Comparison of Browning in Wheat Glutens Enriched by Covalent Attachment and Addition of Lysine

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Wheat gluten was fortified by simple addition or by carbodiimide-mediated covalent attachment of lysine, N^{ϵ}-acetyllysine, or N^{ϵ}-benzylidenelysine. Samples with and without added glucose were compared for color and lysine destruction after baking (190 °C; 10 min). In general, covalently enriched products had lighter color and higher percentages of total, DNBS-available, and pepsin–pancreatin-digestible lysine contents than freely enriched products, especially in the crust portion. N^{ϵ}-Benzylidenelysine-enriched gluten was particularly stable. Relative nutritive values by *Tetrahymena* bioassay were 44, 58, and 88 for baked gluten and freely and covalently N^{ϵ}-benzylidenelysine (1%) enriched glutens, respectively, compared to 100 for casein. It is concluded that covalently attached lysine is more stable than free lysine for enrichment of food products susceptible to Maillard reaction.

The production of color and flavor compounds by the Maillard browning reaction plays an important role in the aesthetic appeal and acceptability of bakery products such as bread. However, careful control is mandatory to prevent excessive browning which could severely impair not only acceptability but also nutritional quality. Moreover, some of the products of the Maillard reaction have been implicated in hepatic disorders, allergic responses, hyperexcitability, and other abnormal symptoms (Adrian, 1974; Ambrose et al., 1961).

Wheat gluten, the major protein fraction in flour, is first limiting in the amino acid lysine. Fortification of gluten with lysine can markedly improve its nutritional quality. However, during baking, especially in the presence of reducing sugars, the added lysine may be rendered unavailable due to participation of its free α- and ϵ-amino groups in the Maillard reaction. Thus, for wheat products in which the Maillard reaction is an important consequence of processing, covalent attachment rather than simple addition of lysine may be the preferred means for nutritional fortification, since the covalently attached lysine may be less susceptible to degradative reactions.

In our previous work, we reported an increase in the protein-bound lysine content of wheat gluten by covalent attachment of lysine or N^{ϵ}-amino-protected lysine derivatives, using a water-soluble carbodiimide as the coupling reagent (Li-Chan et al., 1979; Li-Chan and Nakai, 1980). This study was carried out to compare the extent of browning and destruction of lysine in baked gluten samples fortified by either covalent attachment or free addition. The effect of using N^{ϵ}-substituted lysine derivatives (N^{ϵ}-acetyllysine and N^{ϵ}-benzylidenelysine) was also investigated. Relative nutritive values of the baked samples were compared by using a bioassay with *Tetrahymena pyriformis* W.

EXPERIMENTAL SECTION

Materials. Vital gluten was obtained commercially as "Whet Pro 75% vital wheat gluten" from Industrial Grain Products, Ltd., Thunder Bay, Ontario. L-Lysine monohydrochloride was obtained from Sigma Chemical Co. N^{ϵ}-Benzylidenelysine and N^{ϵ}-acetyllysine were synthesized as previously described (Li-Chan and Nakai, 1980). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was from Sigma Chemical Co., as were the enzymes pepsin (from hog stomach mucosa, 2 times recrystallized) and pancreatin (from porcine pancreas, grade VI). Pronase (B grade) was a product of Calbiochem. Animal Nutrition Research Council (ANRC) casein was a product of Humko Sheffield Chemical, Norwich, NY. *T. pyriformis* W (ATCC) was purchased from the American Type Culture Collection, Rockville, MD.

Carbodiimide Reaction. Covalently enriched gluten samples were prepared as previously described (Li-Chan et al., 1979; Li-Chan and Nakai, 1980) by coupling lysine, N^{ϵ}-acetyllysine, or N^{ϵ}-benzylidenelysine to gluten by using EDC. Covalently enriched "acid gluten" samples were prepared similarly by using gluten previously solubilized by autoclaving (120 °C; 15 min) in 0.05 N HCl, according to Wu et al. (1976). Covalently enriched samples were designated as "lysine–gluten (x%)", representing gluten covalently enriched with x% lysine (weight/weight ratio of lysine equivalent to gluten). Gluten samples enriched by free addition of x% lysine were designated as "gluten + x% lysine".

Baking Procedure. Model baking studies were conducted to investigate the effects of heating lysine or N^{ϵ}-lysine derivative enriched glutens in the absence or presence of a reducing sugar. Covalently enriched gluten samples were formed into "doughs" by mixing 3 g of the freeze-dried samples with water (76% w/w), with or without addition addition of 10% (w/w) glucose. Corresponding samples enriched by simple addition were prepared similarly, after dry mixtures of gluten and lysine or lysine derivative were thoroughly mixed and ground with mortar and pestle in the appropriate ratio. These doughs were baked at 190 °C for 10 min. The resulting "breads" were cooled, separated into crust and crumb fractions, and freeze-dried.

Measurement of Color. Clear extracts of the brown pigments formed during baking were obtained by enzymatic digestion, followed by precipitation and filtration. The methods of Tinkler et al. (1955) and Choi et al. (1949) were modified to give the following procedure. To 0.1 g of ground sample in 5 mL of pH 8, 0.1 M phosphate buffer was added 0.5 mL of 3% Pronase solution. The mixture was incubated at 50 °C for 10 min. After addition of 2 mL of 60% trichloroacetic acid, the mixture was filtered on Whatman No. 1 filter paper with the aid of 0.2 g of Celite filter aid. The filtrate was refiltered if necessary to obtain a clear filtrate, and the absorbance was then read at 420 nm against a reagent blank. [When Whatman No. 50 (hardened) filter paper was used, no filter aid or refiltering was required to obtain a clear filtrate.] Similar procedures

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have been used by other workers to measure nonenzymatic browning pigment production [e.g., Warren and Labuza (1977) and Warmbier et al. (1976)].

Determination of Lysine Content. Samples were prepared for amino acid analysis by first blocking cysteine residues by reaction with 4-vinylpyridine as described by Cavins et al. (1972) and then hydrolyzing at 110 °C for 24 h with *p*-toluenesulfonic acid according to Liu and Chang (1971). Amino acids were analyzed on a single-column system (Durrum Chem Corp., Palo Alto, CA) attached to a Phoenix M 6800 amino acid analyzer. The lysine content was reported as "total" lysine (g/100 g).

"Available" lysine content was determined by the spectrophotometric method of Concon (1975) using reaction with dinitrobenzenesulfonate (DNBS).

"Digestible" lysine content was determined by amino acid analysis after an in vitro digestion using pepsin and pancreatin, by the method of Stahmann and Woldegiorgis (1975), as modified by Holguin and Nakai (1980).

Nitrogen content was analyzed on a Technicon Auto Analyzer II system, after prior digestion of samples by the micro-Kjeldahl digestion method of Concon and Soltess (1973). Protein content was calculated from nitrogen values by using a conversion factor of 5.7 for gluten.

Determination of Relative Nutritive Value by *Tetrahymena*. *T. pyriformis* W culture was routinely maintained at 27 °C in the dark in *Tetrahymena* medium (5 g of proteose peptone, 5 g of tryptone, 0.2 g of K_2HPO_4, and 1 L of distilled water) and transferred every 3–4 days. For the assay, a 3-day-old broth culture was centrifuged twice (3000 *g*; 10 min), the pellet being resuspended in fresh 0.067 M phosphate buffer (pH 7.2) each time.

Samples were predigested with pepsin essentially according to Evancho et al. (1977) and then neutralized to pH 7.1 and frozen until assayed. Vitamin stock solution A, mineral stock solutions B and C, buffer solution D, nucleotide solution E, and dextrin solution G were prepared according to the original method of Stott et al. (1963) as modified by Evancho et al. (1977). Aliquots of predigested samples were used to provide 0.3 mg of N/mL of final culture medium, and assay procedures were carried out as described by Evancho et al. (1977).

Tetrahymena growth was determined by direct microscopic count. An aliquot of the 4-day-old culture suspension was transferred to preserving fluid as described by Evancho et al. (1977), and organisms were counted on a J. F. Hartz double-cell hemacytometer. After the 2-fold dilution into preserving fluid was accounted for, the mean number of organisms per square millimeter for eight alternate millimeter squares gave the test culture population in units of 10^4 organisms/mL. Relative nutritive values were calculated as (organism count for sample/organism count for ANRC casein) × 100.

RESULTS AND DISCUSSION

Comparison of Crust Portions. Table I shows the total lysine content in crusts of gluten samples before and after baking. For the control gluten sample, baking in the absence of glucose caused relatively little destruction of the protein-bound lysine. In the presence of 10% glucose, however, over 30% of the lysine in the crust was destroyed. This suggests the predominant role of Maillard reaction with reducing sugars in the destruction of lysine. When gluten samples were enriched by free addition of lysine, N^{ϵ}-acetyllysine, or N^{ϵ}-benzylidenelysine, very high percentages of the added lysine in the crust were lost after baking in the presence of glucose. In contrast, in samples which were enriched by covalent attachment of lysine or lysine derivative, very high percentages of the added lysine were recoverable. The percentages of added lysine remaining after baking in the presence of glucose were 92, 81, and 108% for glutens covalently enriched with lysine, N^{ϵ}-acetyllysine, and N^{ϵ}-benzylidenelysine, respectively.

Table I. Loss of Lysine in Crust Portions of Enriched Glutens (0% or 10% Glucose Added) after Baking at 190 °C for 10 min

sample (crust only)	lysine, g/100 g before	lysine, g/100 g after	% of added lysine remaining after baking
gluten	1.3	1.2	
gluten + glucose	1.3	0.9	
gluten + 1% lysine	2.5	1.3	8
gluten + 3% lysine	4.9	4.1	81
gluten + 1% lysine + glucose	2.5	0.7	0
gluten + 3% lysine + glucose	4.9	1.4	14
lysine-gluten (1%) + glucose	2.5	2.0	92
gluten + 3% AL[a]	4.0	3.1	70
gluten + 3% AL + glucose	4.0	1.7	30
AL-gluten (3%) + glucose	4.4	3.4	81
gluten + 1% BL[b]	2.5	1.8	50
gluten + 3% BL	4.5	3.3	66
gluten + 1% BL + glucose	2.5	0.7	0
gluten + 3% BL + glucose	4.5	1.1	6
BL-gluten (1%) + glucose	2.6	2.3	108

[a] AL = N^{ϵ}-acetyllysine. [b] BL = N^{ϵ}-benzylidenelysine.

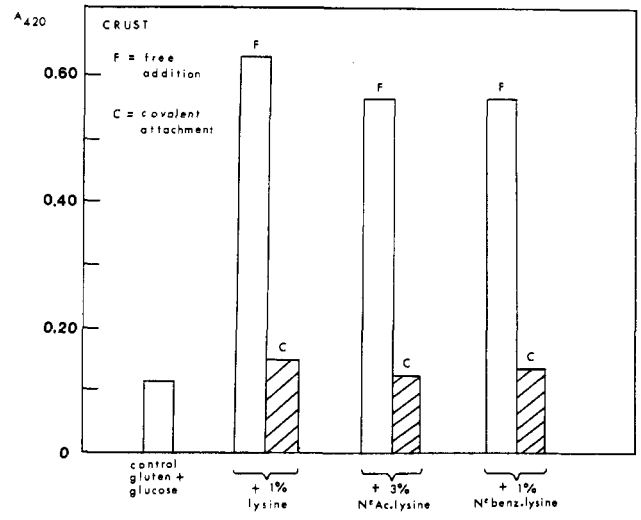

Figure 1. Absorbance of extracts of crust portions of free and covalently enriched glutens (baked with 10% glucose).

Figure 1 shows the absorbance of extracts obtained from the crust portion of different samples after baking in the presence of 10% glucose. The trend in color (A_{420}) of samples parallels the destruction of lysine. Samples which were enriched by covalent attachment showed only slightly higher absorbance readings than the control gluten, whereas samples which were enriched by free addition had much higher absorbance values. The latter samples were very dark brown in color when visualized with the eye and were not acceptable aesthetically by the usual standards of bread crust color.

Lysine possesses an ϵ-amino group in addition to its α-amino group, and both groups can participate in the Maillard reaction with reducing sugars. Lysine causes the formation of more color in glucose–amino acid solutions than do monoaminomonocarboxylic acids such as alanine or monoaminodicarboxylic acids such as glutamic acid, and it can produce color over a wider pH range (Underwood et al., 1959). The pronounced increase in color production by diamino acids cannot be attributed only to the doubling of the number of the amino groups nor to an algebraic sum

Table II. Total, DNBS-Available, and Pepsin-Pancreatin-Digestible Lysine Contents in Crusts of Enriched Glutens after Baking

sample (crust only)	lysine, g/100 g of total amino acids		
	total	DNBS	PPD
unbaked gluten control	1.4	1.2	1.0
gluten	1.2	1.1	0.6
gluten + glucose	0.9	0.5	0.4
gluten + 1% lysine	1.3	1.0	1.0
gluten + 3% lysine	4.1	3.5	2.8
gluten + 1% lysine + glucose	0.7	0.5	0.3
gluten + 3% lysine + glucose	1.4	0.9	0.6
lysine-gluten (1%) + glucose	2.0	0.9	0.6
gluten + 3% AL[a]	3.1	1.5	1.0
gluten + 3% AL + glucose	1.7	<0.2	0.1
AL-gluten (3%) + glucose	3.4	0.7	0.5
gluten + 1% BL[b]	1.8	1.6	1.2
gluten + 3% BL	3.3	2.9	3.2
gluten + 1% BL + glucose	0.7	0.7	0.4
gluten + 3% BL + glucose	1.1	1.0	2.1
BL-gluten (1%) + glucose	2.3	1.4	1.1

[a] AL = N^{ϵ}-acetyllysine. [b] BL = N^{ϵ}-benzylidenelysine.

of the color formed by the corresponding monoamino acids. Lysine produces 6 times more intense coloration than norleucine, an analogue with only an α-amino group. These observations may explain the much greater extent of browning and lysine destruction in the case of samples enriched by free addition, in which case both amino groups of lysine are available for reaction. In the covalently enriched sample, there is a net reduction of one amino group (α- or ϵ-amino of either the attached lysine or gluten) for every lysine residue attached by an amide bond. Thus, the available amino groups for the Maillard reaction are reduced in number in the covalently enriched sample. When N^{ϵ}-substituted derivatives such as N^{ϵ}-acetyllysine or N^{ϵ}-benzylidenelysine are used, less browning should occur since the lysine derivative has only one available amino group. The N^{ϵ} substituent may, in addition, have either a stimulatory or an inhibitory effect on reactivity of the available α-amino group.

Finot et al. (1978) studied the reactivity of various N-substituted lysines toward browning and found that all the lysine derivatives were less reactive than free lysine. Dipeptides such as lysylalanine, alanyllysine and glycyllysine were ~2 times less reactive than lysine, while glutamyllysine was 4 times less reactive. α-Acyl derivatives were 2 times less reactive and ϵ-acyl derivatives were 4 times less reactive than lysine. N^{ϵ}-(α-Glutamyl)lysine was least reactive, being 7 times less reactive than free lysine. These findings are in agreement with the ones reported in the present study. For example, as shown in Figure 1, gluten enriched by free addition of 3% N^{ϵ}-acetyllysine had lower absorbance (implying less browning) than gluten enriched by free addition of only 1% lysine.

The total lysine content of the crust portions is compared to the contents of DNBS-available and pepsin–pancreatin-digestible lysine in Table II. Although the total lysine content in covalently enriched samples was higher than the content in samples enriched by free addition, this trend was not adhered to in the DNBS-available and pepsin–pancreatin-digestible lysine contents. Covalently enriched lysine and N^{ϵ}-acetyllysine–gluten samples had low values of both "availability" and "digestibility" by these in vitro test indexes.

The added lysine residues in lysine–gluten may be covalently attached through either peptide (α–α) or isopeptide (α–ϵ) bonds. In the latter type of linkage, the ϵ-amino group is not available for reaction with DNBS, nor is the isopeptide amide bond hydrolyzed by the proteases pepsin or pancreatin. Thus, available and digestible lysine contents are relatively low in comparison to total lysine content. Yet these values are still higher than the corresponding values for the freely enriched sample or the control gluten sample, indicating that some of the lysine residues are attached by peptide bonds.

Table III. Loss of Lysine in Enriched Gluten and Acid (0.05 N HCl) Solubilized Gluten Samples after Baking

sample (whole)	lysine, g/100 g		% of added lysine remaining after baking
	before	after	
gluten	1.4	1.3	
gluten + glucose	1.4	0.8	
acid gluten + glucose	1.4	0.7	
gluten + 1% lysine + glucose	2.5	1.3	45
lysine-gluten (1%) + glucose	2.5	1.6	73
gluten + 1% AL[a] + glucose	2.5	1.6	73
AL-gluten (1%) + glucose	2.5	2.0	110
gluten + 1% BL[b] + glucose	2.5	1.7	82
BL-gluten (1%) + glucose	2.5	2.0	110
acid gluten + 1% lysine + glucose	2.8	1.4	50
acid gluten + 4% lysine + glucose	5.6	2.2	36
lysine-acid gluten (1%) + glucose	2.8	2.0	93
lysine-acid gluten (4%) + glucose	5.6	2.8	50

[a] AL = N^{ϵ}-acetyllysine. [b] BL = N^{ϵ}-benzylidenelysine.

N^{ϵ}-Acetyllysine–gluten showed virtually no increase in either DNBS-available or pepsin–pancreatin-digestible lysine content when compared to the control gluten, although its total lysine content was significantly higher. Since the N^{ϵ}-acetyl groups were not intentionally removed after the carbodiimide reaction, they would still protect the lysine ϵ-amino groups and render them inaccessible to the DNBS reagent as well as the Maillard reaction. The low pepsin–pancreatin-digestible lysine content in this case may be deceiving. In fact, the N^{ϵ}-acetyllysine residues may have been readily released by enzymatic digestion. However, since N^{ϵ}-acetyllysine emerged at the same position as proline in the amino acid analysis ion-exchange elution profile, it was not possible to resolve the two peaks to give a reliable estimate of either amino acid.

In contrast to N^{ϵ}-acetyllysine–gluten, N^{ϵ}-benzylidenelysine–gluten showed high digestibility and availability of its lysine residues. Although the benzylidene group was not intentionally removed in this case, this group is very sensitive to acidic conditions, being a Schiff's base type of linkage (Bodanszky et al., 1976). It is possible that partial removal of the protecting group occurred during addition of acetic acid to stop the carbodiimide reaction and during subsequent dialysis against 1 N acetic acid. If unintentional removal of the protecting group did occur, one would expect that the exposed ϵ-amino groups would be susceptible to the browning reaction. The higher content of DNBS-available lysine after baking in the presence of glucose for 1% covalently enriched N^{ϵ}-benzylidenelysine–gluten (1.4 g/100 g) compared to the control gluten (0.5 g/100 g) suggests that the presence of the benzylidene substituent on lysine during the carbodiimide reaction may have encouraged attachment of lysine residues in a hydrophobic, relatively inaccessible region in the gluten molecule, thus rendering those residues less reactive to Maillard reaction.

Comparison of Whole (Crust plus Crumb) Samples. Table III shows the total lysine content of gluten and enriched gluten samples (whole) before and after baking

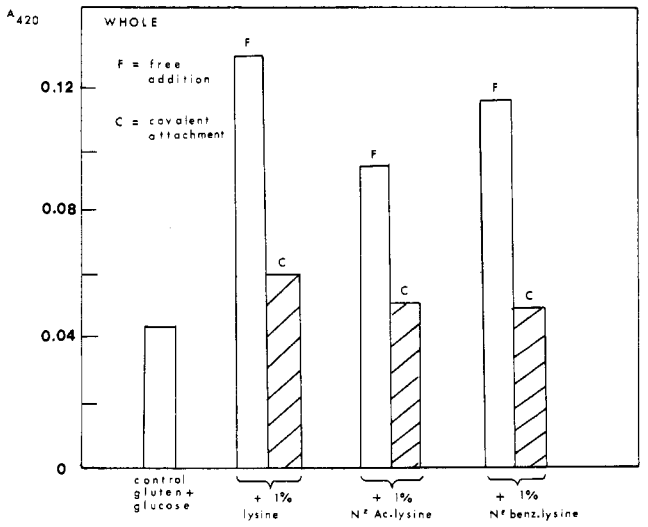

Figure 2. Absorbance of extracts of whole (crust plus crumb) samples of free and covalently enriched glutens (baked with 10% glucose).

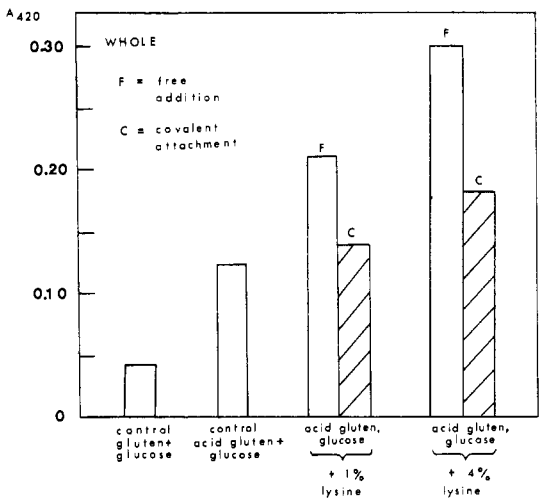

Figure 3. Absorbance of extracts of whole (crust plus crumb) samples of free and covalently enriched acid-solubilized glutens (baked with 10% glucose).

in the presence of glucose, while Figure 2 depicts the absorbance of extracts obtained from these samples. Gluten samples covalently enriched with lysine, N^{ϵ}-acetyllysine, and N^{ϵ}-benzylidenelysine all had higher percentages of lysine remaining after baking when compared to the corresponding samples enriched by free addition. The smaller loss of lysine was accompanied by lighter color (lower absorbance) of extracts for covalently enriched samples. These trends for the whole (crust plus crumb) gluten samples thus reflect findings observed from the crust fraction only of samples. Although, in general, much greater loss of lysine and darkening occurred in the crust and the differences between covalent and free enrichment were more marked when observing the isolated crust fraction, the results from the whole sample support the hypothesis that the covalently enriched lysine residues are more stable against destruction than lysine added in free form, during heating in the presence of a reducing sugar such as glucose.

The doughs prepared for the present study were mixtures of wheat gluten, glucose, and water. The other ingredients usually included in a typical formulation for bread or other bakery products were not included to simplify interpretation of results. It is likely that components such as skim milk powder and wheat starch would affect the destruction of lysine during baking. Greater loss of supplemental lysine was observed in breads containing nonfat dry milk than breads lacking this ingredient (Jansen et al., 1964a,b). However, the conclusions obtained from the model studies presented here should be representative of the trends expected for more complex formulations of bakery products.

Lysine addition has been well documented as a cause of significant darkening of crust color and occasionally changes in loaf volume (Ericson et al., 1961; Jansen et al., 1964a,b; Ehle and Jansen, 1965). The loss of lysine (both protein-bound and added lysine) has been shown to be proportional to the baking time and to the percentage of crust which resulted (Jansen et al., 1964a,b). The nutritional value of bread after toasting decreases with increased extent of toasting (Tsen and Reddy, 1977), and high temperatures cause greater destruction of amino acids (Sabiston and Kennedy, 1957). When bread was fortified with 0.48% L-lysine (0.6% L-lysine hydrochloride) and 0.3% L-threonine, the baking losses of lysine and threonine were 5 ± 6 and 3 ± 2%, respectively, in the crumb, 46 ± 11 and 54 ± 8%, respectively, in the crust, and 14 ± 8 and 15 ± 5%, respectively, in the whole loaf (Murata et al., 1979). The results reported in the present study confirm the greater percentage loss of lysine in the crust than in the whole loaf. Relatively large losses of lysine even in the whole loaf may be attributed to the small size of loaf (since doughs were prepared by using 3 g of gluten), which resulted in a large ratio of crust to crumb in the loaf.

Table III also shows the loss of lysine in baked samples (whole) prepared from acid-solubilized gluten and acid-solubilized gluten enriched with 1 or 4% lysine. Covalently enriched lysine was more stable than freely added lysine. When 1% lysine enrichment was used, the percentage of added lysine remaining after baking was higher in the acid-solubilized gluten samples than in the corresponding gluten samples (50 vs. 45% for freely added lysine, and 93 vs. 73% for covalently added lysine). When lysine enrichment was undertaken at the 4% level, only 36 and 50% of the freely added and covalently added lysine, respectively, remained after baking of the acid-solubilized gluten.

Comparison of absorbance of extracts (Figure 3) shows that the baked acid-solubilized gluten was darker than baked gluten control. Covalently enriched acid gluten samples were lighter in color than freely enriched acid gluten samples, both at the 1 and 4% levels of enrichment. However, both covalently and freely enriched acid gluten samples were darker than corresponding enriched gluten samples.

It is possible that the amide bond formed between lysine and acid-solubilized gluten, primarily an isopeptide bond (γ–α or γ–ϵ), offered greater resistance of the attached lysine residues against Maillard browning reaction than when the amide bond was of the peptide or isopeptide type found between lysine and gluten (α–α or α–ϵ). It is known that reactivity toward Maillard browning destruction of amino acids depends on the side chain of the amino acid (Underwood et al., 1959), and different N substituents of lysine have varying degrees of reactivity (Finot et al., 1978). The decreased loss of lysine in acid-solubilized gluten may have been a result of changed conformation of the gluten molecule by acid treatment, effects of the increased content of carbonyl groups on the proton transfer in reaction sequences such as the Amadori rearrangement or on the Strecker degradation of amino acids, or the protective effect of isopeptide linkages. Although lysine loss was reduced, the acid-solubilized gluten was darker in color,

Table IV. Total, DNBS-Available, and Pepsin-Pancreatin-Digestible Lysine Contents in Enriched Gluten Samples after Baking

sample (whole)	lysine, g/100 g of total amino acids		
	total	DNBS	PPD
unbaked gluten control	1.4	1.2	1.0
unbaked acid gluten control	1.4	1.3	0.6
gluten	1.3	1.2	0.9
gluten + glucose	0.8	0.6	0.6
acid gluten + glucose	0.7	0.6	0.5
gluten + 1% lysine + glucose	1.3	0.7	0.6
lysine-gluten (1%) + glucose	1.6	0.8	0.8
gluten + 1% AL[a] + glucose	1.6	<0.2	0.5
AL-gluten (1%) + glucose	2.0	0.7	0.7
gluten + 1% BL[b] + glucose	1.7	1.0	0.8
BL-gluten (1%) + glucose	2.0	1.4	1.3
acid gluten + 1% lysine + glucose	1.4	0.7	0.6
acid gluten + 4% lysine + glucose	2.2	0.8	0.8
lysine-acid gluten (1%) + glucose	2.0	1.5	0.9
lysine-acid gluten (4%) + glucose	2.8	1.9	1.5

[a] AL = N^{ϵ}-acetyllysine. [b] BL = N^{ϵ}-benzylidenelysine.

a result of perhaps changes in the other amino acids or of the gluten molecule as a whole. Even before baking, the acid-solubilized gluten was darker in color than gluten control.

Total, DNBS-available, and pepsin–pancreatin-digestible lysine contents are compared in Table IV. Among gluten samples, highest contents were obtained for the covalently enriched N^{ϵ}-benzylidenelysine–gluten, while covalently enriched N^{ϵ}-acetyllysine–gluten had high total lysine content but low available and digestible lysine contents. The covalently enriched lysine acid-solubilized gluten (1 and 4% levels of enrichment) had high values of total, available, and digestible lysine contents. In general, these results are in agreement with those reported on the crust fractions of samples, and conclusions drawn regarding N^{ϵ} blocking group lability and isopeptide bonds are similar.

For comparison of the nutritional quality of the different samples after baking, samples were assayed by using the *Tetrahymena* bioassay. The results are shown in Table V. In the absence of glucose, the baked gluten, with a relative nutritive value of 57, had a protein quality approximately equivalent to that of unbaked gluten. However, in the presence of added glucose, the relative nutritive value of gluten after baking decreased to 44. In a study comparing chemically measured lysine availability to *Tetrahymena* relative nutritive value, Warren and Labuza (1977) showed that the protozoan *Tetrahymena* is sensitive to the loss of protein quality due to nonenzymatic browning at advanced stages of browning, when the *Tetrahymena* test showed equivalent or greater losses of nutritional value compared to the fluorodinitrobenzene chemical assay. At early stages of browning, however, less loss was detected by *Tetrahymena* than by the chemical test, suggesting possibly that some of the early Maillard reaction products may be biologically available to *Tetrahymena* or that some loss of lysine and other amino acids may not be detrimental to its growth.

Enrichment of gluten samples by free addition of lysine, N^{ϵ}-acetyllysine, N^{ϵ}-benzylidenelysine before baking resulted in some improvements in protein quality, the relative nutritive values being increased to 50–58. Covalent enrichment with lysine or lysine derivatives improved relative nutritive values more effectively, with the best growth occurring in the test culture medium containing covalently enriched N^{ϵ}-benzylidenelysine–gluten. Relative nutritive value in this case was 88, in comparison to a value

Table V. Relative Nutritive Values (RNV) of Enriched Gluten Samples after Baking

sample (whole)	RNV[a]
gluten	57
gluten + glucose	44
acid gluten + glucose	45
gluten + 1% lysine + glucose	50
lysine-gluten (1%) + glucose	63
gluten + 1% AL[b] + glucose	53
AL-gluten (1%) + glucose	75
gluten + 1% BL[c] + glucose	58
BL-gluten (1%) + glucose	88
acid gluten + 1% lysine + glucose	52
acid gluten + 4% lysine + glucose	81
lysine-acid gluten (1%) + glucose	61
lysine-acid gluten (4%) + glucose	91

[a] Relative nutritive value = 100 × (organism count for sample/organism count for casein). [b] AL = N^{ϵ}-acetyllysine. [c] BL = N^{ϵ}-benzylidenelysine.

of 100 for an unbaked casein reference protein. Covalent enrichment of acid-solubilized gluten also significantly improved protein quality. At a 4% level of enrichment before baking, the baked sample had a relative nutritive value of 91.

The improvement of relative nutritive values by addition of lysine derivatives as well as of lysine, and by enrichment of acid-solubilized gluten as well as of gluten, demonstrates the biological availability of these N^{ϵ} substituents of lysine and of lysine attached by isopeptide bonds, at least in terms of availability as sources of lysine for growth of the protozoan *Tetrahymena*. The biological availability of these products in conjunction with their reduced sensitivity toward degradative reactions such as Maillard browning demonstrates great potential advantages in using covalently attached N^{ϵ}-substituted lysine derivatives for stable enrichment of food proteins.

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Physicochemical Properties of Maize Glutelins As Influenced by Their Isolation Conditions

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The influence of various parameters (presence of sodium acetate in alcohol solutions, pH, concentration and nature of reductant, and blocking of disulfide bonds) upon the distribution and the quality (amino acid composition and electrophoretic mobility at pH 3.5) of G_1-, G_2-, and G_3-glutelins in maize grain was investigated. G_2-Glutelins were divided into two fractions by extraction at pH 3 and 10; acid-soluble G_2-glutelins were also coextracted with G_1-glutelins when sodium acetate was added to alcohol solution containing 2-mercaptoethanol. The possible contamination of zein by G_1- and G_2-glutelins is discussed. Maize proteins can be classified as alcohol-soluble (zein and G_1-glutelins and acid-soluble G_2-glutelins) and alcohol-insoluble proteins (salt-soluble proteins, acid-insoluble G_2- and G_3-glutelins, and insoluble glutelins). The extension of such a classification to grain proteins of other cereals is examined.

Maize grain proteins can be separated into five fractions by a selective extraction method (Landry and Moureaux, 1970). Thus the successive contact of six media with a grain meal results in the release of salt-soluble proteins and zein, referred to as fractions I and II, and of three glutelin subgroups, denoted by G_1, G_2, and G_3 and corresponding to fractions III, IV, and V. Numerous investigations concerning the distribution and the composition of these five protein fractions as well as their accumulation in developing grain, their disappearance in endosperm during germination, their localization in histological parts, and the influence of genetic factors upon their accumulation rate afforded a further insight into the physicochemical and biological properties of glutelins.

However, literature data show that the six media described by Landry and Moureaux (1970) do not always lead to isolation of well-delineated protein fractions (Landry, 1979a; Landry and Moureaux, 1980). The variations in resolution seemed to be due to slight alterations in experimental conditions which can modify efficiency and selectivity of a given medium. For example, 0.5% sodium acetate in aqueous ethanol and 2-mercaptoethanol (2ME) is sufficient to cause simultaneous extraction of fractions III and IV (Paulis and Wall, 1977), whereas without salt only fraction III is isolated. Therefore it appears necessary to assess the validity of the selective extraction method previously developed (Landry and Moureaux, 1970) by examining the possibilities of cross contamination of protein fractions.

The present paper reports the results of such a study. Several sequences of solvents were investigated to determine the influence of various experimental methods upon the distribution and the nature of glutelin subgroups. Glutelin subgroups were identified both by amino acid composition and by electrophoretic behavior on starch gel at pH 3.5, since similar amino acid compositions of two extracts may hide differences in their protein compositions. The data presented here and in other investigations demonstrate the existence of several protein sets in maize grain, each containing constituents with unique physicochemical properties.

MATERIALS AND METHODS

Maize samples were seeds from a normal hybrid (INRA 260). Samples were designated I_1, I_2, and I_3 indicating three different harvest years.

Extraction of Proteins. General Scheme. The experimental conditions for protein extraction (Landry and Moureaux, 1970, 1980) are briefly summarized. Meal was defatted at −10 °C with anhydrous acetone and then with diethyl ether. Samples (3.5 g) were suspended in 35 mL of extractant. The solid material was isolated from extractants; the duration (minutes) and number of extractions and the identification of protein fractions are reported in Table I for two extraction sequences, A_0 and D_0; "A" and "D" refer two extraction schemes tested previously (Landry and Moureaux, 1970).

Specific Extraction Schemes of Protein Groups. The diverse sequences differ from the sequences A_0 or D_0 by alterations of experimental conditions at one or several steps. Thus, at step 1, for sequences D_3 and D_4, salt extraction was performed at 10 °C. At step 3, for sequence D_4, an additional extraction (step designated by 3s) was made with 55% 2-PrOH plus 0.5% sodium acetate (60 min). At step 4, for sequence D_4, extraction was carried out in the presence of sodium acetate (0.5% w/v). At step 5, for sequences A_2, A_3, D_1, and D_4, glutelins were successively extracted at step 5a with a pH 3 buffer (0.04 M citric acid plus 0.02 M Na_2HPO_4) plus 0.6% 2ME for 60, 30, and 30 min and at step 5b with a pH 10 buffer (0.125 M boric acid plus 0.02 M NaOH) plus 0.6% 2ME for 60, 30, and 30 min. At step 6, for sequence A_2, phenol–acetic acid–water (1:1:1 w/v/v) without 2ME was used. On the

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