HPLC Determination of the Reactive Lysine Content of Cottonseed Protein Films To Monitor the Extent of Cross-Linking by Formaldehyde, Glutaraldehyde, and Glyoxal

Catherine Marquié,* Anne-Marie Tessier, Christian Aymard,[†] and Stéphane Guilbert[‡]

Laboratory of Cotton Technology, CIRAD-CA, CIRAD, 2477 Avenue du Val de Montferrand, B.P. 5035, 34032 Montpellier Cedex 1, France

A sensitive and reproductible HPLC method to determine the reactive lysine content of films made from cottonseed flour was developed. This method is applied to follow the cross-linking of cottonseed proteins by formaldehyde, glutaraldehyde, or glyoxal. The cross-linking of these proteins resulted in the decrease of reactive lysine content within the film. The maximum puncture force of the films is correlated with the modified reactive lysine content after cross-linking treatments. Of the three cross-linking agents, formaldehyde was the most effective to enhance the maximum puncture force of the films despite its moderate reaction with only 50% of reactive lysine in the films. By contrast, glutaraldehyde, which reacted with nearly 100% of lysine, led to less resistant films. The results were interpreted as the impact of the molecular structure of the cross-link bridges on the mobility between protein chains.

Keywords: *Cross-linking; HPLC; cottonseed proteins; films; reactive lysine*

INTRODUCTION

Biopolymers could be used to formulate biodegradable packaging, e.g., replace short-life plastics, in conjunction with plastic recycling programs. Of these biopolymers, proteins, which are nonmonotonous polymers, offer high potential to enhance film structure by forming intermolecular and intramolecular linkages. Improved functional properties were obtained by either choosing low-solubility proteins such as corn zein (Aydt et al., 1991; Guilbert and Biquet, 1989; Park et al., 1994), wheat gluten (Gennadios and Weller, 1990; Gontard, 1994), and fish myofibrillar proteins (Cuq et al., 1996a,b) or using chemical cross-linking treatments (Gennadios and Weller, 1992; Jane et al., 1993; Marquié et al., 1995, 1996). The rheological comportment of materials depends mainly on the structure of the polymer, on the chemical bonds involved in film cohesion, and on the interaction of the polymer with the environment. Frequently, a plasticizer such as sorbitol or glycerol is added to the protein film formulation to reduce internal hydrogen bonding and to decrease the intermolecular forces along polymer chains (Lieberman and Gilbert, 1973), thus imparting increased film flexibility while decreasing film maximum puncture force.

Recently, Marquié et al. (1995) prepared cast films from cottonseed flours. Chemical cross-linking of cottonseed proteins by formaldehyde or glutaraldehyde increased maximum puncture force and decreased solubility of films in water. Glutaraldehyde, formaldehyde, and glyoxal are cross-linking agents capable of reacting with amino acid side chains, particularly with the lysine ϵ-NH$_2$ group, to form Schiff bases (Fraenkel-Conrat et al., 1947; Means and Feeney, 1968; Galembeck et al., 1977; Monsan et al., 1975; Peters and Richards, 1977).

Chemical cross-linking of proteins leads to important conformational changes and to chemical and physical property modifications in macromolecules. As a first approach, Marquié et al. (1995) used simple criteria (maximum puncture force, puncture deformation, and film solubility) to assess the efficiency of chemical treatments. In the present study the amount of lysine that did not react with cross-linking agents (reactive lysine) during cottonseed protein film formation was used to assess the degree of protein cross-linking.

The widely used method described by Carpenter (1960) involves 1-fluoro-2,4-dinitrobenzene (FDNB) to tag lysine residues and then measurement of N-ϵ-dinitrophenyled lysine (DNP-lysine) spectrophotometrically after acid hydrolysis. The presence of carbohydrates in the sample may lead to formation of interfering compounds and therefore cause the amount of reactive lysine to be overestimated (Matheson, 1968). Attempts have been made to use chromatography to separate DNP-lysine from interfering amino acids and from other compounds formed during hydrolysis of carbohydrates contained in foods (Holm, 1971). The use of high-pressure liquid chromatography (HPLC) to determine reactive lysine was studied by Peterson and Warthesen (1979) with a μBondapack C18 column and a mobile phase composed of 20% acetonitrile and 80% 0.01 M acetate buffer, pH 4.0. The wavelength used to detect DNP-lysine was 436 nm.

In this study we have first modified the Peterson and Warthesen (1979) procedure and established new experimental conditions that provide a quantitative determination of reactive lysine in films made from cottonseed flour. Subsequently, this methodology was used to investigate the cross-linking of cottonseed proteins by formaldehyde, glutaraldehyde, or glyoxal in films and to analyze the relation between protein cross-linking and film maximum puncture force.

* Author to whom correspondence should be addressed (fax +33-467-61-56-67; e-mail marquie@cirad.fr).

† Present address: Laboratoire de Chimie Biomoléculaire, URA CNRS, Université de Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.

‡ Present address: UFR Technologie des Aliments et des Bioproduits, ENSA, 9 Place Viala, 34060 Montpellier, France.

Reactive Lysine Content

J. Agric. Food Chem., Vol. 45, No. 3, 1997 **923**

MATERIALS AND METHODS

Raw Material. Glandless delipidated flour was obtained after oil extraction (extrusion−solvent process) from glandless cottonseed kernels (variety CIRAD 411) at the TRITURAF factory (Ivory Coast)**.**

Preparation and Dry Matter Content Determination of the Film-Forming Solutions. Glandless delipidated cottonseed 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 content of the film-forming solution was then determined by drying an aliquot of about 2 g of solution at 105 °C for 2 h. By subtraction of the weight of sample after drying from the weight of initial wet sample, the dry matter content was calculated in g 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, Suisse), or glyoxal 40% w/v (Sigma chemical CO, St Louis) were conducted in the centrifuged film-forming solution at a room temperature for 1 h up to 44:1 molar ratio with reference to reactive lysine.

Film Preparation. Glycerol (Carlo Erba, Rodano, Italy), used as a plasticizer, was added to the film-forming solution at a concentration of 20 g per 100 g of dry matter content of the film-forming solution. Defined volumes of the film-forming solution were then poured into polystyrene crystal Petri dishes to obtain 20 mg/cm² of dry matter. The solvent was first evaporated at room temperature for 24 h and then at 60 °C for 24 h.

Mechanical Tests. The maximum puncture force of the film was evaluated using a RHEO TA.XT2 texturimeter (Champlan, France). Films for measurement were equilibrated in a 56% relative humidity atmosphere (NaBr saturated solution, 25 °C, 72h) cut in 6 cm diameter disks fixed in an annular device. A cylindrical probe (12.6 mm² surface) was moved perpendicularly at the film surface at constant speed (1 mm/s) until it passed through the film, and the force versus deformation curve was recorded. For each type of film, puncture tests were replicated three times with specimens taken from different cast films. Maximum puncture force was determined at the break point and expressed in newtons (N) per 100 μm thickness. The mean variation coefficient never exceeded 5%. The film thickness was measured at 10 points with a micrometer (Braive Instrument, Checy, France) to the nearest 10^{-6} m.

Reactive Lysine Analysis by HPLC. We initially applied the Peterson and Warthesen (1979) procedure by using a Spherisorb ODS2 (250 mm × 4.5 mm, 5 μm) column to determine the reactive lysine content of films. To perform the reactive lysine content determination by HPLC, we then developed a new method as described below. Approximately 500 mg of finely cut film was accurately weighed and placed in a 500 mL boiling flask. Quantities of 20 mL of 8% (w/v) sodium bicarbonate in water and 30 mL of ethanol solution containing 2.5% of fluorodinitrobenzene (Merck Schuchardt, Hohenbrunn, Germany) were added. The sample was then shaken for 2 h at room temperature, and the ethanol was boiled off in a hot water bath until a weight loss of 30 g was obtained. A quantity of 60 mL of 8 M HCl was added, and the sample was refluxed for 18 h. After hydrolysis, the sample was filtered while hot into a volumetric flask and brought to volume with water. Approximately 4 mL was then filtered through a 0.2 μm membrane filter (Waters Associates, Millipore, Milford, MA). The DNP-lysine was then separated and quantified by HPLC.

The HPLC equipment consisted of a Waters chromatograph Model 7010 connected to a high-pressure pump Model 510 (Waters Associates, Milford, MA), a Rheodyne injector (Cotati, CA,) equipped with a 20 μL loop, and an UV 481 spectrophotometer (Waters Associates, Milford, MA). The separation was accomplished at room temperature on a column (Touzard et Matignon, Les Ulis, France) packed with Spherisorb ODS-2 (250 mm × 4.5 mm, 5 μm) with a mobile phase of 20% acetonitrile and 80% 0.1 M potassium hydrogen phthalate

buffer at pH 3.9. The flow rate of the mobile phase was 1 mL/ min. The wavelength used to detect DNP-lysine was 364 nm. Four external standard solutions of 2,4-DNP-lysine (Sigma Chemical, CO, St Louis, MO) at concentrations of 5, 10, 25, and 50 μg/mL were used for peak identification and quantification. Detector output was recorded and integrated using a CR 6A recorder-integrator (Shimadzu, Kioto, Japan).

The effect of formaldehyde, glutaraldehyde, or glyoxal concentration in the film-forming solution on the extent of the cross-linking reaction was calculated as the percentage of lysine that reacted with the cross-linking agent (% modified reactive lysine) during film formation:

$$\% \text{ modified reactive lysine} = \frac{(\% \text{ Lys}_C - \% \text{ Lys}_R) \times 100}{\% \text{ Lys}_c} \tag{1}$$

where % Lys_C is the percentage of reactive lysine in noncross-linked films and % Lys_R is the percentage of reactive lysine in cross-linked films.

The limits of quantification and detection were determined as the amount of injected DNP-lysine that resulted in peaks that were 10-fold and 2-fold the baseline noise (Huber, 1993).

The absorption spectrum of DNP-lysine was obtained using a diode array detector (HP 1050) connected to a Chemstation HP 1050 for data analysis (Hewlett-Packard, Palo Alto, CA).

RESULTS AND DISCUSSION

Reactive Lysine Analysis by HPLC. The chromatographic conditions recommended by Peterson and Warthesen (1979) were first applied to analyze a hydrolysate obtained from dinitrophenyled cottonseed proteins. Under these conditions DNP-lysine is not well separated (Figure 1a). In addition, Figure 2 illustrates that DNP-lysine shows maximum absorption of UV radiation at 364 nm. Thus, to improve sensitivity, we measured DNP-lysine at 364 nm instead of 436 nm as recommended by Peterson and Warthesen (1979). To separate each coumpound, we modified the Peterson and Warthesen procedure by using a mobile phase composed of 20% acetonitrile and 80% 0.1 M potassium hydrogenophthalate buffer, pH 3.9. Figure 1b shows a chromatogram at 364 nm for a hydrolysate of dinitrophenyled cottonseed proteins obtained with this buffer. Under these conditions good separation was obtained between DNP-lysine and other compounds. A linear relationship was observed between the peak area and concentration injected into the column from 0.285 to 2.85 nmol of DNP-lysine (R^2 = 0.999). The limits of detection and quantification for DNP-lysine were 0.0057 and 0.029 nmol injected, respectively. We investigated the repeatability of the method by analyzing the DNP-lysine content of two samples of film hydrolysates with three repetitions by sample. The mean variation coefficient observed was 0.5%. Following this study, the DNP-lysine content of each sample of film was determined twice. Under these conditions, the mean variation coefficient obtained for 22 film hydrolysate samples was 1.9%.

Impact of Protein Cross-Linking Treatment on Maximum Puncture Force and Reactive Lysine Content of Films. Reaction with formaldehyde, glutaraldehyde, or glyoxal was conducted with a cross-linking agent/reactive lysine molar ratio ranging from 0 to 44 in consideration of the 1.69 mmol of potentially reactive lysine contained in 100 g of cottonseed film-forming solution at pH 10. Figure 3 gives the maximum puncture force values of films obtained from glandless delipidated flour after treatment with formaldehyde, glutaraldehyde, or glyoxal. A formaldehyde or glyoxal/ reactive lysine molar ratio of 8 or a glutaraldehyde/
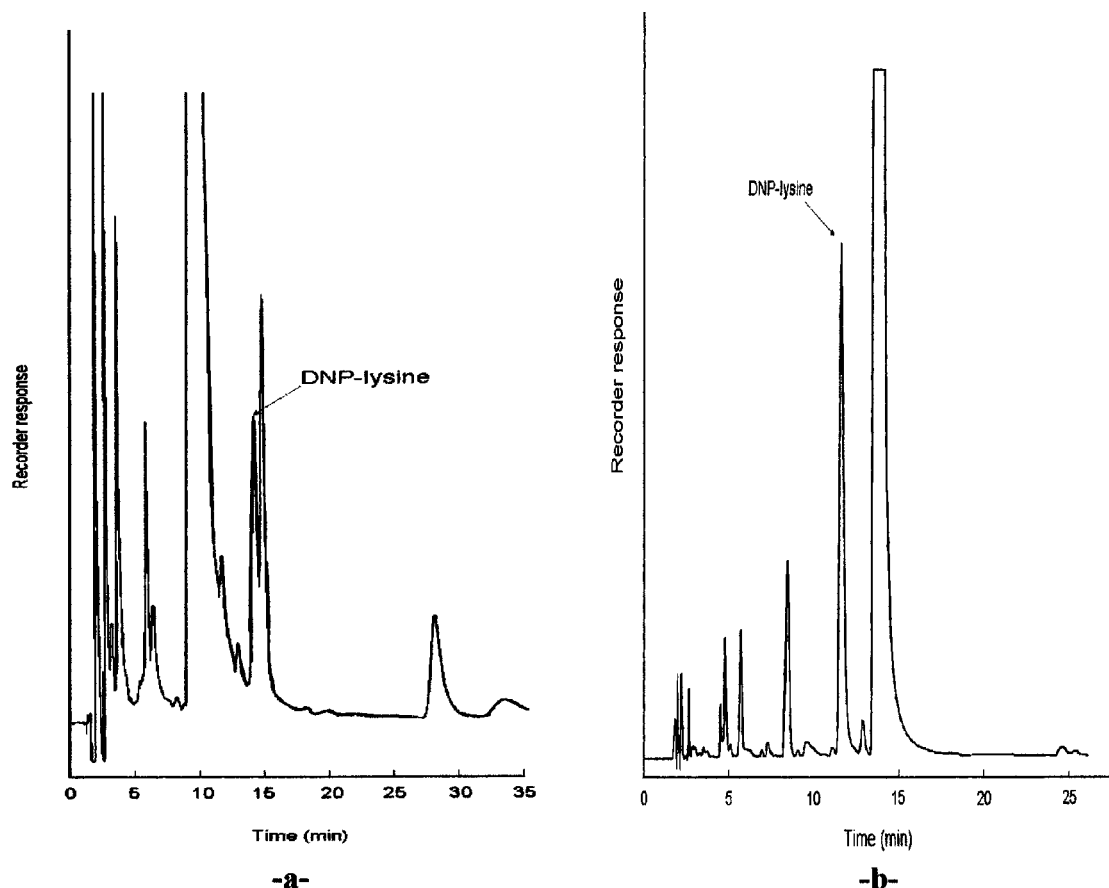
924 *J. Agric. Food Chem.,* Vol. 45, No. 3, 1997

Marquié et al.

Figure 1. HPLC chromatogram of dinitrophenyled cottonseed protein hydrolysate according (a) to the Peterson and Warthesen (1979) procedure and (b) to the proposed procedure. The separation was accomplished at room temperature on a Spherisorb ODS-2 (250 mm × 4.5 mm, 5 μm). The flow rate of the mobile phase was 1 mL/min. For part a the mobile phase was composed of 20% acetonitrile and 80% 0.01 M acetate buffer, pH 4.0. The wavelength was 436 nm. For part b the mobile phase was composed of 20% acetonitrile and 80% 0.1 M potassium hydrogen phthalate buffer at pH 3.9. The wavelength was 364 nm.
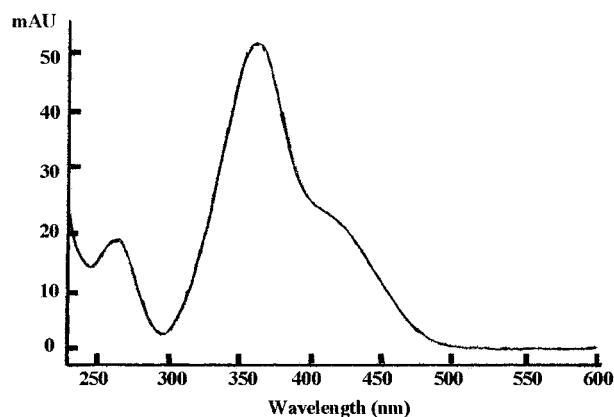

Figure 2. UV absorption spectrum of DNP-lysine measured using a diode array detector.

Figure 3. Changes in film maximum puncture force (N per 100 μm thickness) as a function of the cross-linking agent/ reactive lysine molar ratio in the film-forming solution. Films were made with delipidated glandless cottonseed flour. The cross-linking agents are formaldehyde, glutaraldehyde, and glyoxal. Values are means of three replicates with a coefficient variation lower than 5%. Curves have not been fitted.

reactive lysine molar ratio of 4 seemed to be optimal for enhancing maximum puncture force. Figure 4 shows the increase observed in the modified reactive lysine content of the film after treatment with formaldehyde, glutaraldehyde, or glyoxal. Paradoxically, formaldehyde, which produced films with the greatest maximum puncture force, reacted with less than 50% of reactive lysine at the highest HCHO/reactive lysine molar ratio in the film-forming solution. By contrast, glyoxal and glutaraldehyde reacted with about 90% and 100% of reactive lysine, respectively.

Chemical treatment of cottonseed proteins by formaldehyde, with a molar ratio cross-linking agent/reactive lysine higher than 5, produced films with greater puncture force than with glyoxal or glutaraldehyde
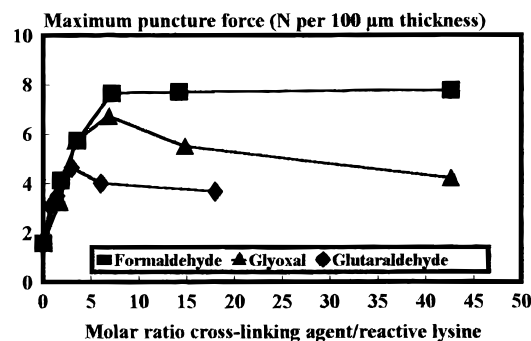
protein cross-linking (Figure 3). In alkaline solution, cross-linking of proteins by formaldehyde led to the formation of short-length methylene cross-links between lysine amino groups (Figure 5a) and also with many additional types of protein groups (Fraenkel-Conrat et al., 1947; Fraenkel-Conrat and Olcott, 1946, 1948a,b; Means and Feeney, 1968; Bizzini and Raynaud, 1974), namely, guanidinyle, amide, and phenol. The formation of the first methylene cross-links between some of the numerous reactive groups in cottonseed proteins with formaldehyde may have established a new protein conformation that dramatically reduced molecular mobility and limited the accessibility of other ε-lysine groups to further reaction with formaldehyde. As a
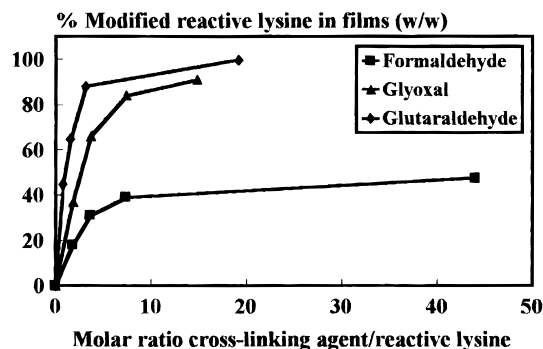
Reactive Lysine Content

J. Agric. Food Chem., Vol. 45, No. 3, 1997 **925**

% Modified reactive lysine in films (w/w)

Legend:
- ■ Formaldehyde
- ▲ Glyoxal
- ◆ Glutaraldehyde

Molar ratio cross-linking agent/reactive lysine

Figure 4. Changes in the percentage of reactive lysine modified in the films by cross-linking with formaldehyde, glutaraldehyde, or glyoxal as a function of the cross-linking agent/reactive lysine molar ratio in the film-forming solution. Films were made with delipidated glandless cottonseed flour.

result, despite the use of a large excess of formaldehyde in comparison to the lysyl group content of the film-forming solution, the reaction of formaldehyde with lysine was limited. However, formaldehyde may have reacted with other protein groups (such as guanidinyl, amide or phenol) to form numerous linkages that may have conferred cohesion into the protein network and resulted in a high maximum puncture force.

Glyoxal is known to condense under mild alkaline conditions with the guanidinyle group in an initial reaction very similar to the Schiff base formation, which undergoes further rearrangement to form cyclicized products (Figure 5b′) unable to cross-link proteins (Shan, 1991). In another reaction glyoxal may also react with lysine (Figure 5b). Figure 4 shows that this reaction is important under our experimental conditions. The increase in the maximum puncture force of the films for a glyoxal/reactive lysine molar ratio of 8 (Figure 3) may be interpreted as the result of protein cross-linking involving lysyl groups. At higher molar concentrations of glyoxal in the film-forming solution, the maximum puncture force of the films decreased despite the decrease of reactive lysine content within the films. In the presence of a large excess of glyoxal in the film-forming solution, we may assume that only a part of this reagent led to protein cross-linking. The glyoxal molecules that reacted with a lysyl group only

or that remained free in the protein network may have decreased the cohesion of the film by reducing the intermolecular forces within the protein network. In addition, chemical modification of the protein by the reaction between arginyl groups and glyoxal, without cross-linking, may have led to the same phenomenon.

Unlike with formaldehyde and glyoxal, glutaraldehyde reacts only with lysyl groups (Monsan et al., 1975). Moreover, this cross-linking agent forms long α,β unsaturated polymers in basic solutions (Monsan et al., 1975) that may include up to eight elementary molecules of glutaraldehyde (Korn et al., 1972). These polymers, which possess numerous aldehyde functional groups, gave probably long and flexible cross-links (Figure 5c) with protein chains that increased molecular mobility and improved the accessibility of the other lysyl groups. The molecular structure of the long bridges formed between the glutaraldehyde cross-linked proteins led to a decrease in the intermolecular forces between polymer chains, resulting in a decrease in maximum puncture force compared with films cross-linked by glyoxal and formaldehyde. As a result, films cross-linked by glutaraldehyde had a lower maximum puncture force than those cross-linked by glyoxal. When higher molar concentrations of glutaraldehyde were used in the film-forming solution, the film maximum puncture force decreased (Figure 3) probably because of a plasticizer effect of the excess glutaraldehyde molecules in the protein network.

Of the three cross-linking agents used in our experiment, formaldehyde was the most efficient in enhancing the maximum puncture force of films made from glandless delipidated flour. These results are illustrated by the graph showing maximum puncture force versus the modified reactive lysine percentage (Figure 6). A clear relation exists between the modified reactive lysine content in the films and the maximum puncture force regardless of the type of cross-linking agent used. These results confirm that a simple evaluation of the maximum puncture force of the films, which is proportional to the decrease of reactive lysine content within the films, gives a good estimate of the extent of protein cross-linking by formaldehyde, glutaraldehyde, or glyoxal as postulated by Marquié et al. (1995).
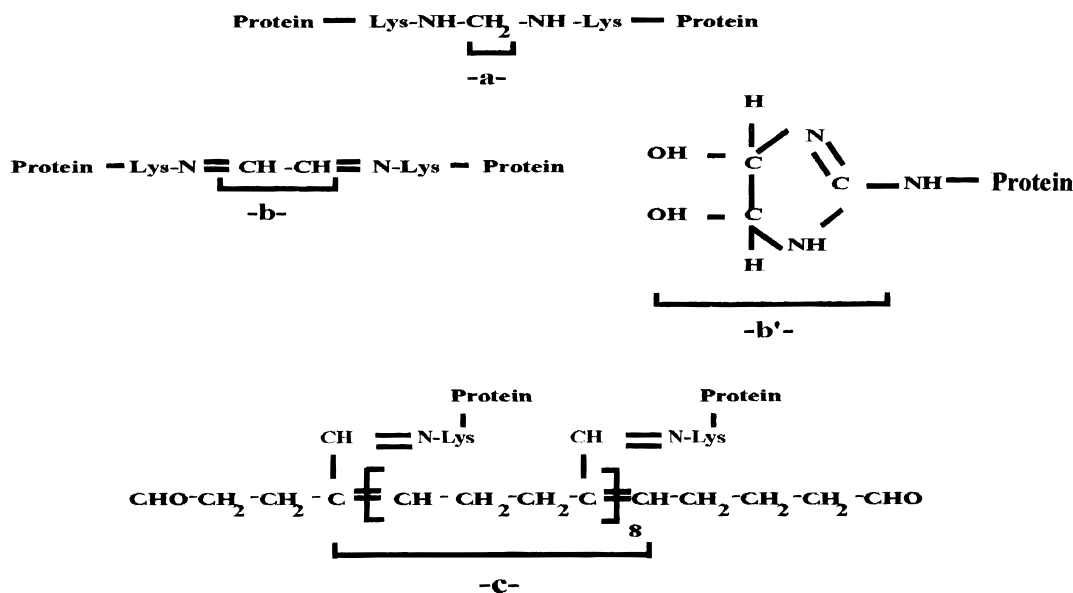

Figure 5. Cross-linking of proteins under basic conditions by formaldehyde, glyoxal, and glutaraldehyde: (a) methylene cross-links resulting from the formaldehyde reaction; (b) cross-links and (b′) cyclized products formed with proteins and glyoxal; (c) polyglutaraldehyde cross-links.
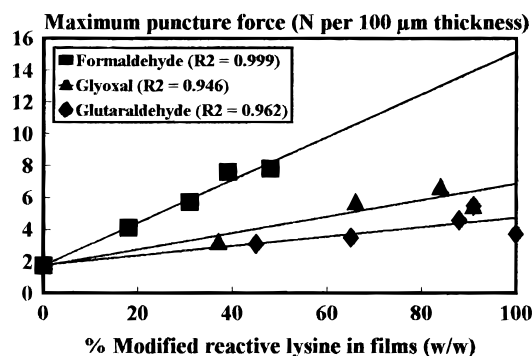
Figure 6. Changes in maximum puncture force (N per 100 μm thickness) as a function of the percentage of reactive lysine modified by protein cross-linking with formaldehyde, glutaraldehyde, or glyoxal. Films were made with delipidated glandless flour. Maximum puncture force values are means of three replicates with a variation coefficient lower than 5%.

In this study we have shown that the HPLC determination of reactive lysine, when adapted according to the modifications described in this paper, may be used to follow the extent of protein cross-linking with formaldehyde, glutaraldehyde, or glyoxal. The sensitivity and reproducibility of the proposed HPLC method enable traces of DNP-lysine to be measured in highly cross-linked films. The impact of protein cross-linking on maximum puncture force of films cannot be explained solely by the number of lysyl groups involved in the reaction but is determined also by the modification of intermolecular interactions between protein chains caused by the chemical structure of the cross-linking agent. This phenomenon that could be qualified as a "protein internal plasticization effect" may be due to the chemical modifications that occur in the protein structure after cross-linking treatments.

ACKNOWLEDGMENT

LITERATURE CITED

Aydt, T. P.; Weller, C. L.; Testin, R. F. Mechanical and barrier properties of edible corn and wheat protein films. *Trans. ASAE* **1991**, *34*, 207−211.

Bizzini, B.; Raynaud, M. Detoxification of proteic toxins by formaldehyde: possible mechanisms and new developments. *Biochimie* **1974**, *56*, 297−303.

Carpenter, K. J. The estimation of the available lysine in animal-protein foods. *Biochem. J.* **1960**, *77*, 604−610.

Cuq, B.; Aymard, C.; Cuq, J. L.; Guilbert, S. Edible packaging films based on fish myofibrillar proteins: formulation and functional properties. *J. Food Sci.* **1996a**, *60*, 1369−1374.

Cuq, B.; Gontard, N.; Cuq, J. L.; Guilbert, S. Rheological model for the mechanical properties of myofibrillar protein-based films. *J. Agric. Food Chem.* **1996b**, *44*, 1116−1122.

Fraenkel-Conrat, H.; Olcott, H. S. Reaction of formaldehyde with proteins: II. Participation of the guanidyl groups and evidence of cross-linking. *J. Am. Oil Chem. Soc.* **1946**, *68*, 34−37.

Fraenkel-Conrat, H.; Brandon, B. A.; Olcott, H. S. The reaction of formaldehyde with proteins: IV. Participation of indole groups, Gramicidin. *J. Biol. Chem.* **1947**, *168*, 99−118.

Fraenkel-Conrat, H.; Olcott, H. S. Reaction of formaldehyde with proteins: VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. *J. Biol. Chem.* **1948a**, *174*, 827−843.

Fraenkel-Conrat, H.; Olcott, H. S. Reaction of formaldehyde with proteins: V. Cross-linking between amino and primary amide or guanidyl groups. *J. Am. Chem. Soc.* **1948b**, *70*, 2673−2684.

Galembeck, F.; Ryan, D. S.; Whitaker, J. R.; Feeney, R. E. Reaction of proteins with formaldehyde in the presence and absence of sodium borohydride. *J. Agric. Food Chem.* **1977**, *25*, 238−245.

Gennadios, A.; Weller, C. L. Edible films and coating from wheat and corn proteins. *Food Technol.* **1990**, *44* (10), 63−69.

Gennadios, A.; Weller, C. L. Tensile strength increase of wheat gluten films. Proceedings of the International Winter Meeting, Nashville, TN, December 15−18, 1992; American Society of Agricultural Engineers: St. Joseph, MI, 1992; Paper No. 92-6517.

Gontard, N. Films comestibles et biodégradables: Etude des propriétés filmogènes du gluten de blé. *C. R. Acad. Agric. Fr.* **1994**, *80*, 109−117.

Guilbert, S.; Biquet, B. In *L'emballage des denrées alimentaires de grande consommation*; Bureau, G., Multon, J. L., Eds.; Technique et Documentation; Lavoisier: Apria, Paris, 1989; Chapter 22.

Holm, H. Micro-method for determination of non-N-terminal lysine in milk and similar products. *J. Sci. Food Agric.* **1971**, *22*, 378−381.

Huber, L. Good Laboratory Practice. Publication 12-5091-6259E; Hewlett-Packard: Berlin, 1993.

Jane, J. L.; Lim, S. T.; Paetau, I. Degradable plastics made from starch and protein. In *Biodegradable Polymers and Packaging*; Ching, C., Kaplan, D. L., Thomas, E. L., Eds.; Technomic: Lancaster, PA, 1993; pp 63−73.

Korn, A. H.; Feairheller, S. H.; Filachione, E. M. Glutaraldehyde, nature of the reagent. *J. Mol. Biol.* **1972**, *65*, 525−529.

Lieberman, E. R.; Gilbert, S. G. Gas permeation of collagen films as affected by cross-linkage, moisture, and plasticizer content. *J. Polym. Sci.* **1973**, *41*, 33−43.

Marquié, C.; Aymard, C.; Cuq, J. L.; Guilbert, S. Biodegradable packaging made from cottonseed flour: Formation and improvement by chemical treatments with gossypol, formaldehyde and glutaraldehyde. *J. Agric. Food Chem.* **1995**, *43*, 2762−2767.

Marquié, C.; Héquet, E.; Guilbert, S.; Vialettes, V.; Tessier, A. M. Biodegradable material from cottonseed flour. 23rd International Cotton Conference, Bremen, March 6−9, 1996.

Matheson, N. A. Available lysine. II. Determination of available lysine in feedingstuffs by dinitrophenylation. *J. Sci. Food Agric.* **1968**, *19*, 496−502.

Means, G. E.; Feeney, R. E. Reductive alkylation of amino groups in proteins. *Biochemistry* **1968**, *7*, 2192−2201.

Monsan, P.; Puzo, G.; Mazarguil, H. Etude du mécanisme d'établissement des liaisons glutaraldehyde-protéines. *Biochimie* **1975**, *57*, 1281−1292.

Park, H. J.; Chinnan, M. S.; Shewfelt, R. L. Edible corn-zein film coatings to extend storage life of tomatoes. *J. Food Process. Preserv.* **1994**, *18* (4), 317−331.

Peters, K.; Richards, F. M. Chemical cross-linking: Reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* **1977**, *46*, 523−551.

Peterson, W. R.; Warthesen, J. J. Total and available lysine determinations using high-pressure liquid chromatography. *J. Food Sci.* **1979**, *44*, 994−997.

Shan, S. H. Reactive groups of proteins and their modifying agents. In *Chemistry of protein conjugation and cross-linking*; Wong, S. S., Ed.; CRC Press: Boca Raton, FL, 1991; Chapter 2.