Da; D4772), all from Sigma. The SE limit of the column was ≈300 kDa. For SE-FPLC a Pharmacia (Pharmacia, Uppsala, Sweden) FPLC system was used with a Superose 6 column (1.0 × 30 cm) (Pharmacia). Before analysis, the dissolved samples were centrifuged (2 min; 12.000*g*) and 100 μL of supernatant was injected onto the column. The components were eluted with a nonreducing buffer consisting of 0.05 M potassium phosphate and 0.15 M sodium chloride (pH 7.0), at 0.4 mL/min, and detected at 280 nm. The whey proteins were identified by analysis of standards. The SE limit of the column was ≈600 kDa.

Gel Electrophoresis. SDS−PAGE was performed as described by Færgemand et al. (1997). The samples were dried by vacuum centrifugation using a Hetovac VR-1 (Heto Lab Equibment) and redissolved in 0.010 M Tris-HCl buffer (pH 8.0) containing 2.0% SDS, 0.5% DTE, 1 mM EDTA, and 0.01% bromphenol blue. The samples were heated in boiling water for 5 min in the SDS buffer, and separation of samples was performed on a Phastgel homogeneous 12.5% gel using Phast-system (Pharmacia, Allerød, Denmark) using the standard separation and staining methods given by the supplier.

Viscometry. The viscosity of 5% WPI solutions (on protein basis) was measured at 25 °C using a Viscotek Y-501 IV capillary viscometer from Viscotek (Basingstoke, U.K.) operating at a typical shear rate of 3000 s^{-1}. The apparent viscosity (η) was determined as

$$\eta = (\eta_{\text{solution/water}}/\eta_{\text{water}})\eta_{\text{water,25°C}}$$

where $\eta_{\text{solution/water}}/\eta_{\text{water}}$ was directly measured as the pressure drop of the solution relative to water.

RESULTS AND DISCUSSION

Microbial peroxidase was assayed for its ability to polymerize WPI using SE-FPLC to detect reaction products. The effect of the hydrogen peroxide concen-

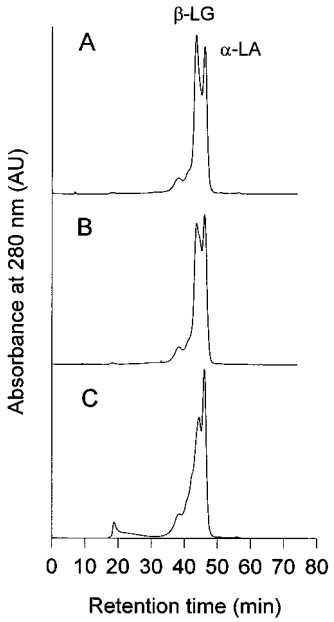

Figure 1. SE-FPLC of 10% WPI solution at pH 7.0 and 40 °C with 50 mM hydrogen peroxide: (A) after 0 min with 0.002% microbial peroxidasee; (B) after 24 h without microbial peroxidase; (C) after 24 h with 0.002% microbial peroxidase. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

tration at pH values from 6.5 to 8.0 was investigated. At peroxide concentrations below 50 mM no polymerization of the whey proteins was detected at peroxidase concentrations of 0.002 or 0.2% [E/S% = (weight of enzyme/weight of substrate protein) × 100) (results not shown).

Figure 1 is the SE-FPLC chromatogram, which shows the formation of polymerized reaction products at pH 7.0 with 50 mM hydrogen peroxide. Without peroxidase, only a small decrease in the area of the β-lactoglobulin peak as well as a little oligomerization of β-lactoglobulin was observed after 24 h at 40 °C (Figure 1B) compared to the sample taken at the beginning of the experiment (Figure 1A). Probably without peroxidase present a little nonenzymatic oxidation by hydrogen peroxide occurs at this peroxide level, resulting in the formation of β-lactoglobulin dimers/trimers, but clearly no polymers. With microbial peroxidase present (Figure 1C), the area of the β-lactoglobulin peak was reduced more significantly and a wide distribution of oligomers and polymers was seen. It is interesting that apparently only β-lactoglobulin was affected by the enzymatic oxidation, the peak area of α-lactalbumin remaining roughly constant during the experiment.

The formation of more polymers in the presence of microbial peroxidase compared to just hydrogen peroxide was verified by SDS−PAGE under reducing conditions. Figure 2 shows how more high molecular weight polymers were formed with microbial peroxidase present (lanes 1 and 2) compared to when only hydrogen peroxide was present (lane 3) and confirms the presence of covalent bonds. It is also clear that more native β-lactoglobulin had disappeared after 24 h of reaction compared to 1 h of reaction. Furthermore, it can be noticed that some oligomers and polymers apparently became so large that they disappeared from the stacking gel after 24 h.

At pH 6.5, which is the optimum pH of the microbial peroxidase (according to the manufacturer), the extent

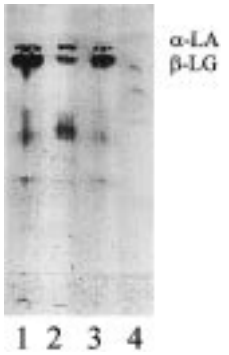

Figure 2. SDS−PAGE gel of a 10% WPI solution at pH 7.0 and 40 °C with 50 mM hydrogen peroxide: (lane 1) after 1 h with 0.002% microbial peroxidase; (lane 2) after 24 h with 0.002% microbial peroxidase; (lane 3) after 24 h without microbial peroxidase; (lane 4) standards [from the top, α-lactalbumin (14 400 Da), soybean trypsin inhibitor (20 100 Da), carbonic anhydrase (30 000 Da), ovalbumin (43 000 Da), bovine serum albumin (67 000 Da), phosphorylase *b* (94 000 Da)]. The bands of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

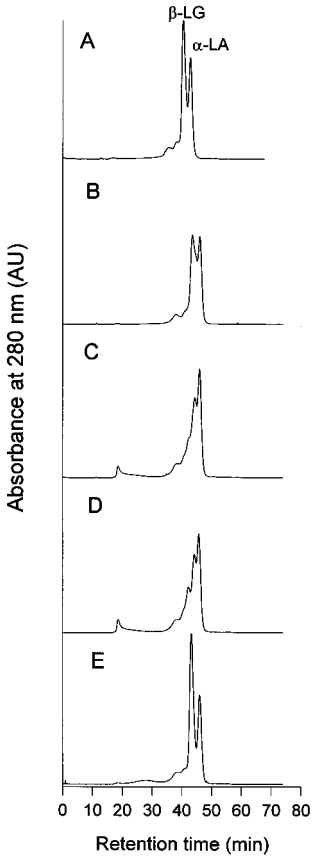

Figure 3. SE-FPLC of 10% WPI solution at pH 8.0 and 40 °C: (A) after 0 min without any additions; (B) after 0 min with 50 mM hydrogen peroxide and 0.002% microbial peroxidase; (C) after 24 h with 50 mM hydrogen peroxide (without peroxidase); (D) after 24 h with 50 mM hydrogen peroxide and 0.002% microbial peroxidase; (E) after 24 h without hydrogen peroxide/peroxidase. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

of the reaction was about the same as at pH 7.0 (results not shown). However, at pH 8.0 the pattern was somewhat different, as shown in Figure 3. Even without peroxidase present, β-lactoglobulin was polymerized to some extent in the presence of 50 mM hydrogen peroxide (Figure 3C), and adding microbial peroxidase to the system increased the amount of

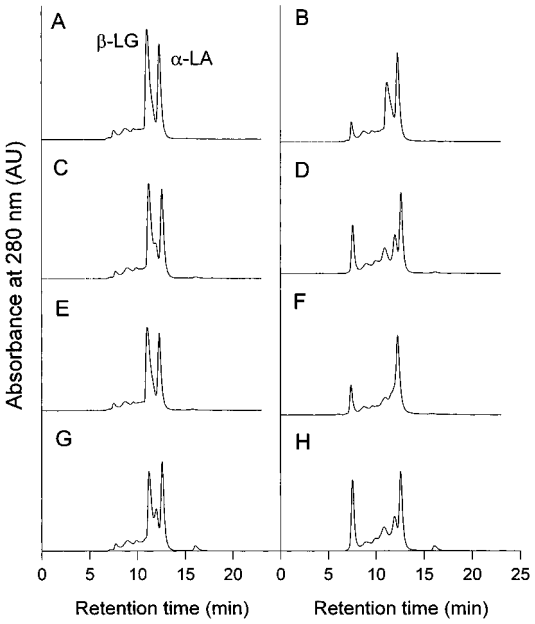

Figure 4. SE-HPLC of 10% WPI solution at pH 7.0 and 40 °C: (A) after 0 min with 100 mM hydrogen peroxide; (B) after 24 h with 100 mM hydrogen peroxide; (C) after 0 min with 100 mM hydrogen peroxide and 0.002% microbial peroxidase; (D) after 24 h with 100 mM hydrogen peroxide and 0.002% microbial peroxidase; (E) after 0 min with 500 mM hydrogen peroxide; (F) after 24 h with 500 mM hydrogen peroxide; (G) after 0 min with 500 mM hydrogen peroxide and 0.002% microbial peroxidase; (H) after 24 h with 500 mM hydrogen peroxide and 0.002% microbial peroxidase. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

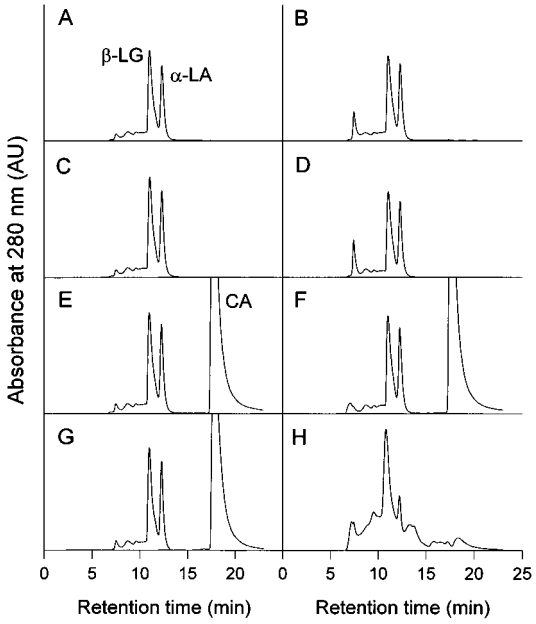

Figure 5. SE-HPLC of 10% WPI solution at pH 7.0 and 40 °C: (A) after 0 min; (B) after 24 h; (C) after 0 min with 0.004% laccase; (D) after 24 h with 0.004% laccase; (E) after 0 min with 25 mM chlorogenic acid; (F) after 24 h with 25 mM chlorogenic acid; (G) after 0 min with 0.004% laccase and 25 mM chlorogenic acid; (H) after 24 h with 0.004% laccase and 25 mM chlorogenic acid. The peaks of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and chlorogenic acid (CA) are indicated.

polymers only slightly (Figure 3D). In the absence of hydrogen peroxide/peroxidase (Figure 3E), only a small concentration of aggregates was detected after 24 h at pH 8.0. The polymerization with hydrogen peroxide was therefore clearly due to the oxidation of β-lactoglobulin. This oxidation is apparently mainly enzymatic at pH 7.0, close to the optimum pH of the microbial peroxidase, and mainly nonenzymatic at pH 8.0. It seems that at pH 8.0 β-lactoglobulin is more susceptible to oxidation with hydrogen peroxide, considering that 50 mM hydrogen peroxide induces oxidative polymerization. This may be due to the reversible changes in β-lactoglobulin at pH 7.5, known as the Tanford transition, leaving the molecule more unfolded at alkaline pH values (Tanford et al., 1959). This unfolding may expose more residues sensitive to oxidation (for example, tyrosine residues). In addition, at alkaline pH values the frequency of disulfide interchanges is increased (Tanford, 1968), and this may expose more sulfhydryl groups to oxidation, again facilitating polymerization. This may also explain why apparently already after 0 min with 50 mM peroxide and 0.002% microbial peroxidase (Figure 3B) β-lactoglobulin has been modified (indicated by the fact that the β-lactoglobulin peak is changed compared to that at pH 7.0), as the oxidation reaction is apparently much faster at pH 8.0, probably because β-lactoglobulin is more unfolded.

Even at pH 7.0 hydrogen peroxide alone in concentrations ≥100 mM was able to induce oxidative polymerization, as shown in Figure 4. The effect of hydrogen peroxide on β-lactoglobulin increased with increasing concentration of hydrogen peroxide, whereas α-lactalbumin was not affected by enzymatic or nonenzymatic oxidation even at 500 mM hydrogen peroxide (Figure 4F,H). Microbial peroxidase is active at pH 7.0, as opposed to its inactivity at pH 8.0, and it is evident that the enzyme induced further polymerization of β-lactoglobulin at both levels of hydrogen peroxide at pH 7.0, as shown by the presence of a large peak at the void volume of the column. In the enzyme-treated samples, a new peak appears with a retention time between those of β-lactoglobulin and α-lactalbumin (Figure 4D,H). This peak is not the microbial peroxidase, which is eluted earlier (as observed by running a sample of the enzyme alone in a high concentration; results not shown). The observed peak could be due to glycosylated α-lactalbumin (D. Chatterton, personal communication), which becomes visible when part of the coeluting β-lactoglobulin peak disappears due to the polymerization process.

It is clear from these experiments that hydrogen peroxide treatment alone may be quite effective in oxidizing β-lactoglobulin, though more polymers are formed when peroxidase is present as well. Increasing the concentration of microbial peroxidase (for example, 100 times) at low hydrogen peroxide levels (for example, 50 mM) did not increase the formation of polymers or result in an effect similar to that of increasing the concentration of hydrogen peroxide, so evidently a quite high concentration of hydrogen peroxide is needed to polymerize WPI.

Laccase uses oxygen for the oxidation, and therefore no other oxidant such as hydrogen peroxide is needed for the oxidation catalyzed by this enzyme. This makes the system simpler and probably more useful in foods. The effect of laccase acting on WPI alone or in the presence of a low molecular weight phenolic substance (chlorogenic acid) is presented in Figure 5.

With laccase alone (Figure 5D) no polymerization was detected apart from the aggregation taking place in the sample without enzyme as well (Figure 5B). This is not surprising considering that laccase is more specific than, for example, peroxidase toward electron donors and

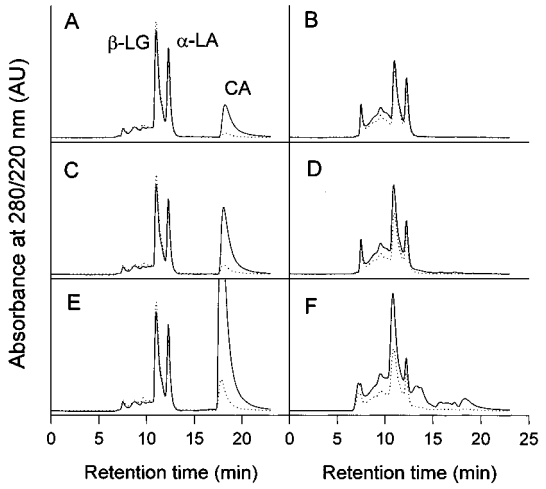

Figure 6. SE-HPLC of 10% WPI solution at pH 7.0 and 40 °C with 0.004% laccase and chlorogenic acid: (A) after 0 min with 5 mM chlorogenic acid; (B) after 24 h with 5 mM chlorogenic acid; (C) after 0 min with 10 mM chlorogenic acid; (D) after 24 h with 10 mM chlorogenic acid; (E) after 0 min with 25 mM chlorogenic acid; (F) after 24 h with 25 mM chlorogenic acid. Solid lines identify absorbance at 280 nm, whereas broken lines represent absorbance at 220 nm, which was reduced 10 times to fit in the figure. The peaks of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and chlorogenic acid (CA) are indicated.

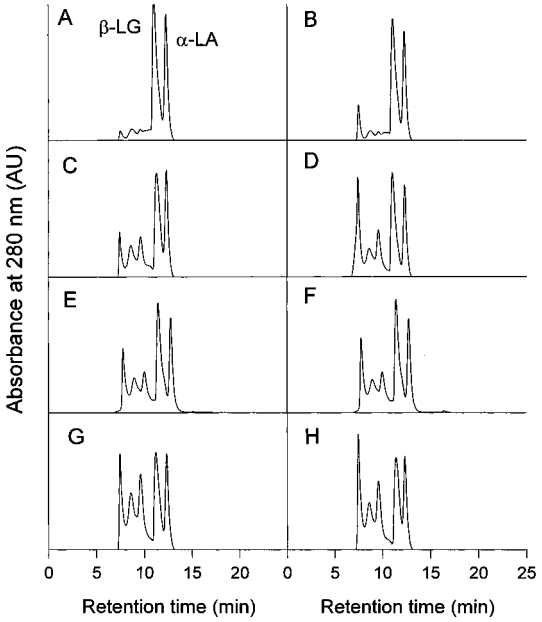

Figure 7. SE-HPLC of 10% WPI solution at pH 7.5 and 40 °C: (A) after 0 min without enzyme; (B) after 24 h without enzyme; (C) after 0 min with 0.02% monoamine oxidase; (D) after 24 h with 0.02% monoamine oxidase; (E) after 0 min with 0.04% monoamine oxidase; (F) after 24 h with 0.04% monoamine oxidase; (G) after 0 min with 0.08% monoamine oxidase; (H) after 24 h with 0.08% monoamine oxidase. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

primarily works on diphenols, which are not present among the amino acid residues. A way of initiating the formation of primary oxidation products (quinones), which can then react further with various amino acid residues, is to add a small amount of a low molecular weight diphenol for laccase to work on. In this work chlorogenic acid was used for that purpose. It is evident from the chromatograms that chlorogenic acid itself did not cause any polymerization of the proteins (Figure 5E,F). With laccase present as well, the peak area of α-lactalbumin especially decreased significantly with time and higher molecular weight products were formed (Figure 5H). These were probably α-lactalbumin oligomers as well as products of α-lactalbumin with chlorogenic acid (oligomers). Also, various oligomers of chlorogenic acid were formed, as indicated by peaks with retention times between those of α-lactalbumin and chlorogenic acid itself. The amount of high molecular weight polymers, as estimated by the area of the peak in the void volume, was not very large compared to what was formed in the absence of chlorogenic acid/laccase. It is interesting to notice that laccase (with chlorogenic acid present) probably worked mainly on α-lactalbumin, and the apparent slight increase in the area of the β-lactoglobulin peak may be due to α-lactalbumin and/or chlorogenic acid products of approximately the same molecular mass as β-lactoglobulin. The preferential polymerization of α-lactalbumin with laccase (in the presence of chlorogenic acid) is opposed to the action of peroxidase, which worked only on β-lactoglobulin, indicating that the two oxidoreductases proceed through different mechanisms in the oxidative polymerization of WPI.

As presented in Figure 6 the concentration of chlorogenic acid was found to influence the final molecular weight distribution of the reaction products. At high concentrations of chlorogenic acid the amount of high molecular weight protein oligomers and polymers formed did not increase (for example, Figure 6F). At these high concentrations, chlorogenic acid was not completely used during 24 h and a broad distribution of chlorogenic acid oligomers and polymers was formed rather than increased polymerization of WPI. This was very clearly the case with 25 mM chlorogenic acid (Figure 6F), for which a comparison of the absorbance at 220 nm with that of 280 nm indicated that a distribution of chlorogenic acid polymers was situated "on top" of the peaks of β-lactoglobulin and α-lactalbumin, explaining why these peaks (at 280 nm) seemed larger in the samples after 24 h than in the sample taken just after enzyme addition. Also, the comparison of the absorbance at 220 nm with that of 280 nm shows that the low molecular mass products formed are not peptides but just aromatic compounds (chlorogenic acid oligomers and polymers), giving rise to high absorbencies at 280 nm. At none of the concentrations of chlorogenic acid used in the presented work, or even with 10 times as much enzyme, was laccase able to induce a very high degree of polymerization of WPI.

The last enzyme assayed for its ability to polymerize WPI was monoamine oxidase. This enzyme is more specific than peroxidase, and as it acts mainly on primary amines, it should be able to act on the proteins in WPI without a low molecular weight phenolic substance present, as opposed to laccase.

Figure 7 shows the polymerization of WPI in the presence of monoamine oxidase. Monoamine oxidase oxidizes primary amines to yield aldehydes—which in turn may condense with other aldehydes (formed by oxidation of primary amines) or attack primary amines, thus forming cross-links. With all of the applied monoamine oxidase dosages, β-lactoglobulin and α-lactalbumin were both oxidized quite rapidly. Already in the sample taken immediately after enzyme addition (Figure 7C) a distribution of oligomeric and polymeric products was present, and the amount of polymers increased only slightly with time. In the sample without

Table 1. Apparent Viscosities (η) of 5% WPI Solutions Treated with Oxidoreductases

enzyme	buffer composition	enzyme dosage (%)	η at 0 min (mPa·s)	η at 24 h (mPa·s)
peroxidase	75 mM Tris-HCl (pH 7.0), 100 mM H_2O_2	0.002	1.17	1.43
none added	75 mM Tris-HCl (pH 7.0), 100 mM H_2O_2		1.18	1.34
laccase	75 mM Tris-HCl (pH 7.0), 5 mM chlorogenic acid	0.0036	1.11	1.14
none added	75 mM Tris-HCl (pH 7.0), 5 mM chlorogenic acid		1.11	1.10
monoamine oxidase	75 mM Tris-HCl (pH 7.5)	0.004	1.05	1.12
none added	75 mM Tris-HCl (pH 7.5)		1.07	1.10

enzyme (Figure 7B) only a small amount of aggregates was found after 24 h. Although the reaction is apparently very fast for all of the used dosages of monoamine oxidase, it is clear that more oligomers and polymers were formed in the sample after 0 min with high enzyme concentrations (for example, Figure 7G) than with low concentrations (Figure 7C), indicating a faster reaction with more enzyme as expected.

Evidently the enzymatic oxidation of WPI with monoamine oxidase is much faster than with either peroxidase/hydrogen peroxide or laccase/chlorogenic acid. A possible explanation for the apparently fast initial reaction and very slow subsequent reaction could be that only a few primary amines are readily available/suitable for the enzyme and that these possible reaction sites are used very quickly. Another explanation might be that the enzyme could be highly unstable and only able to survive for a short time in the given environment, as supported by the slightly larger amount of polymers at higher enzyme dosages. Analysis of a sample of enzyme alone in the highest concentration used in the experiments (results not shown) showed no large aggregates present in the enzyme preparation alone, so the oligomers and polymers must be due to the action of monoamine oxidase on WPI. The presence of polymerized protein already in the samples taken after 0 min of reaction may also indicate that the enzyme was incompletely inactivated and continued to slowly oxidize β-lactoglobulin and α-lactalbumin during HPLC analysis.

The effect of the three oxidoreductases on the viscosity and gelling ability of WPI was investigated. Table 1 summarizes the apparent viscosities of 5% WPI solutions. It is clear that among the investigated oxidoreductases only peroxidase with hydrogen peroxide increased the apparent viscosity slightly. The main part of this increase in apparent viscosity was due to the action of hydrogen peroxide, as the viscosity of the sample without peroxidase (but with hydrogen peroxide) increased almost as much as for the enzyme-treated sample. The peroxidase/hydrogen peroxide system mainly acted on β-lactoglobulin, which was not affected so much by either laccase or monoamine oxidase. This may explain why this system was the only one to show increased viscosity after oxidation, since β-lactoglobulin is, for example, also considered mainly responsible for heat-induced gelation of WPI. Higher WPI concentrations were applied to investigate if the polymerizing effect of any of the oxidoreductases could induce gelling of WPI. No gelling was observed (visually by turning test tubes upside down) with any of the oxidoreductases at (WPI) protein concentrations up to 20% at 40 °C. In contrast, transglutaminase has been shown to induce gelation by cross-linking of, for example, 10% WPI or just 8% β-lactoglobulin solutions in the presence of dithiothreitol, which unfolds the whey proteins (Færgemand et al., 1997).

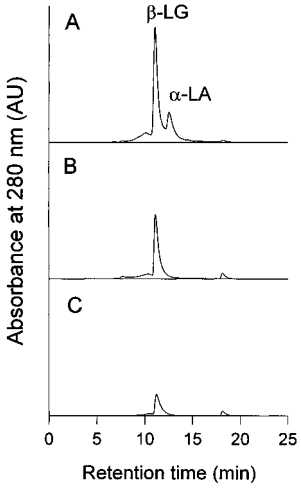

Figure 8. SE-HPLC of 10% WPI solution at pH 7.5 and 40 °C with 10 units of transglutaminase/g of protein and 20 mM dithiothreitol: (A) after 0 min; (B) after 60 min; (C) after 2 h. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

If we compare the SE-HPLC chromatogram of WPI cross-linked with transglutaminase (as shown in Figure 8) with the SE-HPLC profiles of the various oxidized WPI samples, we find some indication of why transglutaminase-catalyzed cross-linking promotes gelling, whereas cross-linking of WPI by enzymatic oxidation does not.

It is evident from Figure 8 that the cross-linking of WPI with transglutaminase is quite fast; after 2 h, 77% of β-lactoglobulin was polymerized, as also reported previously (Færgemand et al., 1997). The chromatogram after 2 h (Figure 8C) indicates that the polymers formed by transglutaminase acting on WPI were quite large, as they were not even able to enter the column. Even when using the Superose 6 column of the SE-FPLC system (results not shown) the polymers did not enter the gel, indicating sizes >600 kDa. In contrast to this, most of the oligomers and polymers formed by the three oxidoreductases were present on the HPLC chromatograms.

Transglutaminase only produces gels with WPI when the whey proteins are partly unfolded, for example, chemically by the action of dithiothreitol. Therefore, to compare the effect of the oxidoreductases with the effect of transglutaminase, the oxidoreductases were incubated with WPI in the presence and absence of dithiothreitol. Table 2 shows results from which it may be concluded that all of the enzymes were able to decrease the peak areas of β-lactoglobulin more in the presence of dithiothreitol. In all cases, except with hydrogen peroxide present, the peak of α-lactalbumin disappeared after incubation with dithiothreitol (with or without enzymes). So apparently hydrogen peroxide protects α-lactalbumin from aggregation in the presence of

Table 2. Decrease (Percent) in Peak Areas of β-Lactoglobulin and α-Lactalbumin in SE-HPLC Chromatograms of 10% WPI Incubated at pH 7.5 and 40 °C

treatment	incubation time (h)	β-lactoglobulin		α-lactalbumin	
		no DTT[a]	20 mM DTT	no DTT	20 mM DTT
none	24	5	10	3	100
peroxidase + 100 mM H_2O_2	24	55	77	0	0
100 mM H_2O_2	24	13	79	0	0
laccase	24	10	31	4	100
laccase + 5 mM chlorogenic acid	24	11	27	22	100
monoamine oxidase	24	45	77	38	100
transglutaminase	2	0	77	29	100

[a] DTT, dithiothreitol.

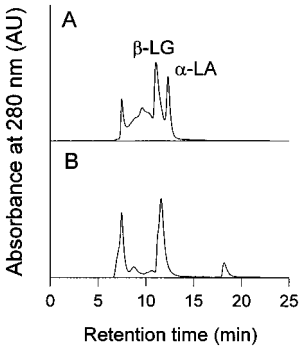

Figure 9. SE-HPLC of 10% WPI solution after 24 h at pH 7.0 and 40 °C with 0.004% laccase and 5 mM chlorogenic acid: (A) no dithiothreitol; (B) 20 mM dithiothreitol. The peaks of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are indicated.

dithiothreitol, probably by preventing the reduction with dithiothreitol. Even though some of the oxidoreductases apparently polymerized at least reduced β-lactoglobulin (and perhaps reduced α-lactalbumin as well, although this is impossible to estimate as the α-lactalbumin peak disappeared in the presence of dithiothreitol) to quite a large extent (judged from the decrease in the peak areas), none of the 10% WPI samples gelled, as opposed to the transglutaminase-treated sample, which gelled in 120 min, as described previously (Færgemand et al., 1997). The larger decrease in the peak area of β-lactoglobulin in the presence of dithiothreitol may be due to better substrate availability upon unfolding, but it may possibly also be due to interactions between the sulfhydryl groups (available after reduction of disulfide bonds) and oxidized tyrosine residues, a type of reaction that has also been suggested by Matheis and Whitaker (1984b).

Figure 9 shows the polymerization of WPI with laccase (in the presence of chlorogenic acid) with dithiothreitol present. It is evident that although apparently the α-lactalbumin peak disappeared totally in the presence of laccase, chlorogenic acid, and dithiothreitol, the polymers formed after 24 h (Figure 9B) were still visible in the chromatogram (in the void volume of the column). It was not possible to determine if α-lactalbumin was actually polymerized by laccase under these conditions, as the presence of dithiothreitol alone resulted in the disappearance of the α-lactalbumin peak (data nor shown). However, it is evident from Figure 9 that apparently enzymatic oxidative polymerization of partly unfolded whey proteins did not produce as polymers as large as transglutaminase-catalyzed polymerization did.

The study shows that oxidation of whey proteins with various oxidoreductases can induce the formation of protein oligomers and polymers. The oxidoreductases that were used differed widely in their specificities and their effects on the individual whey proteins: microbial peroxidase acted primarily on β-lactoglobulin, laccase worked well on α-lactalbumin, and monoamine oxidase acted on both main whey proteins. No gelation of WPI was observed, although increases in viscosities were found in some cases after polymerization. The lack of gelation was ascribed to the rather low extent of formation of high molecular weight polymers as compared to the result when using transglutaminase for enzymatic cross-linking and gelation.

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