Properties of Deamidated Gluten Films Enzymatically Cross-Linked

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Films were prepared at neutral pH from deamidated gluten by casting with or without enzymatic treatment by transglutaminase in the presence of various concentrations of diamines added to the film-forming solution. Variation in the glycerol/deamidated gluten ratio from 0.2 to 0.5 had a major effect on the film mechanical properties, which is characteristic of a plasticizing effect. A ratio of 0.35, producing a tensile strength of 1.14 ± 0.12 MPa and an elongation at break of 376 ± 62%, was chosen for most of the enzymatic modifications. The action of transglutaminase with or without the addition of external diamines induced a simultaneous increase in tensile strength and elongation at break of the films but tended to decrease the contact angle between the film surface and a water droplet. The presence of diamines in the film solution affected the elongation at break more than the tensile strength of the films. These diamines, able to react at their two extremities, probably acted as spacers between gluten proteins. The decrease in solubility was related to the formation of high molecular weight polymers in the film. The film properties were unaffected by the type of diamine added as secondary substrate in the transglutaminase reaction.

Keywords: *Wheat; gluten; deamidation; transglutaminase; protein films; mechanical properties*

INTRODUCTION

A wide variety of biopolymers have been used, alone or in mixtures, to obtain edible films and coatings (Kester and Fennema, 1986; Krochta and De Mulder-Johnson, 1997; Gennadios, 1990). Among them, several plant proteins have been investigated (Cuq et al., 1998) including soy proteins (Gennadios et al., 1991, 1993; Kunte, 1997), corn zein (Yamada et al., 1995; Parris and Coffin, 1997; Rayas, 1997), wheat proteins (Gennadios, 1990; Gontard et al., 1992, 1993; Sanchez et al., 1998), cotton seed proteins (Marquié et al., 1995), and pea proteins (Guéguen et al., 1995, 1998). Unlike synthetic polymers or polysaccharides, which can be considered to be homopolymers, proteins are structured heteropolymers. Two classes of proteins can be distinguished, globular or pseudoglobular proteins such as globulins or gliadins and fibrous or "polymerized " proteins such as collagen or glutenins. Gluten is a mixture of monomeric proteins (gliadins) and polymerized proteins (glutenins) linked through intermolecular disulfide bridges. These glutenins, largely implicated in the viscoelastic character of gluten, may play an important role in film formation. Wheat gluten films have been studied extensively (Gennadios and Weller, 1990; Gontard et al., 1992; Herald et al., 1995; Roy et al., 1999). Films were cast from gluten protein dispersions in water at acidic or alkaline pH values or in the presence of ethanol (Ali et al., 1997). The addition of plasticizing agents improves film flexibility and overcomes the brittleness of the films. Gluten films obtained in these conditions presented good elongation properties and were quite flexible.

The use of chemically or enzymatically modified glutens has also been investigated. Krull and Inglett (1971) cast films from gluten hydrolysates alone or in mixtures with chemically modified polypeptides. Good tensile strength, comparable with those of poly(vinyl chloride), were obtained with a mixture of polypeptides and ethylene-treated derivatives (6:1, w/w). Only a few studies have been reported on the effect of cross-links on film properties. Chemical cross-linkers such as formaldehyde, glyoxal, or glutaraldehyde were effective at enhancing the tensile strength and puncture strength of films made from cottonseed proteins (Marquié et al., 1997) or soy proteins (Ghorpade et al., 1995). Brault et al. (1997) introduced cross-links between aromatic compounds (i.e., dityrosine) in caseinate films by exposing them to γ-ionization. Puncture strength and deformation were both increased, but this effect was very dependent on the glycerol/protein ratio.

Two types of enzyme have been used to introduce cross-links in protein films: peroxidase and transglutaminase. A treatment by peroxidase in the preparation of films of thermally denaturated soy proteins or gluten reduced both tensile strength and elongation at elastic limit (Stuchell and Krochta, 1994; Michon et al., 1999). The effects of transglutaminase treatment on film properties have also been studied for many proteins: α_{s1}-casein (Motoki et al., 1987), whey proteins (Mahmoud and Savello, 1992; Yildirim and Hettiarachchy, 1997), 11S globulin (Yildirim and Hettiarachchy, 1997), and egg white proteins (Lim et al., 1998). The enzymatic cross-linkage of α_{s1}-casein film increased its tensile strength and strain. The solubility of the constitutive proteins of the film decreased, but they were still susceptible to proteolysis. Yildirim and Hettiarachchy reported in 1997 similar results for films from whey proteins, 11S globulins, or a mixture and showed that they exhibited higher mechanical properties and higher water vapor permeability.

Transglutaminase catalyzes the self-polymerization of proteins through ϵ-(γ-glutamyl)lysine bonds but can

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also introduce cross-links between external primary amines and glutamine residues of proteins. In the case of gluten in a doughy state, this enzyme was also shown to increase the proportion of high molecular weight polymers and to strengthen the protein network (Larré et al., 1998). Despite its very low lysine content, some intermolecular ϵ-(γ-glutamyl)lysine bonds were generated in gluten.

On the basis of these studies, our objective was to produce films with deamidated gluten enzymatically modified by transglutaminase. The effects of the introduction and type of covalent bonds on the films were studied. These bonds were first formed between glutaminyl and lysyl residues, and then, by adding various reactive diamines that could act as spacers between two glutamines, new bonds such as *N*,*N*-bis(glutamyl diamine) were introduced into the system. The mechanical properties of the resulting films were investigated as well as their solubility in water.

MATERIALS AND METHODS

Materials. Industrial gluten deamidated at 20% was provided by Amylum (Aalst, Belgium). Putrescine, cadaverine, hexyldiamine, 1,8-diaminooctane, 1,10-diaminododecane, and glycerol were purchased from Sigma Chemical Co. (St Quentin Fallavier, France). Transglutaminase (1 unit/mg) was supplied by Ajinomoto (Tokyo, Japan). Transglutaminase (Tgase) activity was determined according to the colorimetric hydroxamate procedure of Folk (1970).

Enzymatic Reaction and SDS—PAGE of the Products. Deamidated gluten (2 mg/mL) was solubilized in 0.05 M Tris-HCl, pH 8, and in 0.05 M Tris-HCl, pH 8, added to 25% glycerol. The solution was heated at 37 °C and incubated with Tgase (40 UI/g) for 4 h. The reaction products were subjected to SDS−PAGE under reducing conditions and analyzed on vertical 12% polyacrylamide gel according to the method of Laemmli (1970).

Preparation of Cross-Linked Deamidated Gluten Films. Film-forming solutions were prepared at 10% protein in 0.05 M Tris-HCl, pH 8, with ratios of glycerol/protein ranging from 0.2 to 0.5, mixed with a polytron (Kinematica) at 25000 rpm, and then centrifuged at 1000*g* for 15 min to remove air bubbles. The enzyme (40 UI/g of protein) was added to this solution immediately prior to casting. The cast film solution was then incubated for 4 h at 37 °C in a covered tray to avoid drying during the incubation. After the incubation time, the film was dried at 70 °C for 60 min. When alkyldiamines were used, they were added at various concentrations to the film-forming solution and the same procedure was followed for preparing films with and without transglutaminase addition.

Mechanical Characterization of the Films. Mechanical properties were analyzed on five duplicates for each film tested. The shape and size of the tested pieces of film were made according to ISO 527-2 standards. Elongation at break and tensile strength were measured at 20 °C and at 57 ± 2% of hygrometry on an Adamel-Lhomargy testing instrument (model DY34, MTS Systems). Five thickness measurements were made along each piece of film with a micrometer (Kafler), and the mean value was taken for calculation. Tensile strength (σ) was calculated by dividing the maximum stress at break by the mean cross-sectional area of the film, elongation (Δl) was expressed in percentage of initial length (l_0 =20 mm) of the piece tested, and the Young modulus (E_0) was calculated from the slope of the function $\sigma = E_0(\Delta l/l_0)$.

Surface Hydrophobicity. The surface hydrophobicity of films was evaluated by an image analysis system Digidrop, GBX, Scientific Instruments (Roman sur Isère, France). A drop of water was deposited on the film; the contact angle between the film surface and the tangent at the base of the drop was measured and expressed in degrees.

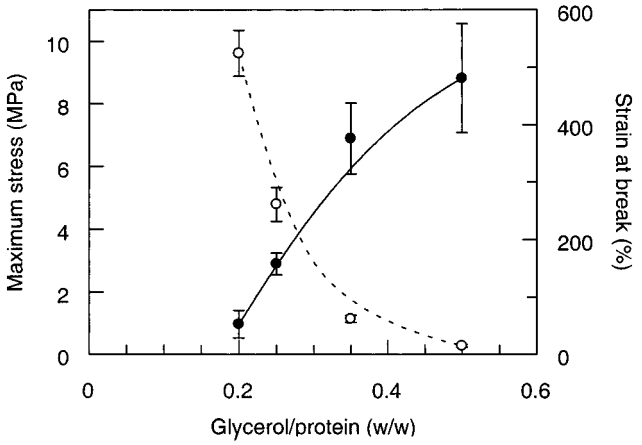

Figure 1. Effect of glycerol concentration on the mechanical properties of deamidated gluten films: (○) maximum stress; (●) strain at break. Gluten concentration: 10%.

Film Protein Solubility. An aliquot (40 mg) of each film was added to 1 mL of water, stirred for 20 h at 20 °C, and then centrifuged at 20000*g*. The supernatant, referred to as the soluble fraction in water, was recovered and diluted in 12.5 mM borate buffer, pH 8.5, 2% SDS before gel filtration analysis. The pellet was recovered and added to 1 mL of 12.5 mM borate buffer, pH 8.5, 2% SDS before being stirred for 20 h at 20 °C. The resulting solution was centrifuged at 20000*g* before further analysis. The absence of pellet was controlled. Following this procedure, supernatants and resolubilized pellets were analyzed by size exclusion chromatography using a Superose 6 (Pharmacia) column eluted with 12.5 mM borate buffer, pH 8.5, 0.2% SDS. The proportion of soluble and insoluble proteins was calculated from the peak areas.

Statistical Analysis. Statistical analyses were performed using Statgraphics Plus. The results were compared by variance analysis and Fisher's least significant difference procedure.

RESULTS AND DISCUSSION

Effect of Glycerol Concentration on the Properties of Films Obtained with Deamidated Gluten. As commonly shown for biopolymers, films obtained from deamidated gluten without the addition of any plasticizer are brittle and cannot be handled. Among the usual plasticizers used, glycerol was chosen and a minimum glycerol/protein ratio of 0.2 was necessary to overcome the brittleness of the film. All films obtained from deamidated gluten were glossy and translucent. The decrease in the maximum stress and the increase in the strain at break when the proportion of glycerol was increased agree with a plasticizing effect of glycerol (Figure 1), which has already been reported on other biopolymers (Gennadios et al., 1993; Sanchez et al., 1998). The mechanical properties of the films obtained from deamidated gluten were lower than those from native gluten. For example, at a glycerol/protein ratio of 0.35, the tensile strength and the elongation at break for native gluten film prepared at pH 10 were 1.87 ± 0.16 MPa and 704 ± 59%, respectively, and for deamidated gluten 1.12 ± 0.11 MPa and 327 ± 32%. This difference could be related to the modification of the gluten. In the case of native gluten, protein−protein interactions are dominated to a large extent by hydrogen bonds and nonpolar interactions in which the amide groups are largely involved. Deamidation enhances the number of negative charges along the protein backbone

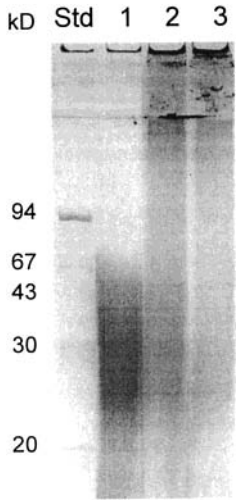

Figure 2. SDS−PAGE in reducing conditions of deamidated gluten and reaction products: (lane 1) deamidated gluten; (lane 2) Tgase-treated deamidated gluten; (lane 3) Tgase-treated deamidated gluten in the presence of 35% glycerol.

by modifying amide groups in carboxylic groups. These charges, by weakening the protein−protein interactions, should induce a decrease in the cohesion of the film. This is consistent with previous data (Ma et al., 1986) on the effect of deamidated gluten on the rheological behavior of dough, where gluten deamidation caused a complete loss of dough extensibility, pointing out the importance of amide groups and hydrogen bonding forces in the interactions between gluten proteins.

Enzymatic Treatment. Wheat gluten has a high proportion of glutamine (40%) and a low proportion of charged amino acids such as lysine (<1%). The Tgase reaction involves two amino acids, glutamine and lysine, in a stoichiometric ratio to establish an ϵ-(γ-glutamyl)-lysine bond. In gluten deamidated at 20%, the residual glutaminyl residues and the lysine accounted for 32 and <1%, respectively, of the total amino acid content. Consequently, the ability of deamidated gluten to be a substrate for transglutaminase was tested in solution with and without glycerol. Electrophoretic patterns of the reaction products (Figure 2, lanes 2 and 3) showed newly formed polymers unable to penetrate in the stacking gel or in the gel and a simultaneous partial disappearance of the original polypeptides corresponding to deamidated gluten before treatment (lane 1). This result indicates that some of the residual glutamines of the deamidated gluten were still reactive in the enzymatic reaction to form ϵ-(γ-glutamyl)lysine bonds.

Effect of Putrescine Concentration in the Film-Forming Solution. Putrescine, like some other polyamines, is a natural substrate for Tgase (Folk et al., 1980). One or both amino groups of these molecules can react with glutaminyl residues leading either to *N*-(γ-glutamyl)putrescine or to N^1,N^4-bis(γ-glutamyl)-putrescine. When putrescine is incorporated through both primary amino groups, a cross-link between two polypeptide chains occurs. As the lysine content of gluten is very low, the addition of putrescine to the mixture potentially increases the number of intermolecular cross-links. The concentrations of added putrescine correspond to ratios of putrescine added/glutamine from 0 to 0.30 mol/mol. The analysis of the mechanical properties of films obtained without Tgase treatment (Table 1) showed that the addition of

Table 1. Tensile Properties of Deamidated Gluten Films at Various Concentrations of Putrescine

putrescine/Gln (mol/mol)		TS (MPa)	EB (%)	contact angle, $t = 0$
0	− TG	1.14 ± 0.12	376 ± 62	96 ± 7
	+ TG	2.34 ± 0.43	455 ± 43	67 ± 15
0.025	− TG	1.29 ± 0.11	323 ± 27	87 ± 6
	+ TG	2.09 ± 0.16	480 ± 35	75 ± 5
0.045	− TG	1.37 ± 0.06	289 ±16	83 ± 8
	+ TG	1.97 ± 0.13	444 ± 78	63 ± 12
0.09	− TG	1.22 ± 0.07	335 ± 21	103 ± 2
	+ TG	1.96 ± 0.11	515 ± 52	84 ± 9
0.18	− TG	0.84 ± 0.09	350 ± 34	95 ± 4
	+ TG	1.36 ± 0.14	598 ± 64	92 ± 15
0.25	− TG	0.90 ± 0.11	318 ± 52	nd
	+ TG	1.27 ± 0.05	514 ± 28	nd
0.30	− TG	0.89 ± 0.08	252 ± 38	nd
	+ TG	1.07 ± 0.13	419 ± 78	nd

putrescine has a significant effect ($p < 0.05$) on both tensile strength (TS) and elongation at break (EB). At low putrescine concentrations EB was slighly affected but higher putrescine concentrations were required to affect both EB and TS. The simultaneous decrease of these two film properties when increasing quantities of putrescine were added indicated that this molecule did not act as a plasticizer.

When deamidated gluten was used as the single substrate in the film-forming solution, the Tgase reaction induced a simultaneous increase in TS and EB, the values of which were multiplied by 2 and 1.2, respectively. In the case of α_{s1}-casein films, Motoki et al. (1987) showed that the TS and strain of a film treated with guinea pig liver Tgase were about twice as high as those of the control film. Yildirim and Hettiarachchy (1997) also described a similar 2-fold increase in the TS of films based on whey protein isolates and soybean 11S globulin after Tgase treatment. These results obtained for various proteins characterized by different amino acid compositions and structures clearly indicate that these effects on TS and EB reflect the formation of new isopeptidic bonds in the film.

Variance analysis of the results obtained on treated or nontreated films showed that the Tgase treatment and putrescine concentration had significant effects on TS and EB ($p < 0.05$). A significant interaction between Tgase treatment and putrescine concentration was found for TS ($p < 0.05$) but not for EB ($p = 0.315$). Tgase action always increased TS and EB simultaneously. This points to the formation of covalent linkages that reinforce the film but are flexible enough to permit a gain in elongation. The addition of increasing concentrations of putrescine to Tgase-treated films induced a slight and progressive decrease in TS. The effect on EB was nonlinear; at lower putrescine concentrations (<0.18 mol/mol) EB increased and then decreased at higher concentrations. Putrescine is able to react at both of its amino groups. When only a few putrescine molecules are present in the film solution, they could act as bifunctional cross-linkers between two glutaminyl residues, but when their concentration is in excess, some of them probably react at only one extremity and therefore do not create any cross-linking between polypeptides. The highest EB was obtained with a ratio of putrescine/Gln of ~0.18, meaning that a maximum of 36% of glutamine had reacted.

Contact angles between 83 and 103° were measured on the untreated films. Similar values were observed for gliadin films (Sanchez et al., 1998) and are charac-

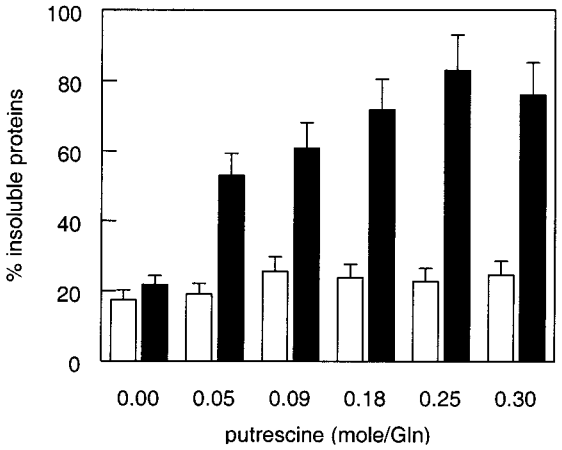

Figure 3. Effect of putrescine concentration on the percentage of insoluble proteins in films: (■) film treated with Tgase; (□) untreated films.

Table 2. Mechanical Properties of Films Prepared with Various Diamine/Gln Ratios (Plasticizer/Protein = 0.35)

diamine/Gln (mol/mol)		EB (%)	TS (MPa)	Young modulus (N/mm^2)	contact angle, $t = 0$
putrescine		327 ± 32	1.12 ± 0.11	6.76 ± 2.99	103 ± 2
0.09	TG	582 ± 57	1.61 ± 0.16	2.15 ± 0.62	84 ± 9
0.3	TG	419 ± 78	1.07 ± 0.13	1.82 ± 0.10	40 ± 7
cadaverine		304 ± 73	0.94 ± 0.06	9.51 ± 3.40	97 ± 4
0.09	TG	522 ± 83	1.24 ± 0.15	2.04 ± 0.34	39 ± 8
diaminohexane		351 ± 81	0.89 ± 0.06	6.50 ± 1.68	44 ± 8
0.09	TG	527 ± 85	1.87 ± 0.25	3.96 ± 0.91	37 ± 5
0.3	TG	302 ± 43	1.02 ± 0.09	8.18 ± 2.60	36 ± 4
diaminooctane		413 ± 118	0.75 ±0.06	4.18 ± 1.43	31 ± 4
0.09	TG	501 ± 73	1.53 ± 0.24	2.92 ± 0.15	
0.3	TG	334 ± 48	0.79 ± 0.06	3.21 ± 0.70	37 ± 6

teristic of hydrophobic surfaces (Extrand and Kumagai, 1997). The enzymatic treatment of the films induced in all cases a decrease in the contact angle of 10−30°, indicating a modification of the surface hydrophobicity of the films. In both types of samples, reference and treated, the angles decreased rapidly with contact time to reach similar values of ∼20−30° after 5 min, suggesting a rapid change in the surface properties. However, Tgase cross-linking induced an initial decrease in the surface hydrophobicity, which could be due to a variation of the protein orientation after cross-linking or to a modification of the number or pore size (Yildrim and Hettiarachchy, 1997).

The solubility of TG-treated films was compared to that of nontreated films (Figure 3). Films obtained from deamidated gluten are soluble at 75% in water, and the presence of putrescine did not modify the film solubility. The action of Tgase induced a decrease in the water solubility of the films. To control the size of proteins in the water-insoluble fractions, they were solubilized in 2% SDS and then analyzed by gel filtration. The selected chromatograms presented in Figure 4 clearly show that a large proportion of the proteins present in these fractions are constituted of proteins of molecular weight $>2 \times 10^6$ eluted in the first peak of the chromatogram. The proportion of high molecular weight polymers increased with the quantity of putrescine added in the film-forming solution until 0.25 mol/mol; therefore, the insolubilization of Tgase-treated deamidated gluten films can be related to the formation of high molecular weight polymers.

Effect of the Addition of Diamines of Various Lengths. The results obtained on films prepared with 0.09 mol/mol of diamine to glutamine (Table 2) were analyzed by a multifactorial analysis of variance for each measured parameter. The effect of Tgase was significant on the mechanical characteristics but not on the contact angle. The enzymatic treatment induced an increase in the TS and the EB and simultaneously a decrease in the Young modulus. The type of diamine affected TS, Young modulus, and contact angle. These results were further analyzed using multiple range tests, which permitted the identification of groups among the types of diamine, but their meaning remains unclear (Table 2). Cadaverine and diaminooctane formed a homogeneous group for TS and Young moduli, and putrescine and diaminohexane constituted a homogeneous group for TS. In the case of the surface hydrophobicity of the films, the addition of diaminohexane or diaminooctane, which have the longest carbon chains, significantly reduced the contact angles in treated or nontreated films. This might suggest that these diamines, by interacting with deamidated gluten, affected the protein orientation in the film and enhanced the proportion of hydrophilic proteic segments at the film surface. As shown by angle contact values, the enzymatic treatment induced in all cases a decrease in the film surface hydrophobicity.

With all types of diamine, the Tgase reaction reduced the water solubility of the films (Figure 5), but no effect of the type of diamine was noted. Polymers of high molecular weight ($MW > 2 \times 10^6$) were formed in treated films as in the case of putrescine.

Even if no effect of the chain length was shown, both types of bond, ϵ-(γ-glutamyl)lysine and N^1,N^4-bis(γ-glutamyl)diamine, induced a simultaneous increase in Eb and TS that was different from the effect of bonds such as $(Tyr)_2$ or bonds formed via formaldehyde treatment, which increased the TS but drastically decreased the EB. At least two differences between these cross-linkings can be noted: their distribution and their nature along the protein chain. The cross-linking position cannot be the only explanation; without diamine, their distribution is related to the position of lysine because the protein is the only substrate for Tgase, whereas when diamines are added, the cross-linking can occur between any glutamines. The nature of the bonds can be implicated and especially their flexibility, which can be related to the length of the side chain of lysine or the length of the carbon chain in the case of diamine. In contrast to dityrosine bonds, they could act like a spacer between the bound polypeptides and may allow a higher mobility of the polypeptides bound together.

The use of higher concentrations of diamines (Table 2) in the film-forming solution did not induce any significant modification of the mechanical properties of the treated films ($p > 0.05$) compared with the untreated films. This confirms the assumption that, at high concentrations of diamines, Tgase produces more diamine grafting than cross-linking.

Conclusion. This study showed that transglutaminase was effective in introducing covalent bonds into films obtained from slightly deamidated gluten. The establishment of these covalent bonds induced the formation of polymers of high molecular weight that were responsible for the greater insolubility of the treated films but a reduced surface hydrophobicity. Mechanical properties showed that the addition of

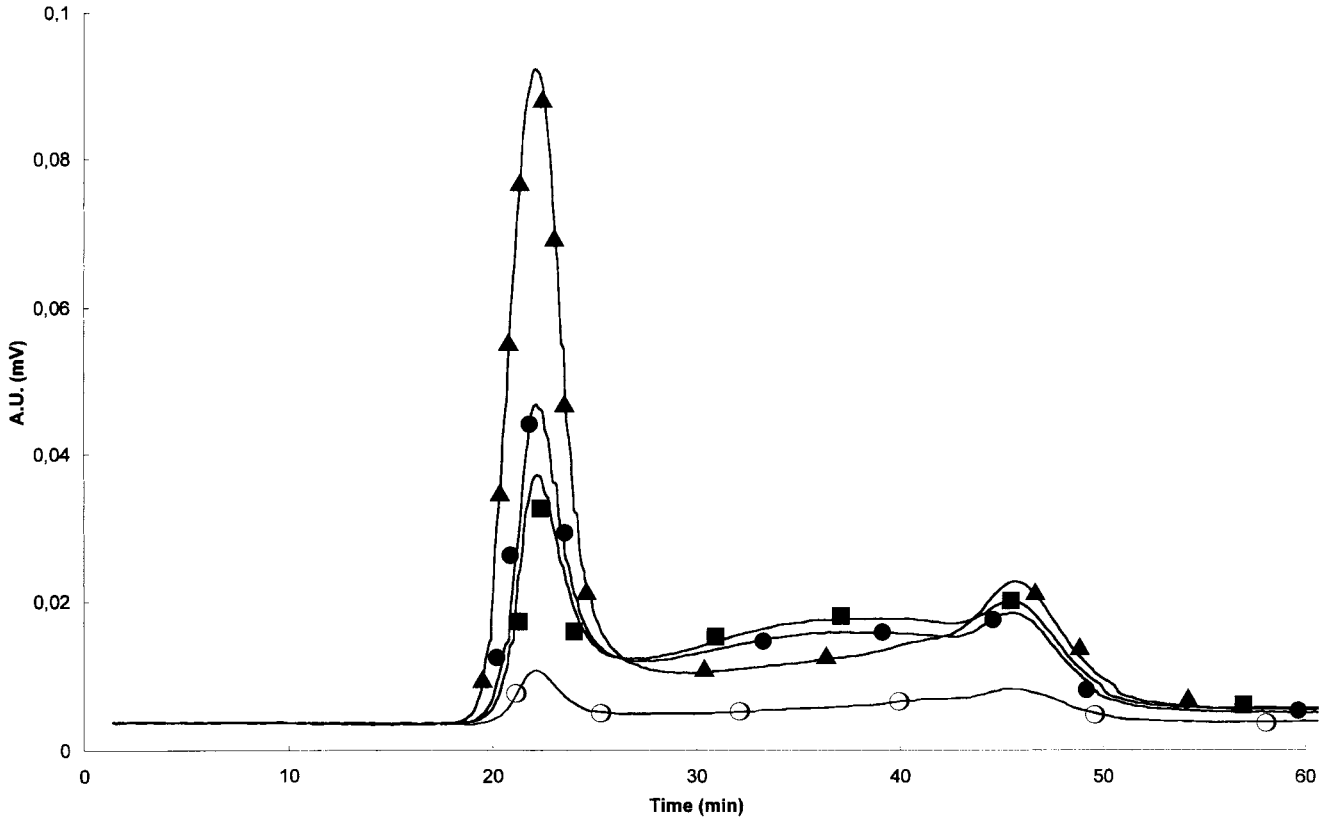

Figure 4. Selected HPLC chromatograms of water-insoluble fractions of deamidated gluten films treated by Tgase on a Superose 6 Prep Grad eluted with 0.025 M borate buffer, pH 8.5, 0.2% SDS. Films were prepared at various putrescine/Gln ratios: (○) 0 mol/mol; (■) 0.05 mol/mol; (●) 0.18 mol/mol; (▲) 0.25 mol/mol.

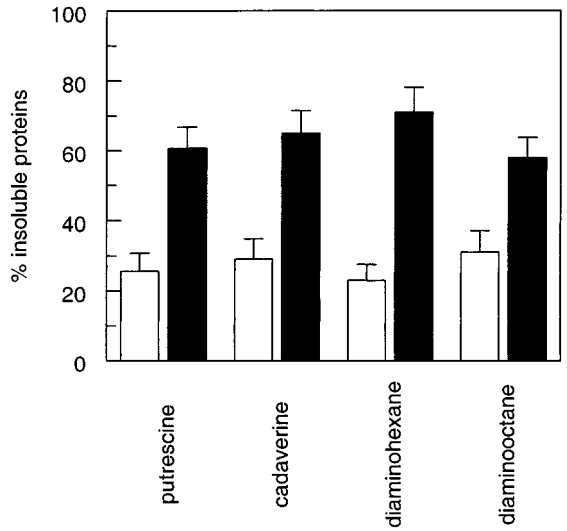

Figure 5. Water solubility of films prepared with amines and enzymatic treatment: (■) film treated with Tgase; (□) untreated films. Percentages are calculated from peak areas obtained by gel filtration.

covalent bonds by way of Tgase increased the film's integrity and heavy-duty capacity as well as its ability to stretch. Moreover, the incorporation of diamines was effective in increasing elongation and preserving tensile strength. These effects can be related to the flexibility of the added bonds due to the length of the lysine side chain or the carbon chain of the diamines. The use of bifunctional chemical agents could provide new information on the effect of the type of bond on the mechanical properties of films. Such studies are in progress.

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