Chemical Reactions in Cottonseed Protein Cross-Linking by Formaldehyde, Glutaraldehyde, and Glyoxal for the Formation of Protein Films with Enhanced Mechanical Properties

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Amino acids involved in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal during protein film formation were identified by an original technique. The entire HPLC amino acid profile (after acid hydrolysis) was studied, along with variations in reactive lysine contents, in films cross-linked or not with increasing quantities of formaldehyde, glutaraldehyde, and glyoxal. This strategy highlighted the formation of acid-resistant lysine derivatives that a simple reactive lysine determination would not have detected. The results—which agree with previously published data—enhance the overall understanding of cross-linking activities that occur in aqueous alkaline solutions during the formation of protein films made with cottonseed flour. Lysine was found to have a key role in protein cross-linking by dialdehydes, with the involvement of tyrosine in the presence of formaldehyde and of arginine in the presence of glyoxal. These results could provide valuable chemical tools for adjusting the mechanical properties of cottonseed protein films.

Keywords: *Protein cross-linking; cross-linking mechanisms; formaldehyde; glutaraldehyde; glyoxal; cottonseed proteins; films*

INTRODUCTION

Cottonseed flour is an interesting raw material for making biodegradable protein films. An aqueous alkaline protein solution is used to form such films by a casting process. Formaldehyde, glutaraldehyde, or glyoxal can be added to the film-forming solution to promote chemical cross-linking of cottonseed proteins (*1*). The percentage of lysine that reacts with these compounds—as determined by assaying the reactive lysine content of cross-linked films—is closely correlated with the puncture strength of the films (*2*). Almost the entire reactive lysine content of cottonseed proteins reacts with glutaraldehyde and glyoxal, but only 50% of this amino acid is involved in cross-linking by formaldehyde (*2*, *3*). Interestingly, the addition of formaldehyde produces films that are more resistant than those obtained after cross-linking by glyoxal or glutaraldehyde. This prompted more in-depth studies on the mechanisms involved in formaldehyde-, glutaraldehyde-, and glyoxal-induced cross-linking of cottonseed proteins by identifying the amino acids involved in the reaction during film formation.

Protein cross-linking by formaldehyde has been used over the past century in leather tanning and other pharmacological applications. In the 1950s, research scientists began investigating the protein cross-linking characteristics of formaldehyde for potential use in the detoxification of proteic toxins in vaccines (*4*, *5*) and to identify amino acids involved in the catalytic reactions of some enzymes (*6*). The first detailed studies on formaldehyde−amino acid reaction mechanisms were undertaken during this period.

Baseline experimental data were obtained by Fraenkel-Conrat et al. (*7*) and Fraenkel-Conrat and Olcott (*8*−*10*), which helped to advance the understanding of the effects of formaldehyde on proteins. The reaction of formaldehyde with lysine is a two-step process. First, ϵ-NH_2 residues and formaldehyde form aminomethylol derivatives. This very quick reversible reaction is optimal at highly alkaline pH (*11*).

An irreversible much slower elimination reaction then leads to the formation of inter- and intramolecular methylene linkages. The formation of irreversible methylene linkages is pH-dependent (*12*).

The formaldehyde−protein reaction is not lysine-specific and depends closely on the experimental conditions. Blass et al. (*4*) obtained evidence of the nonspecificity of formaldehyde by isolating two compounds, one comprising a lysyl residue and a tyrosyl residue linked by a methylene bond, and the other combining two lysine molecules and a tyrosine molecule, also linked by a methylene bond, in an anatoxin hydrolysate (nontoxic immunogen). These products are the result of a Mannich reaction and resistant to acid hydrolysis. The correlated decreases in lysine and tyrosine levels in formaldehyde-treated protein hydrolysate are of interest for understanding how these compounds are formed. The formaldehyde−protein reaction does not always give rise to stable derivatives in acidic media. Bizzini and Raynaud (*5*) identified acid-labile derivatives comprising methylol groups or resulting from methylene linkages between two amine functions. These derivatives are regenerated from formaldehyde by acid hydrolysis (*6*).

The acid hydrolysis stability of formaldehyde in association with residues other than tyrosine and lysine is not fully understood. Some authors have pointed out the involvement of serine and threonine in the formation of acid-resistant bonds with tyrosine and histidine (*9*, *10*). Bizzini et al. (*13*) demonstrated a slight decrease

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in arginine and histidine levels in tetanus anatoxin acid hydrolysates. Lysine contents in unstable derivatives cannot be determined simply on the basis of total amino acids in cross-linked proteins after acid hydrolysis. They could be indirectly determined by assaying reactive lysine after protein cross-linking by formaldehyde.

Protein cross-linking by glutaraldehyde has often been used to stabilize proteins (*14*−*18*). The results of a very sophisticated study by Monsan et al. (*16*) explain the reactivity of glutaraldehyde with proteins**.** In highly acidic commercial solutions (pH 3.1), glutaraldehyde is in equilibrium with its cyclical hemiacetal form and cyclical hemiacetal polymers.

In neutral or basic medium, dialdehyde condenses and gives rise to α,β-unsaturated polymers of various lengths, with more polymers formed as the pH becomes more basic. Most glutaraldehyde-induced protein cross-linking reactions are carried out at highly alkaline pH, with α,β-unsaturated molecules predominating. The aldehyde functions of these molecules react with primary amines to form imines (Schiff bases) stabilized by resonance and resistant to acid hydrolysis (*16*). Terminal aldehyde functions of polyglutaraldehyde, which are not conjugated due to their position at the end of the chain, cannot form acid hydrolysis resistant products. Nevertheless, nonconjugated imine bonds can be stable in hydrophobic microenvironments (*16*). A secondary Michael reaction can occur when the protein concentration is higher than that of the cross-linking agent; that is, amine is added to the ethylene bond of α,β-unsaturated polymers. Michael addition products are not resistant to acid hydrolysis (*17*).

Protein cross-linking by glyoxal involves a key reaction with arginine guanidyl groups. This reagent is preferentially active at alkaline pH. The first step of the reaction resembles Schiff base formation and then gives rise to different products. Secondary reactions can also occur with primary amines and thiol groups. Reaction of glyoxal with bovine serum albumin (BSA), at pH 9, modifies more than 80% of arginyl residues and 30% of lysyl groups (*19*). Most glyoxal-induced modifications of arginyl residues at alkaline pH are reversible. Glyoxal is not commonly used as a protein cross-linking agent. Glyoxal derivatives such as phenylglyoxal and azidophenylglyoxal are usually inserted in cross-linking agent molecules directed against arginine (*20*).

The experimental strategy aimed at identifying amino acids active in protein cross-linking was based on the studies of Blass et al. (*4*). This involved detecting the formation of covalent bonds resistant to acid hydrolysis between certain amino acids present in films cross-linked to various extents by formaldehyde, glutaraldehyde, or glyoxal. Reactive lysine in the same films was also assayed while total amino acid analyses were performed.

MATERIALS AND METHODS

Raw Material. Delipidated glandless cottonseed flour was obtained after oil extraction (extrusion−solvent process) from glandless cottonseed kernels (cv. CIRAD 411) at the Trituraf factory (Ivory Coast).

Protein Determination of Film-Forming Solutions or Cottonseed Flour. The total nitrogen content of film-forming solution dry matter or cottonseed flour was determined by combustion with a Leco FP 428 analyzer (Leco Corp. 3000). Protein content was calculated by using the nitrogen conversion factor 5.3 recommended by De Rham (*21*).

Preparation and Dry Matter Content Determination of Film-Forming Solutions. Glandless delipidated flour was soaked for 1 h in water (20% w/v), at pH 10, with triethylammonium (Merck, Schuchardt, Hohenbrunn, Germany) at 40 °C. The mixture was then centrifuged (500*g*, 2 × 5 min). The dry matter (dm) content of the film-forming solution was then determined by drying an aliquot of ∼2 g of solution at 105 °C for 2 h. By subtracting the weight of sample after drying from the weight of initial wet sample, the dry matter content was calculated in grams per 100 g of film-forming solution.

Protein Cross-Linking Treatments. Protein treatments with formaldehyde (HCHO) 40% w/v (Carlo Erba, Rodano, Italy), glutaraldehyde 5.6 M (Fluka Chimie AG, Buchs, Switzerland), or glyoxal 40% w/v (Merck) were conducted in the centrifuged film-forming solution obtained from glandless delipidated flour at room temperature for 1 h, up to 1.20 mol/100 g of dispersed proteins.

Film Preparation. Glycerol (Carlo Erba) was added as plasticizer to the film-forming solution at 20 g/100 g of dm in the film-forming solution. Specific volumes of the film-forming solution were then poured into polystyrene crystal Petri dishes to obtain 20 mg/cm^2 dm. The solvent was first evaporated at room temperature for 24 h and then at 60 °C for 24 h.

HPLC Reactive Lysine Analysis. Approximately 500 mg of finely cut film or 300 mg of cottonseed flour was analyzed using the HPLC method of Marquié et al. (*2*). The HPLC equipment consisted of a Waters (Waters Associates, Milford, MA) chromatograph model 7010 connected to a high-pressure pump (model 510), a Rheodyne injector (Cotati, CA) equipped with a 20 μL loop, and a UV 481 spectrophotometer (Waters Associates). Detector output was recorded and integrated using a CR 6A recorder−integrator (Shimadzu, Kioto, Japan).

HPLC Amino Acid Analysis. Acid hydrolysates obtained from flours or ground film samples were analyzed by HPLC after precolumn derivatization of amino acids by 4-(dimethylamino)azobenzene-4′-sulfonyl chloride (dabsyl chloride). The HPLC equipment included a Spectra Physics chromatograph (Thermo Separation Products, Darmstadt, Germany), including a P200-330 pump (for analyses over a binary gradient), an automatic AS3000-024 injector, a precolumn autosampler (AS 3000 VIV/OVEN/OREP), and a UV 150-511 detector.

Protein hydrolysis was performed as follows: around 20 mg of dried cottonseed flour or 15 mg of dried ground film was accurately weighed in a hydrolysis tube (Pierce Chemical Co., Rockford, IL). The hydrolysis medium included the test sample, 1 mL of 4 N methanesulfonic acid containing tryptamine (Pierce Chemical Co.), and 50 μL of an aqueous norleucine solution (3×10^{-2} M) used as internal standard. Oxygen was flushed several times from the system under slight vacuum conditions and nitrogen injection. The airtight tube was then heated at 110 °C for 24 h. The hydrolysate was then neutralized with 1 mL of 4 N NaOH, poured into a graduated flask and topped up to 5 mL with HPLC grade water, and finally filtered through 0.2 μm membrane filter (Waters Associates, Millipore) before derivatization.

Amino acid derivatization with dabsyl chloride (Sigma Chemical Co., St. Louis, MO) was carried out using an automatic precolumn autosampler (Thermoseparation Product AS 3000).

The reaction mixture was composed of 50 μL of 0.1 M sodium bicarbonate buffer (pH 9), 100 μL of a dabsyl chloride solution (4 mM) in acetonitrile, and 5 μL of hydroysate containing ∼30 nmol/μL of amino acids. The mixture was stirred and heated at 70 °C for 10 min, and then 345 μL of phosphate buffer (5 mM, pH 7), was added. Then, 20 μL of mixture was injected in the column.

A column (Merck) packed with Spherisorb ODS 2 (250 mm × 4.0 mm, 5 μm), thermostated at 40 °C, was used for the chromatography analysis. The amino acids were clearly separated over the elution gradient described in Table 1. The flow rate of the mobile phase was 1.2 mL/min. At the column outlet, dabsylated amino acids were detected by spectrophotometry at 436 nm. They were quantified against an external standard with three solutions prepared with amino acid standards (Sigma Chemical Co.) solubilized in 0.1 M sodium hydrogeno-

Table 1. Characteristics of the Elution Gradient for the Separation of Amino Acids Derivatized with Dabsyl Chloride on a Spherisorb ODS-2 Column (250 mm × 4.0 mm, 5 μm)

time (min)	solvent A[a] % (vol)	solvent B[b] % (vol)
0	97	3
1	97	3
4	81	19
8	79	21
12	77	23
16	70	30
27	45	55
28	0	100
31	97	3
45	97	3

[a] Solvent A was composed of acetonitrile/water/glacial acetic acid (180:820:1.4, v/v. [b] Solvent B was composed of an acetonitrile/isopropanol mixture (400:600, vv).

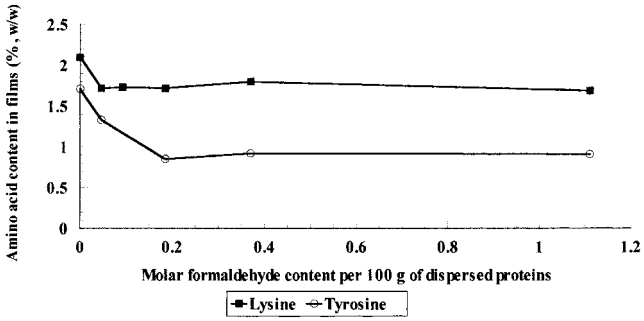

Figure 1. Changes in total lysine and tyrosine contents in cottonseed protein based films cross-linked by formaldehyde as a function of the molar formaldehyde content in the film-forming solution (per 100 g of dispersed proteins). Films were made with delipidated glandless cottonseed flour.

carbonate (pH 9), respectively containing 20, 75, and 150 μg/mL of each of 17 different amino acids—aspartic acid (Asp), glutamic acid (Glu), serine (Ser), threonine (Thr), glycine (Gly), alanine (Ala), proline (Pro), arginine (Arg), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), cysteine (Cys), lysine (Lys), histidine (His), and tyrosine (Tyr)—and 8, 30, and 60 μg/mL of norleucine (tryptophan was not assayed, whereas asparagine and glutamine were assayed as aspartic and glutamic acids). The chromatograms were processed with a Hewlett-Packard integrator model 35900 (Hewlett Packard Intercontinental, Palo Alto, CA).

The effect of formaldehyde, glutaraldehyde, and glyoxal concentrations in the film-forming solution on the extent of the cross-linking reaction was calculated as the percentage of amino acid reacting with the cross-linking agent (percent modified amino acid) during film formation

$$\%\ \text{modified amino acid} = \frac{(\%\ AA_i - \%\ AA_c) \times 100}{\%\ AA_i} \quad (1)$$

where % AA_i is the percentage of an amino acid in non-cross-linked films and % AA_c is the percentage of the same amino acid in cross-linked films.

RESULTS AND DISCUSSION

Protein Cross-Linking by Formaldehyde. Comparison of the amino acid contents of films cross-linked or not by formaldehyde revealed variations only in lysine and tyrosine concentrations. Figure 1 shows the concentration patterns for these two amino acids (percent, w/w, dry basis) relative to the molar formaldehyde content in the film-forming solution (moles per 100 g of dispersed proteins). The percentage of lysine decreased slightly at 0.05 mol of formaldehyde and then remained unchanged for higher cross-linking agent concentrations. Tyrosine content declined by >50% within a formaldehyde content range of 0−0.18 mol/100 g of dispersed proteins and then leveled off. For a formaldehyde concentration of 0.05 mol/100 g of dispersed proteins in the film-forming solution, the molar lysine concentration that triggered the formation of acid-resistant compounds in the film was identical to that of tyrosine.

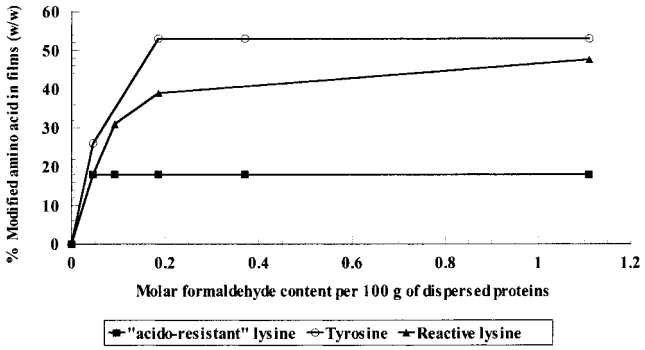

Figure 2. Changes in the percentage of total lysine, tyrosine, and reactive lysine modified by formaldehyde in cottonseed protein based films as a function of the molar formaldehyde content in the film-forming solution (per 100 g of dispersed proteins). Films were made with delipidated glandless cottonseed flour.

Figure 2 compares the percentage of modified reactive lysine with that of tyrosine and lysine involved in the formation of acid-resistant compounds relative to the molar formaldehyde content in the film-forming solution (moles per 100 g of dispersed proteins). For a molar formaldehyde concentration of 0.05, which corresponds to a molar ratio of ∼1 (HCHO/reactive Lys + Tyr) in the film-forming solution, the percentages of modified reactive lysine and of lysine involved in the formation of acid-resistant compounds were identical (18%). Acid-resistant covalent bonds were formed after reaction of lysine with formaldehyde.

Because equivalent quantities of lysine and tyrosine were involved at this cross-linking stage but not detected in the amino acid assay, protein cross-linking probably involved a lysyl residue and a tyrosyl residue to form acid-resistant Mannich compounds, as demonstrated by Blass et al. (*4*). At molar formaldehyde contents >0.05, the percentage of modified reactive lysine in films gradually increased while the total lysine content remained steady. Lysine reacted with formaldehyde under these conditions to produce acid-labile compounds. Formaldehyde released during acid hydrolysis of these compounds can subsequently react with tyrosine, as shown by Blass (*22*). This explains the decline in tyrosine content (∼50%) in the corresponding hydrolysates (Figure 1). These compounds can be aminomethylol derivatives or molecules with a methylene bond between two lysyl groups, that is, acid-labile molecules as noted by Monsan et al. (*16*).

Previous studies revealed a linear correlation between the mechanical resistance of cross-linked films and the percentage of reactive lysine modified by formaldehyde (*2*). The puncture strength of films could be explained by the formation of lysine−tyrosine Mannich derivatives and of covalent bonds between two lysyl residues, as indicated in Figure 3. In these conditions, the lysine−tyrosine reaction seemed to prevail because it was the only reaction that occurred at lower formaldehyde

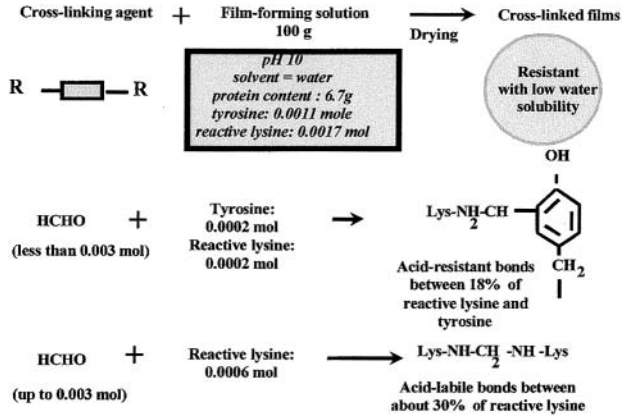

Figure 3. Postulated mechanism of protein cross-linking by formaldehyde under alkaline conditions during the preparation of cottonseed protein based films.

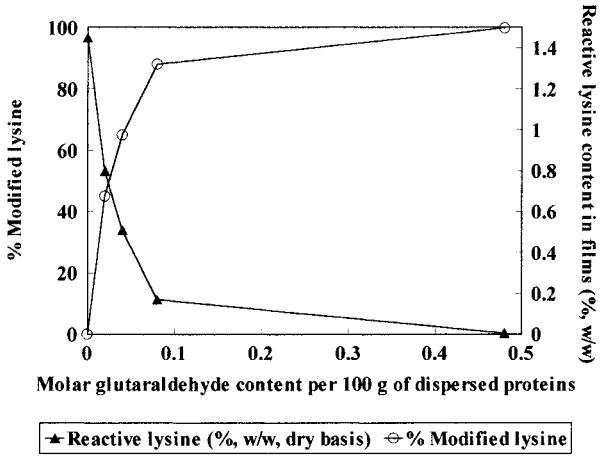

Figure 4. Changes in the percentage of reactive lysine modified by cross-linking with glutaraldehyde and of reactive lysine content in films as a function of the molar glutaraldehyde content in the film-forming solution (per 100 g of dispersed proteins). Films were made with delipidated glandless cottonseed flour.

concentrations. Conformational changes in protein chains likely occurred thereafter as a result of lysine−tyrosine linkages, thus reducing potential encounters of other reactive species with formaldehyde (Lys and Tyr). The level of reactive lysine modified by the cross-linking treatment was therefore never >50%, even in the presence of a high excess level of formaldehyde relative to the quantity of these two reactive amino acids.

Protein Cross-Linking by Glutaraldehyde. Comparison of amino acid profiles of cross-linked and non-cross-linked films revealed that glutaraldehyde reacted chiefly with lysine. The lysine content that was not detected by total amino acid assay paralleled the decrease in reactive lysine content in cross-linked films. This seemed to indicate that all of the lysine reactive to glutaraldehyde induced the formation of acid hydrolysis resistant compounds.

Figure 4 shows changes in reactive lysine contents in films (percent, w/w, dry basis) and the percentage of reactive lysine modified according to the molar glutaraldehyde concentration (moles per 100 g of protein) in the film-forming solution. A 0−0.08 mol increase in the glutaraldehyde content led to the formation of Schiff bases with 88% of the reactive lysine. Because the quantity of lysine that reacts with glutaraldehyde is proportional to the film puncture strength (*2*), it was

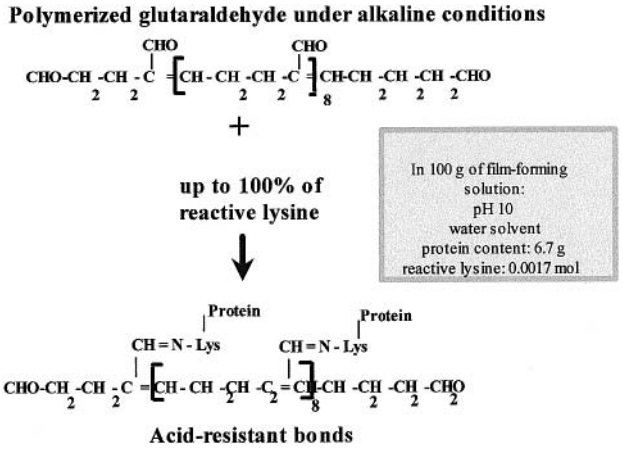

Figure 5. Postulated mechanism of protein cross-linking by glutaraldehyde under alkaline conditions during the preparation of cottonseed protein based films.

concluded that the Schiff bases formed were involved in protein chain cross-linking.

In film-forming solutions containing 0.08 mol of glutaraldehyde/100 g of dispersed proteins, 0.022 mol of lysine (per 100 g of proteins) actually reacted with the cross-linking agent. Around 4-fold higher amounts of glutaraldehyde were injected in the film-forming solution relative to the lysine content modified by the cross-linking treatment. Note that glutaraldehyde polymerizes in alkaline conditions to form unsaturated compounds that could subsequently react with reactive lysine. Korn et al. (*23*) found that the mean molar concentration of glutaraldehyde consumed during glutaraldehyde treatment of proteins was 4-fold higher than the molar concentration of lysine involved. The results obtained, matched with those of Korn et al. (*23*), suggest that the polyglutaraldehyde molecule that reacted with cottonseed proteins was generally composed of eight glutaraldehyde molecules forming covalent bonds between two lysyl groups (Figure 5).

The experimental stoichiometry would have to be boosted to 18 by using 0.48 mol of glutaraldehyde/100 g of protein (Figure 4) to mobilize the residual lysine in the film-forming solution (∼12% of the initial reactive lysine).

Protein Cross-Linking by Glyoxal. Comparison of the amino acid profiles of cross-linked and non-cross-linked films showed that arginine and lysine were modified by glyoxal, leading to the formation of acid hydrolysis resistant compounds. Changes in the total contents of these two amino acids in films and in the reactive lysine content (percent, w/w, dry basis) according to the glyoxal content in the film-forming solution (moles per 100 g of dispersed proteins) are shown in Figure 6.

An increase in the glyoxal concentration in the film-forming solution led to a steady decrease in the lysine content, whereas the arginine content seemed to level off at a molar glyoxal concentration of 0.1 and higher. The results also highlighted similar variations in the total lysine and reactive lysine contents as a function of the cross-linking agent concentration. This indicated that the overall lysine−glyoxal reaction led to the formation of acid-resistant compounds. This was a total reaction in the presence of 0.4 mol of glyoxal (per 100 g of dispersed proteins). Amino acid analysis in glyoxal cross-linked films also revealed a compound that had coeluted with cysteine, the content of which content

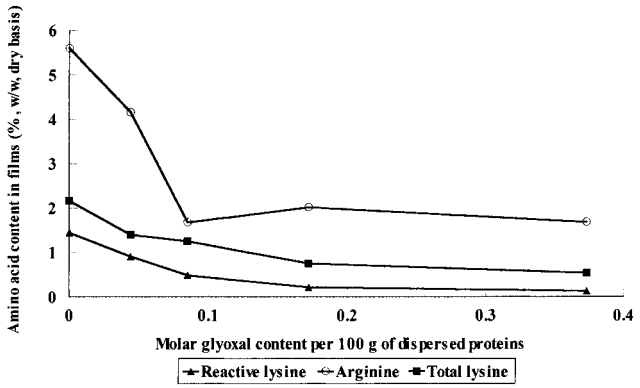

Figure 6. Changes in the reactive lysine, arginine, and total lysine contents in cottonseed protein based films cross-linked by glyoxal as a function of the molar glyoxal content in the film-forming solution (per 100 g of dispersed proteins). Films were made with delipidated glandless cottonseed flour.

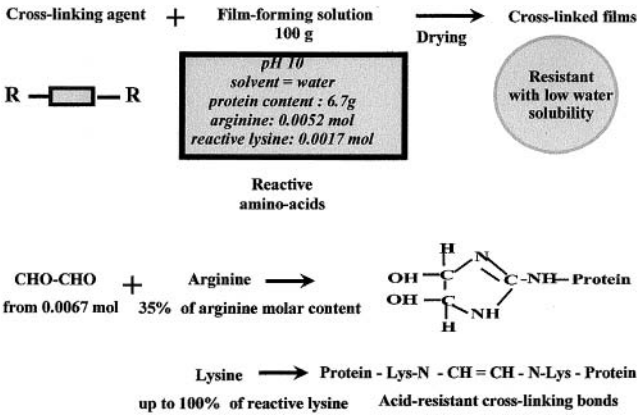

Figure 7. Postulated mechanism of protein cross-linking by glyoxal under alkaline conditions during the preparation of cottonseed protein based films.

increased proportionally with the percentage of arginine modified by the cross-linking treatment.

Arginine was probably not involved in cross-linking because of a probable glyoxal−arginine reaction mechanism (Figure 7). The correlation between the percentage of reactive lysine modified during cross-linking and the film puncture strength (*2*) also indicates that lysyl residues play a key role in protein cross-linking by glyoxal. Glyoxal, which is mainly known for its reactivity with arginine, is thus an excellent protein cross-linking agent because of its reactivity with lysine, as noted here at highly alkaline pH.

A strategy that includes the analysis of amino acids involved in acid hydrolysis resistant compounds, combined with a reactive lysine assay, in protein films cross-linked to various extents by formaldehyde, glutaraldehyde, and glyoxal enabled the indirect determination of the main reactive amino acids. Under the experimental conditions used here, the molecular basis for the strengthening of mechanical properties of cottonseed protein based films was determined by using reagents, concepts, and their implementation described in the 1960s for studies on the reactivity of pure proteins in diluted solution. The key role of tyrosine in the early formation of covalent bonds in formaldehyde-induced cross-linking of cottonseed proteins was thus highlighted. The resulting formation of Mannich derivatives with tyrosine and lysine then governed the extent of the reaction with lysine. It was then demonstrated, contrary to the results reported by Tunnicliff and Ngo (*19*), that there was a preferential reaction of lysine with glyoxal.

This discrepancy could be explained by differences in the nature of the proteins and/or the experimental conditions. Morever, these results and others reported previously (*2*) could be valuable chemical tools for modifying the mechanical properties of cottonseed protein films.

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