Aging Properties of Films of Plasticized Vital Wheat Gluten Cast from Acidic and Basic Solutions

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In order to understand the mechanisms behind the undesired aging of films based on vital wheat gluten plasticized with glycerol, films cast from water/ethanol solutions were investigated. The effect of pH was studied by casting from solutions at pH 4 and pH 11. The films were aged for 120 days at 50% relative humidity and 23 °C, and the tensile properties and oxygen and water vapor permeabilities were measured as a function of aging time. The changes in the protein structure were determined by infrared spectroscopy and size-exclusion and reverse-phase high-performance liquid chromatography, and the film structure was revealed by optical and scanning electron microscopy. The pH 11 film was mechanically more stable with time than the pH 4 film, the latter being initially very ductile but turning brittle toward the end of the aging period. The protein solubility and infrared spectroscopy measurements indicated that the protein structure of the pH 4 film was initially significantly less polymerized/aggregated than that of the pH 11 film. The polymerization of the pH 4 film increased during storage but it did not reach the degree of aggregation of the pH 11 film. Reverse-phase chromatography indicated that the pH 11 films were to some extent deamidated and that this increased with aging. At the same time a large fraction of the aged pH 11 film was unaffected by reducing agents, suggesting that a time-induced isopeptide cross-linking had occurred. This isopeptide formation did not, however, change the overall degree of aggregation and consequently the mechanical properties of the film. During aging, the pH 4 films lost more mass than the pH 11 films mainly due to migration of glycerol but also due to some loss of volatile mass. Scanning electron and optical microscopy showed that the pH 11 film was more uniform in thickness and that the film structure was more homogeneous than that of the pH 4 film. The oxygen permeability was also lower for the pH 11 film. The fact that the pH 4 film experienced a larger and more rapid change in its mechanical properties with time than the pH 11 film, as a consequence of a greater loss of plasticizer, was presumably due to its initial lower degree of protein aggregation/polymerization. Consequently, the cross-link density achieved at pH 4 was too low to effectively retain volatiles and glycerol within the matrix.

Introduction

Wheat gluten (WG) is an interesting alternative to synthetic plastics in food packaging applications due to its combination of attractive mechanical, oxygen barrier, and film-forming properties and renewability. From an industrial point of view, the advantage of vital WG is that it is readily available at a low price (∼1 euro/kg), with only a small variation in quality.[1] Several studies have dealt with the film-forming properties of WG proteins, mostly involving water−ethanol dispersions/solutions.[2−10]

Packaging materials must have time-stable properties in order to protect the foodstuff and give a long shelf life. However, biopolymers, including WG films, suffer from aging. To limit aging, it is important to identify and understand the mechanisms and reasons for the time-dependent physical and chemical changes. Only a limited amount of studies have been reported on this topic despite that it is perhaps the most important problem that has to be solved before WG films can be of commercial interest for the packaging industry. Examples of factors that have an impact on the aging of protein films are phase separation, diffusion/migration and loss of additives,[10−12] and thiol oxidation.[7,13] In the case of WG films the migrating species can be water and ethanol, originating from the solvent, and added plasticizer (e.g. glycerol). Since these components plasticize the film, migration or phase separation results in a time-induced brittleness.[10] Other time-dependent changes may possibly also occur, including protein aggregation and oxidation that increase the brittleness. Vital wheat gluten contains a small amount of starch that inevitably also ages.

The film and film-forming properties are strongly dependent on the pH of the dispersion, and they are normally inferior close to the isoelectric point, which for WG is of the order of 7.5.[14] Gennadios et al.[15] obtained homogeneous WG films at pH 2−4 and pH 9−13, whereas films were of poor quality at pH 5−6 and did not form at pH 7−8.

This study aims toward an understanding of the mechanisms responsible for the aging of films of plasticized vital/commercial wheat gluten cast from water−ethanol solutions at pH 4 and pH 11. The choice of these pH values was based on the idea of having acidic and basic solutions far from the isoelectric point.

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Casting was used since it is closely related to dispersion coating, an interesting potential application technique for vital wheat gluten on e.g. paperboard in packaging applications. The films were stored at 50% relative humidity (RH) on a blotting paper. The aim was to reveal if the plasticizer migrates to the paper during aging. This may be a critical issue in case wheat gluten is to be laminated with paper in future applications. This is, to our knowledge, the first study on the correlation between protein structure, film "homogeneity", volatile mass loss, plasticizer migration, and mechanical and permeation properties during aging of flexible wheat gluten films.

Experimental Section

Materials. The commercial vital WG powder was kindly supplied by Reppe AB, Lidköping, Sweden. The powder consisted of 84.8 wt % WG proteins, 8.1 wt % wheat starch, 5 wt % water, 1.34 wt % fat, and 0.76 wt % ash. Glycerol with a purity of 99.5% was supplied by Karlshamns Tefac AB, Karlshamn, Sweden. The ethanol (95%) was obtained from Kemetyl AB, Sweden. Acetic acid (99%) was supplied by Merck Eurolab GmbH (Germany). Sodium hydroxide was supplied by Sigma-Aldrich Chemie GmbH, Germany. Iodine, resublimed grade for analysis, was supplied by Merck KGaA, Germany.

Methods. *Film Formation.* The films were prepared by first stirring 30 g of WG powder and 9.9 g of glycerol in 135 g of ethanol with a magnetic stirrer. After a homogeneous solution had been attained, 90 g of deionized water was slowly added. pH 4 or pH 11 was reached by adding, respectively, acetic acid or sodium hydroxide. The solution was thereafter heated to 75 °C during 20 min (2.5 °C/min) and kept at that temperature for 10 min under gentle stirring. The solution was thereafter poured into Petri dishes and dried for 2 days at 23 °C and 50% relative humidity to yield 300−400 μm thick films. The Petri dishes were coated with a release agent layer of poly(tetrafluoroethylene) supported by an aluminum foil (Bytac Type AF-21; Norton Performance Plastics Corp., Wayne, NJ). If not stated otherwise, the films were analyzed with the different methods without being cleaned on the surfaces.

Aging of Films. Films were conditioned in a climate chamber at 23 °C and 50% RH for 48 h and then placed, with the side that had faced the Petri dish down, on a porous paper support (Blotting Paper 1600, 220 diameter from VWR International). The films were aged at 23 °C and 50% RH.

Film Thickness. Film thickness was measured using a Mitutoyo 10C-1128 micrometer. The mean thickness was calculated from measurements at five different locations on each film sample.

Tensile Testing. The WG films were tensile tested at 50% RH and 23 °C in a Zwick Z010 tensile tester controlled by a testXpert 7.1 computer program supplied by Zwick GmbH & Co., Germany. Dumbbell shaped specimens punched from the films had a length and width of the narrow section of, respectively, 16.0 ± 1.0 mm and 4.0 ± 0.1 mm (ISO 37:1994(E)). The measurements were performed as described in ASTM 882-01 with a crosshead speed of 100 mm/min and a clamp distance of 40 mm. Ten replicates of each sample were measured. The tensile strength was defined as the maximum stress in the nominal stress−strain curve.

Water Vapor Permeability. The water vapor transmission rate (WVTR) was measured on three replicates of each sample using a Mocon Permatran-W Twin according to ASTM F 1249-90. Water vapor permeabilities were evaluated by applying a small RH gradient (11%) across the film samples. As explained by Gennadios et al.,[5] this low RH was used in order to avoid swelling and non-Fickian behavior of the films. Specimens were tightly sandwiched between two pieces of aluminum foil leaving a 5 cm^2 exposure area for the WVTR measurements. Before the measurements, the specimens were conditioned in isolated diffusion cells with one side in contact with an atmosphere of a saturated LiCl solution (giving 11% RH) and the other side facing dry nitrogen gas. The WVTR was normalized with respect to water vapor pressure and film thickness to yield the water vapor permeability (WVP).

Oxygen Permeability. The oxygen transmission rate was determined on two replicates of each sample at 23 °C and 0% RH, using a Mocon Ox-Tran 2/20 apparatus, according to ASTM D 3985-95. The specimens, tightly sandwiched between two pieces of aluminum foil, were mounted in isolated diffusion cells that were subsequently purged with nitrogen gas (2% hydrogen) to obtain the background oxygen leakage. The films were conditioned for at least 24 h before background correction. After background correction, the specimen was exposed to flowing oxygen gas (99.95%) at atmospheric pressure. Measurements were interrupted when a steady state was attained. The oxygen transmission rate was normalized with respect to oxygen pressure and film thickness to yield the oxygen permeability (OP). The observed steady-state permeation values assured that the film was in equilibrium with 0% RH.

Infrared Spectroscopy (IR). Fourier transform infrared spectra (12 scans) were recorded on a Perkin-Elmer Spectrum 2000 FTIR spectrometer equipped with a normal single reflection ATR accessory, Golden Gate from Specac Ltd. (Kent, England). Spectra were recorded on WG films placed tightly between a sapphire anvil and the ATR germanium crystal.

Glycerol Content by Gas Chromatography (GC). The procedure is to a large extent based on the method by Graça and Pereira.[16] It is indeed possible to use HPLC methods,[17] but our equipment was more suited for GC analysis. Wheat gluten films were placed in a circular flask, and 50 mL of methanol was added. The solution was gently stirred at 300 rpm, and the glycerol was eluted for 3 days. Each eluted solution (0.5 mL) was decanted into a 1.5 mL vial, and 1 mg of erythritol dissolved in 0.5 mL of methanol was added as internal standard. The liquid was then dried under nitrogen. Thereafter glycerol and erythritol were acetylated with a mixture of pyridine (400 μL) and acetic anhydride (500 μL) for 50 min at room temperature. The glycerol analysis after the acetylation was carried out on a Hewlett-Packard 5890 gas chromatograph system with a DB5-MS column (J&W, Folsom, CA, U.S.A.; 60 m × 0.25 mm i.d.; 0.25 μm film thickness). High-purity helium was used as carrier gas, and the flow rate was held at 1.5 mL/min. The injection temperature was 230 °C, and the flame ionization detector (FID) temperature was 250 °C. The oven temperature program was as follows: 95 °C for 2 min, increase to 150 °C at 5 °C/min, and then a raise to 300 °C at 20 °C/min. The quantitative analysis was performed with Turbochrom Workstation software (Perkin-Elmer Co.). The calibration for glycerol using erythritol as internal standard was performed as follows: mixtures of glycerol and erythritol (weight ratios: 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, and 10:1) were dissolved in methanol, and the latter was evaporated in a nitrogen atmosphere. After evaporation, the mixtures were treated with 400 μL of pyridine and 500 μL of acetic anhydride reagents to acetylate both glycerol and erythritol. The acetylated solutions were injected into the GC-FID under the same conditions as used for the glycerol analysis above. The ratio of the peak areas was proportional to the ratio of the mixture of glycerol and erythritol ($R^2 = 0.9994$).

Protein Solubility. The amounts and size distributions of proteins extractable with sodium dodecyl sulfate (SDS) and SDS + sonication of cast films were obtained using size-exclusion high-performance liquid chromatography (SE-HPLC), together with a three-step extraction procedure according to Gällstedt et al.[18] Proteins soluble in dilute SDS were extracted in the first step, and additional proteins were extracted with repeated SDS and sonication treatments. The "unaged" films were films analyzed 6 days after preparation. Aged films were at least 120 days old.

In the first extraction (ext 1), 16.5 mg of each film was suspended in 1.5 mL of 0.5% SDS-phosphate buffer (pH 6.9) and vortexed for 10 s. The suspension was then stirred for 5 min at 2000 rpm and centrifuged for 30 min at 8160*g* to obtain the supernatant protein. In the second extraction (ext 2), the pellet was re-suspended in the SDS

buffer and sonicated in an ultrasonic disintegrator (Soniprep 150, Tamro, Mölndal, Sweden) for 30 s, amplitude 5, fitted with a 3 mm exponential microtip. The samples were then centrifuged (30 min, 8160*g*) to obtain a supernatant of proteins. In the third extraction (ext 3), the pellet was again re-suspended in the SDS buffer and sonicated as above for 30 + 60 + 60 s.

The extracts were filtered through 0.45 μm filters (Millipore, Durapore Membrane Filters) before the SE-HPLC operation. SE-HPLC analyses were performed on a Waters HPLC system using a BIOSEP SEC-4000 Phenomenex column. Separation was obtained during 30 min by loading 20 μL of sample into an eluant of 50% (v/v) acetonitrile and water containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.2 mL min^{-1}. Proteins were detected by UV absorption at 210 nm. The amount of proteins extracted after each extraction step was normalized with respect to the total protein solubility of unprocessed WG exposed to the three extraction steps.[18] The SE-HPLC chromatograms were divided into polymeric and monomeric fractions.[19] The polymeric proteins were referred to as those that were eluted before 17 min and consisted of essentially glutenin and albumin moieties, and the monomeric proteins, eluted later, consisted of mainly gliadins, albumins, globulins, peptides, and amino acids.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Wheat proteins were extracted from 100 mg of the respective WG films and the WG dough and were analyzed with RP-HPLC. The extraction was performed in nine steps.[20] The extraction buffers during the different steps were as follows: (1) 0.6 mL of H_2O, (2) 0.6 mL of 0.5 M NaCl, (3) 0.3 mL of 0.5 M NaCl, (4) 0.6 mL of 70% ethanol, (5) 0.6 mL of 50% 1-propanol, (6) 0.3 mL of 50% 1-propanol and 1% 1,4-dithio-DL-threitol (DTT), (7) 0.6 mL of 50% 1-propanol and 1% DTT and 1% glacial acetic acid, (8) 0.6 mL of 0.5% SDS and 1% DTT, (9) 0.6 mL of 6 M urea, 0.5% SDS, and 1% DTT.

After each extraction, the supernatant was collected and a new buffer was added to the pellet. The sample was suspended in the buffer, stirred for 30 min at 2000 rpm, and centrifuged for 30 min at 8160*g* to obtain the supernatant. Extractions 2 and 3 were performed at 4 °C, extractions 6 and 7 were performed at 60 °C, and for extractions 8 and 9 the samples were heated to 100 °C for 5 min. The other extractions were performed at room temperature. The samples were rinsed with H_2O between extractions 3 and 4 in order to remove the salt. After the protein extractions (nine steps) from the gluten films and the dough, protein fractionation was carried out using RP-HPLC.[20] It was performed on a Waters HPLC system using a Supelcosil LC-308 column with 300 Å pore size, 5 μm particle size, 250 × 4.6 mm i.d. The solvent flow rate was 0.8 mL/min using a column temperature of 70 °C, and the effluent was monitored at 210 nm. Elution was achieved using a gradient system formed from two solvents: A, water containing 0.1% (v/v) trifluoroacetic acid (TFA), and B, acetonitrile containing 0.1% (v/v) TFA. The fractions from extractions 1−3 were analyzed with HPLC using a gradient of 30−60% solvent B from 1 to 20 min. The gradient used for the separation of the other extractions was 28−56% solvent B from 1 to 30 min.[19] Three replicates of each gluten film or dough were analyzed for the investigation of protein composition.

Scanning Electron Microscopy (SEM). Au/Pd (60%/40%) sputtered film surfaces and fracture surfaces from tensile specimens were characterized using a JEOL JSM-5400, scanning electron microscope.

Optical Microscopy. The presence of starch was revealed by staining the films with iodine vapor. Iodine powder was dispersed in the bottom of a hermetically closed recipient, and the WG films were hung inside the recipient in order to expose the entire surface to the sublimed iodine. The samples were kept in iodine vapor for 24 h. The structure of the films was analyzed with a Leitz Ortholux POL BK II optical microscope, and the starch was visible as dark-blue particles.

Results and Discussion

Mass Loss. The films lost mass continuously during the entire aging period. The pH 4 film lost more mass than the pH 11 film. The reason for the mass loss could be loss of residual water and ethanol and/or loss of glycerol.[10−12] Ethanol was, however, absent or below the IR detection limit, in the films. In the first 20−22 days of aging, especially for the pH 4 film, some volatiles/moisture were lost, as observed by the fact that the film mass loss was greater than the loss to the paper support, the latter considered to be due primarily to loss of glycerol. The reason to this was presumably that the film equilibrated slowly from the casting conditions to the 50% RH surrounding. Nevertheless, since the films were aged at constant relative humidity and ethanol, due to its high vapor pressure, had left the film during the casting procedure, it is assumed here that changes in the glycerol content played a key role for the film properties during aging. Therefore the GC method, described above, was developed to determine the glycerol content in the films. Figure 1 shows that the loss of glycerol from the pH 4 and pH 11 films was small and basically the same after 9 days but that the glycerol loss was significantly greater from the pH 4 film after 120 days. Considering that the glycerol content was 25 wt % in the new films, the glycerol content after 120 days was 12 wt % and 17 wt % for, respectively, the pH 4 and pH 11 films. This, together with the mass loss measurements, shows that the pH 11 film retained glycerol and volatile mass, since glycerol binds water, better than the pH 4 film.

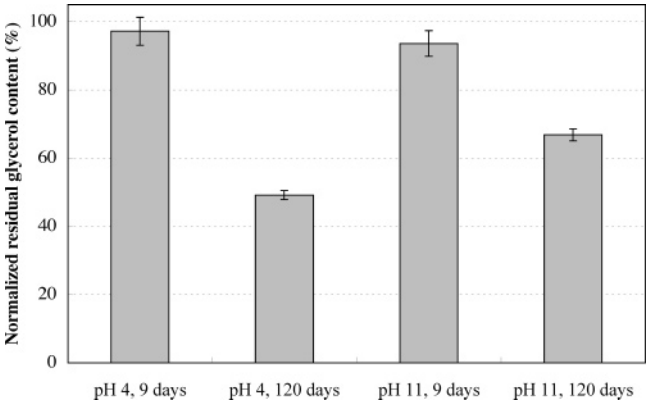

Figure 1. Residual glycerol content in the films with respect to the new pH 4 and pH 11 films.

General Film Characteristics. The films prepared from the basic solution (pH 11) appeared to be more homogeneous and smooth than the films prepared from acidic solutions (pH 4). SEM revealed an undulating pH 4 film upper surface and an uneven film thickness (Figure 2). The lower surface, i.e., the surface that faced the Teflon-coated Petri dish during casting, was always flat for both film types. IR spectroscopy on unaged samples showed that the pH 4 film surface was richer in glycerol than the corresponding pH 11 film, as indicated by a more pronounced 850 cm^{-1} peak, associated with the C−C−O stretch or CH_2 twist of glycerol, relative to the amide-II ∼1535 cm^{-1} peak,[21] in the former IR spectrum. IR, which penetrates to a depth of a few micrometers, also indicated that there was more glycerol on the lower side than on the upper side of the cast films. These two trends in glycerol distribution were small (the differences in the 850 cm^{-1}/1535 cm^{-1} peak ratios between the two pH's and between the upper and lower surfaces were both on the order of 20%) but always present (cf. Figure 3a,b). Gravitational forces probably caused a limited amount of glycerol to migrate downward through the solidifying film during the casting process. Both the pH 4 and pH 11 films contained particles at the surface and also in the bulk. Iodine staining indicated that these were starch granules.

Tensile Properties of Unaged and Aged WG Films. The unaged pH 11 film had a higher Young's modulus, a higher

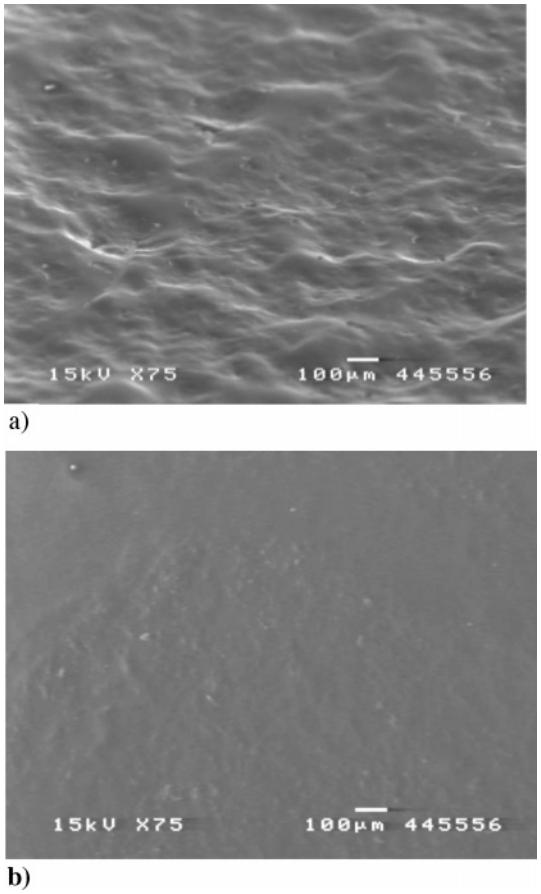

Figure 2. SEM micrographs of the upper surface of the pH 4 film (a) and the pH 11 film (b).

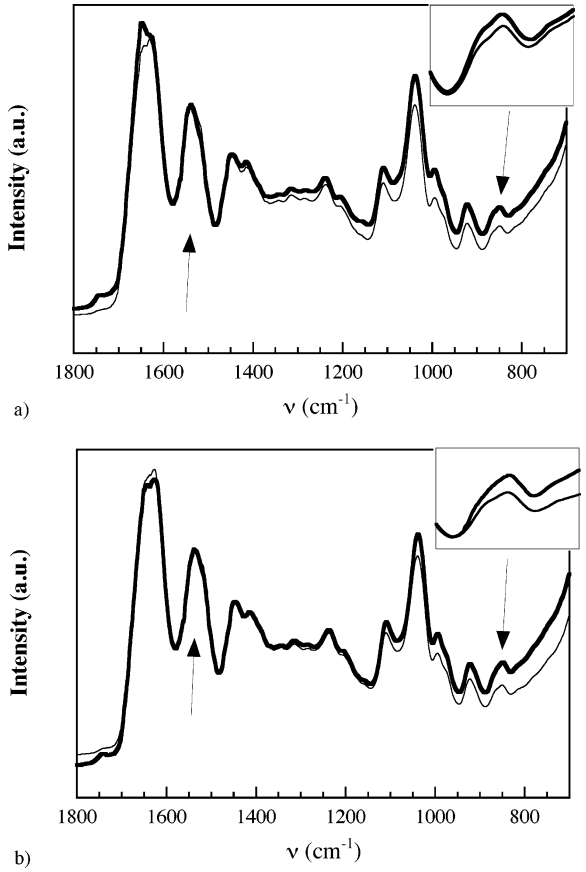

Figure 3. (a) IR spectra of unaged pH 4 (thick line) and PH 11 (thin line) upper film sides. (b) IR spectra of the lower (thick line) and upper (thin line) sides of unaged pH 11. The spectra were normalized with respect to the amide-II peak. The insert figure is a magnification of the 850 cm^{-1} peak region with the curves positioned closer together to facilitate a comparison.

Table 1. Mechanical Properties of the WG Films[a]

aging	ϵ_b (%)		*E* (MPa)	
(days)	pH 4	pH 11	pH 4	pH 11
0	213 (15)	95 (7)	23 (2)	91 (5)
3	172 (29)	106 (12)	53 (19)	68 (22)
9	112 (5)	98 (13)	95 (8)	111 (15)
30	72 (20)	119 (12)	117 (71)	128 (14)
60	46 (24)	117 (7)	263 (95)	89 (8)
90	22 (17)	116 (3)	413 (103)	123 (16)
120	2 (1)	100 (15)	745 (132)	176 (48)

[a] The values within parentheses are the standard deviations.

tensile strength, and a lower elongation at break than the unaged pH 4 film (Table 1 and Figures 4 and 5). This was in agreement with the results of Gennadios et al.[15] and of Kayserilioglu et al.,[2] who reported stronger films under alkaline conditions. The unaged pH 4 film was very ductile. However, the ductility dropped, the stiffness and strength increased monotonically, and the film became brittle with time (Table 1 and Figure 4). In contrast, the ductility and stiffness of the pH 11 film changed only marginally with time (Table 1 and Figure 5). The stiffness of the pH 4 film increased 32-fold during 120 days of storage whereas there was only a 2-fold increase for the pH 11 film. The corresponding factors for the tensile strength were 6.5 and 2. The pH 11 film always yielded and showed a post-yield strain-hardening as indicated by an increase in the slope of the stress−strain curve after yielding. As shown in Figure 4, this also occurred in the pH 4 film but only up to 30 days of storage. As a comparison, Hernandez et al.[12] reported a 1.7-fold increase in strength for glutenin films prepared at pH 5 and aged for 112 days. It is tentative to suggest that their reported small changes in strength with aging was due to that glutenin is less susceptible to cross-linking with aging than vital WG, which contains a large amount of readily cross-linkable gliadin.

The changes in mechanical properties were accompanied by changes in the film structure as observed by SEM. The structure of the pH 4 film became coarser and more heterogeneous during aging whereas the structure of the pH 11 film was unchanged (Figures 6 and 7). The same type of time-induced coarsening has been observed for cast whey films plasticized with either glycerol or diethylene glycol.[22]

Protein Solubility of Unaged and Aged WG Films. The SE-HPLC data revealed that the protein solubility of the unaged pH 4 film was considerably higher than that of the corresponding pH 11 film (Figure 8). The protein solubility of the pH 4 film was high already after the SDS treatment which dissolved protein molecules primarily by breaking secondary bonds. However, the subsequent sonication, which is considered to also break disulfide bonds, increased the protein solubility significantly. Interestingly, sonication had a greater impact on the pH 4 film than on the pH 11 film. Figure 8 also shows that a significant part of the dissolved matter of the pH 4 film was so-called "polymeric" proteins, i.e., proteins of large molecular size. In fact, after the last sonication extraction, more than one-third of the dissolved protein from the unaged pH 4 film was of the polymeric protein type. Only a small part of the dissolved pH 11 film proteins were of this type. Since it is known that alkaline conditions promote protein denaturation[23] and that the conformation of the protein molecule is more stable in acidic

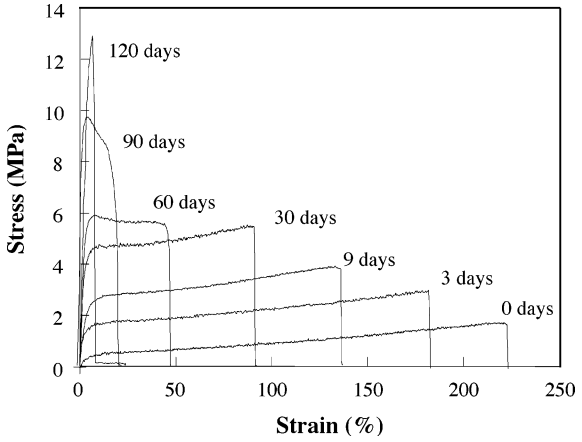

Figure 4. Stress−strain curves of the pH 4 film.

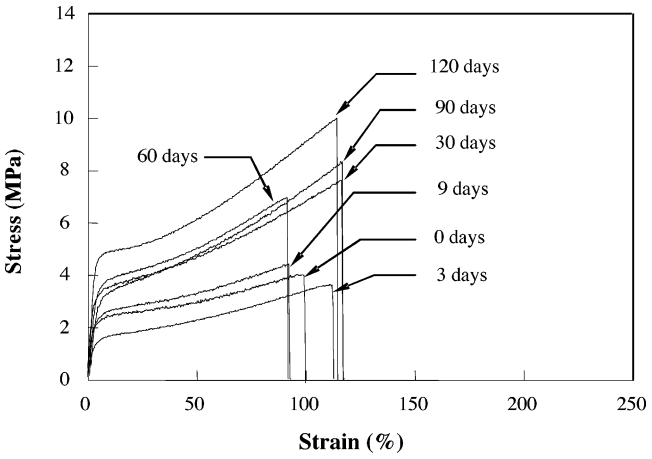

Figure 5. Stress−strain curves of the pH 11 film.

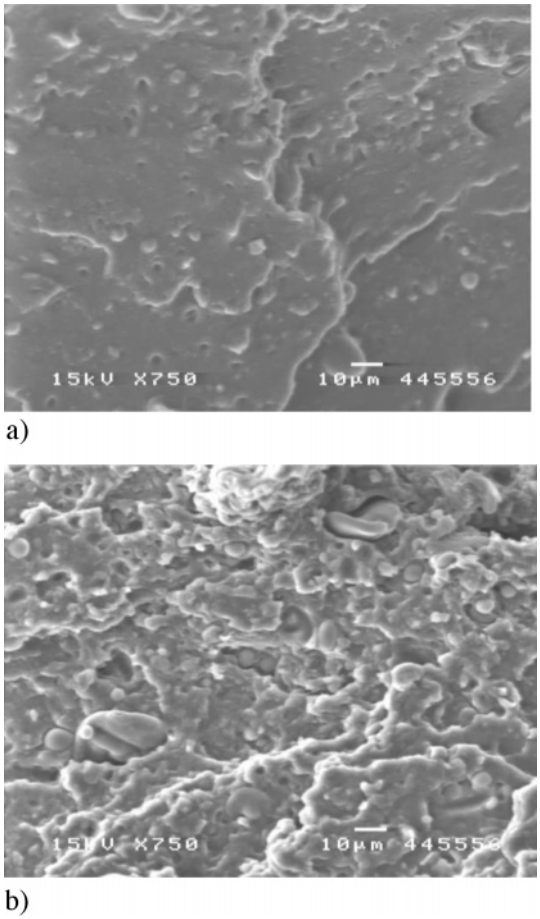

Figure 6. SEM micrographs of cross-sections of an unaged (a) and aged (b) tensile-fractured pH 4 film.

conditions,[24] it is likely that the pH 11 film experienced a greater degree of denaturation during film formation than the pH 4 film. In addition, as will be discussed later, other amino acid cross-linking reactions not involving thiol groups, e.g. the formation of lysinoalanine, occur more readily at high pH.[2] After the more extensive denaturation, the pH 11 film proteins would then show a more extensive aggregation and polymerization than those in the pH 4 film. This would explain the low protein solubility of the pH 11 film. The higher ductility of the unaged pH 4 film than of the pH 11 film was therefore a consequence of its lower degree of denaturation.

Whereas the protein solubility was relatively constant during aging under basic conditions, it decreased significantly with time under acidic conditions. It became more difficult to dissolve proteins both by attempting to break secondary bonds (SDS) and by attempting also to break disulfide bonds (sonication). The amount of soluble pH 4 film "polymeric" proteins was also reduced during aging. Thus the pH 4 film experienced a time-dependent protein aggregation. However, during the aging period, the extent of protein aggregation in the pH 4 film did not reach the level of that in the pH 11 film, as indicated by the overall higher protein solubility of the pH 4 film (Figure 8). On the basis of the protein solubility results, it was clear that changes in the protein structure had an impact on the aging properties of the films. Comparing Figures 1 and 8 it is evident that plasticizer migration is reduced by a more aggregated protein structure (pH 11), which then explains why the mechanical properties of the pH 11 films were more stable in time as compared to the pH 4 film.

IR Spectroscopy. From the IR amide-I region (in the vicinity of 1650 cm^{-1}), it is possible to obtain information on the conformation of the protein molecules.[25−27] It is suggested that the amide-I absorption in the 1580−1720 cm^{-1} region consists of at least four peaks. The peaks located in the vicinity of 1616, 1632, and 1672 cm^{-1} correspond, respectively, to the amounts of amide groups in intermolecular β-sheet networks, in extended β-sheets, and in β-turns.[28] The 1616 cm^{-1} peak in particular has been associated with aggregated protein structures. The peak at 1652 cm^{-1} corresponds to the amount of amide groups in disordered or α-helix molecular conformations. Because of the uncertainty in deconvoluting the amide-I region, the experimental curves were instead compared directly. The unaged pH 11 film had a significantly higher 1616 cm^{-1} peak than the unaged pH 4 film, indicating, in agreement with the solubility tests, that the pH 11 film proteins were the more aggregated already after film formation (Figure 9). During aging, again in agreement with solubility tests, the pH 4 film experienced a significant aggregation (increase in the 1616 cm^{-1} intensity, Figure 9) whereas further aggregation in the pH 11 film was small or absent.

RP-HPLC. Figure 10 shows the "accumulated" protein solubility of the pH 4 and pH 11 films relative to the wheat gluten dough, assessed by RP-HPLC, after different treatments. The solubility in water, due to the presence of albumins, peptides, amino acids, perhaps residual starch, and other water-soluble entities was highest for the unaged pH 4 film, the gluten dough, and surprisingly the aged pH 11 film. Deamidation, transforming glutamine amide groups to glutamate carboxylate groups, can occur at high pH[29,30] and yields more polar and therefore more water-soluble protein units. In addition, deamidation can lead to protein chain scission, which adds to the protein solubility.[30] Apparently, comparing the solubility of the

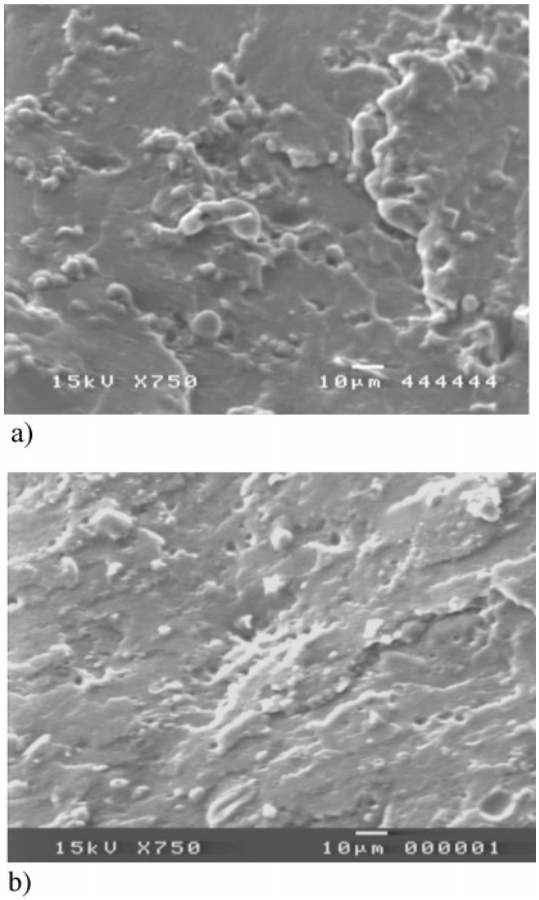

Figure 7. SEM micrographs of cross-sections of an unaged (a) and aged (b) tensile-fractured pH 11 film.

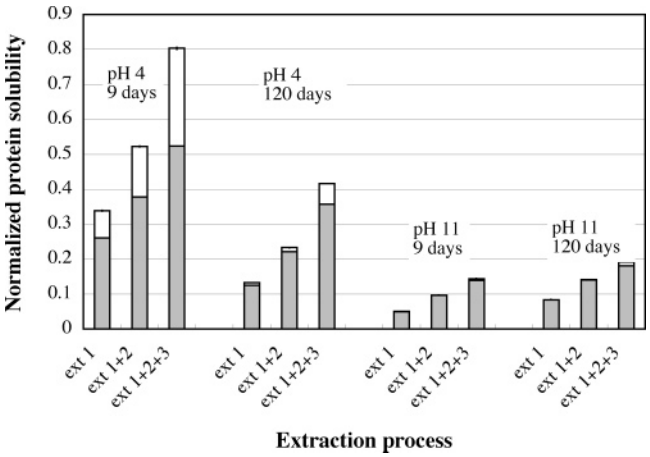

Figure 8. Normalized protein solubility, i.e., the ratio of the protein solubility of the film to the protein solubility of the wheat gluten dough for films exposed to the following: SDS (ext 1); SDS solution and 30 s sonication (ext 2), and SDS and 150 s (30 s and 60 + 60 s) sonication (ext 3). The gray and white column sections refer to the amount of "monomeric" and "polymeric" proteins.

unaged and aged pH 11 films, deamidation seemed to have taken place to a large extent during aging. Also, the polarity of the protein in a certain extraction can be observed in the RP-HPLC chromatograms. RP-HPLC elutes due to polarity, by the use of the gradient system. With the present gradient system, the most polar proteins were eluted early in the chromatogram, as observed for the water-soluble proteins in the pH 11 films (Figure 11b) compared to the proteins in the gluten dough (Figure 11a). The protein profiles in the chromatograms of the pH 4 films were more similar to that of gluten dough (results

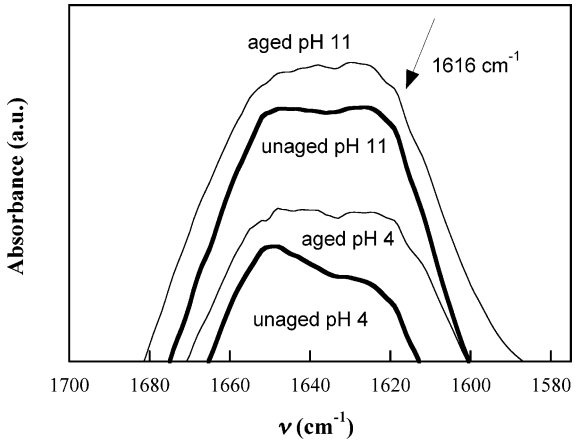

Figure 9. Infrared spectra showing the amide-I region of dried films.

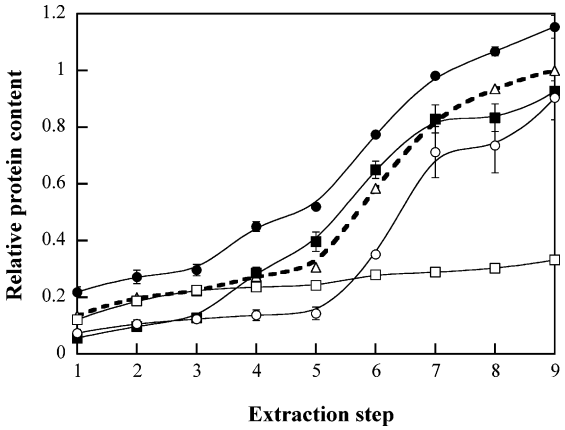

Figure 10. Amount of extracted protein relative to that of wheat gluten dough (△) after extraction step 9 as determined by RP-HPLC: (●) unaged pH 4 film; (■) aged pH 4 film; (○) unaged pH 11 film; (□) aged pH 11 film. The lines illustrate the trends.

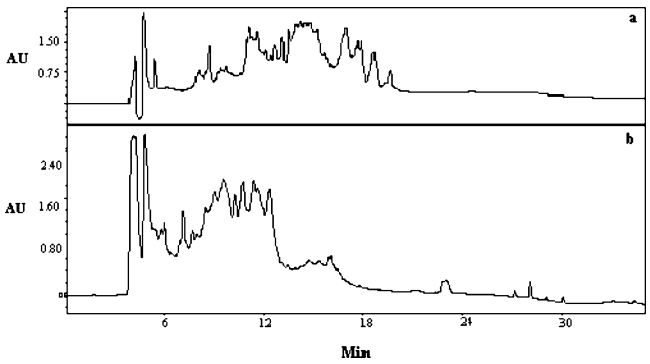

Figure 11. RP-HPLC chromatograms for (a) wheat gluten dough and (b) aged pH 11 film, both extractad with water (extraction buffer 1). AU = absorbance units of the UV detector.

not shown). The solubility increased only marginally and with the same rate for all films, and the gluten dough, after the subsequent extractions 2 and 3. After extractions 4 (ethanol) and 5 (1-propanol), the pH 4 film solubilities increased significantly, indicating that those contained a sizable amount of alcohol-soluble proteins. Also the protein solubility of the gluten dough increased somewhat but the pH 11 films did not contain any significant amount of alcohol-soluble proteins (Figure 10). All samples except the aged pH 11 film experienced a significant increase in protein solubility when the disulfide cross-links were broken by the DTT reducing agent (extraction 6) and when DTT was combined with acetic acid (extraction 7). The addition of the acid was shown previously to yield

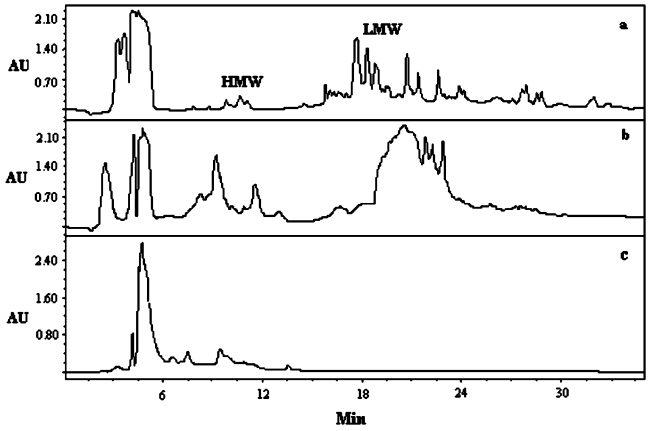

Figure 12. RP-HPLC chromatograms of (a) glutenins extracted from flour, cv Dragon, (b) gluten dough extracted with buffer 8, and (c) aged pH 11 extracted with buffer 8. AU = absorbance units of the UV detector.

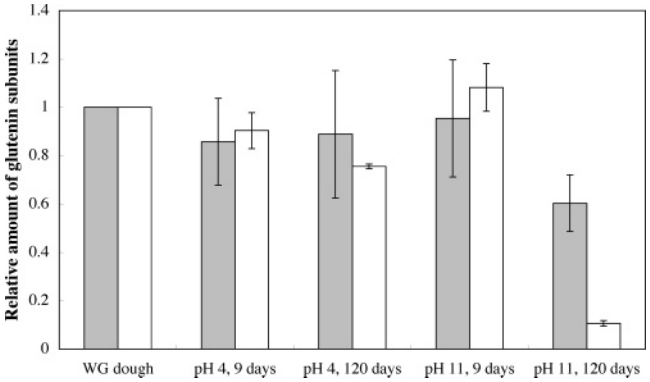

Figure 13. Accumulated amount of extracted HMW (gray column) and LMW (white column) glutenin subunits relative to those of the gluten dough from extractions 6−9. Error bars indicate standard deviations.

additional protein solubility in combination with the reducing agent.[20] The combination of SDS, breaking secondary bonds, DTT, and high temperature only increased the unaged pH 4 and gluten dough solubility (extraction 8). In the last extraction, urea was added, and it is remarkable that even at this severe treatment the protein solubility of the aged pH 11 film remained small. The insensitivity to DTT suggests that the aged pH 11 film has cross-linked to a certain extent with nondisulfide bonds. It is possible that those are isopeptide bonds. After extraction of the albumins, globulins, and gliadins by the use of different extraction buffers in wheat, the glutenin subunits can be extracted and observed individually in the HPLC chromatogram based on the different elution times (Figure 12a).[20] Analyzing the chromatograms containing DTT reduced proteins (from extractions 6−9) that had been separated into elution time intervals corresponding to HMW and LMW glutenin subunits, it was striking to see that the aged pH 11 film chromatogram did not contain any LMW glutenin subunits (Figure 12c and 13). Thus, based on the RP-HPLC data it is tentative to suggest that mainly LMW's are taking part in the cross-linking through isopeptide bonds. It is known that isopeptide formation occurs in wheat gluten during heat treatment at high pH.[31] In addition Dosaka[32,33] has shown, on casein and soy protein dope solutions, that isopeptide formation can occur during aging. Deamidation of the pH 11 film was also indicated since the HMW proteins were eluted earlier in the chromatograms compared to, for example, the gluten dough (Figure 13). The present data consequently indicate that isopeptide formation occurs in parallel with deamidation during aging of the pH 11 system.

Table 2. Oxygen Permeability[a]

days of storage	oxygen permeability (cm^3 mm/(m^2 day atm)) pH 4 film	pH 11 film
0	1.5 (0.1)	0.5 (0.2)
90	2.5 (1.0)	0.9 (0.6)

[a] The values within parentheses are the standard deviations.

Permeation. The permeability to water vapor and oxygen provide additional information on the differences in the structure and homogeneity of the different films. It is expected that if the film loses plasticizing molecules during aging the oxygen permeability would decrease. However, the change in OP with aging was insignificant (all-paired Student-*t* test ($p \leq 0.1$)) (Table 2). This suggested that the expected decrease in OP with the loss of plasticizer was compensated for by the less dense and more permeable structure generated by the same plasticizer loss and aggregation. The OP of the pH 4 film was significantly higher than that of the pH 11 film (all-paired Student-*t* test ($p \leq 0.1$)), indicating that the more heterogeneous pH 4 film structure was more permeable. In addition, the scatter in OP data (standard deviation, Table 2) increased with aging and was greatest for the pH 4 film, reflecting its greater heterogeneity. Even though the undulating surface of the pH 4 film led to an overestimation of the thickness, and consequently an overestimation of the OP, simply because the caliper might not have reached the bottom of every valley, it could not explain the large OP differences between the pH 4 and pH 11 films. The water vapor permeability, averaged over all films, were WVP = 700 g mm/(m^2 day atm) and did not change significantly with aging time or pH (all-paired Student-*t* test ($p \leq 0.1$)). If it is assumed that the more heterogeneous structure of the pH 4 film is associated with a larger amount of voids and capillaries than in the pH 11 film, the small difference in WVP between the pH 4 and pH 11 films is expected. WVP is less sensitive to the presence of voids than OP,[34] which is explained by the fact that condensed moisture, because of high surface energy, adheres to the surfaces of capillaries, cavities, and voids, whereas oxygen fills them. Consequently the concentration of oxygen (and therefore also the OP) increases more rapidly than the concentration of water (and WVP) with increasing amounts of capillaries and voids.

Conclusion

The properties of the cast films depended strongly on the pH of the solution. A pH of 11 yielded, in general, better properties. The change in film stiffness, strength, and ductility with aging time was also significantly less than at pH 4. In fact, the latter became brittle after 120 days. In addition, the pH 11 film was more homogeneous and more uniform in thickness. The greater film homogeneity gave better oxygen barrier properties. The time-induced decrease in ductility of the pH 4 film was due to the loss of plasticizing molecules, mainly glycerol, which, in turn, was due to a lower degree of protein aggregation than in the pH 11 film. The higher degree of aggregation (more complete protein network) seemed to be more effective in retaining the plasticizing molecules within the film. RP-HPLC results suggested that the pH 11 film experienced a certain degree of deamidation and that it increased with aging, in parallel with isopeptide cross-linking. These processes did not alter the overall initially high degree of aggregation of the

pH 11 film and consequently did not alter the mechanical properties. The results show that in laminated structures of gluten and paper, plasticizer migration into the paper can be a problem.

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