Covalent Attachment of Lysine to Wheat Gluten for Nutritional Improvement

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The lysine content of wheat gluten was increased up to fourfold after reaction of pepsin-hydrolyzed wheat gluten with N^{ϵ}-benzylidenelysine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Although partially acid-hydrolyzed gluten reacted with L-lysine hydrochloride, thus increasing the lysine content up to 20-fold, the available lysine determined by the dinitrobenzenesulfonate method and the liberated lysine by the pepsin pancreatin digestion method were low, 48 and 41%, respectively. This result suggests formation of γ–α, γ–ϵ, and α–ϵ isopeptide bonds. When a reversibly ϵ-amino protected lysine, N^{ϵ}-benzylidenelysine, was used for binding, the available lysine content increased to 91%. However, the liberated lysine value stayed the same, suggesting the possibility of γ–α bond formation. When pepsin hydrolysis instead of acid was used for solubilization of gluten, the liberated lysine value was improved to 65% compared to 31 and 45% for egg and casein, respectively. The bound lysine residues in the pepsin-hydrolyzed gluten were readily digestible and available.

The nutritional value of lower quality proteins such as cereal and oilseed proteins may be improved by fortification with the limiting essential amino acids. Direct addition of the limiting amino acids in free form is the simplest but not necessarily best approach, due to possible losses of the amino acids during processing, undesirable flavor changes, increased susceptibility to browning or degradative reactions, or differing rates of in vivo digestion and absorption. Covalent attachment of the limiting amino acids through chemical or enzymatic modification could circumvent these problems and improve protein nutritional quality.

To date, relatively little work has been published on the use of chemical modification of food proteins for this purpose. Covalent introduction of phenylalanine, tyrosine, methionine, and isoleucine into whey protein by the *N*-carboxyanhydride method was accomplished by Bjarnason-Baumann et al. (1977). The relative nutritive values by the slope ratio assay with young rats were 0.75 for whey protein and 0.92 for the fortified whey protein.

Casein was covalently modified via the ϵ-amino group of lysine residues using a series of active *N*-hydroxysuccinimide esters of amino acids (Puigserver et al., 1978). In vitro digestibility using bovine α-chymotrypsin, pancreatin, or rat bile–pancreatic juice was lower than the untreated protein, but plasma amino acid patterns for rats fed a 10% protein diet of highly modified glycyl- or methionylcaseins suggested good in vivo digestibility.

In our laboratory, limiting amino acids have been covalently attached by a coupling reaction using carboxyl group activation with a water-soluble carbodiimide. The use of carbodiimides as coupling reagents for peptide synthesis was first proposed by Sheehan and Hess (1955). The use of water-soluble carbodiimides is advantageous in that excess reagents and possible urea and acylurea products can be easily removed (Sheehan and Hlavka, 1956). Voutsinas and Nakai (1977) used this approach to increase methionine and tryptophan contents of soy protein isolate to 6.3-fold and 11.3-fold, respectively. An in vitro pepsin pancreatin digestion test demonstrated that the bound amino acids were easily hydrolyzable.

The objective of the present work was to investigate the use of the carbodiimide method for covalent attachment of lysine to wheat gluten. Although the reaction is basically simple, several problems had to be overcome to enable the carbodiimide method feasible for this purpose. Firstly, wheat gluten is highly insoluble in water, forming a cohesive and elastic mass of dough; pepsin and partial acid hydrolysis were therefore assessed for solubilization of gluten prior to lysine attachment. Peptide bond cleavage by pepsin hydrolysis results in increased contents of α-amino and α-carboxyl groups, which is accompanied by an increase in gluten dispersibility (Yang and McCalla, 1968; Oka et al., 1965). Mild acid hydrolysis increases gluten dispersibility by selective hydrolysis of amide groups of glutamine residues to γ-carboxyl groups (Wu et al., 1976). Secondly, lysine contains a side chain ϵ-amino group in addition to α-amino and α-carboxyl groups. Since the question of digestibility of ϵ-blocked lysine residues has not been clearly resolved, it was necessary to consider reversible procedures for protecting the ϵ-amino group of lysine prior to reaction with gluten to stimulate formation of α–α peptide bonds.

EXPERIMENTAL SECTION

Materials. Vital gluten was obtained commercially as "Whet Pro 75% vital wheat gluten" from Industrial Grain Products Ltd., Thunder Bay, Canada. L-Lysine monohydrochloride was obtained from Sigma Chemical Co. N^{ϵ}-Benzylidenelysine was synthesized by the method of Begas and Zervas (1961); cleavage and removal of the protecting groups after the carbodiimide reaction was accomplished by heating in 1 N HCl at 50 °C for 15 min, followed by ether extraction. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was from Sigma Chemical Co., as were the enzymes pepsin (from hog stomach mucosa, two times recrystallized) and pancreatin (from porcine pancreas, grade VI).

Gluten Solubilization. Vital gluten was solubilized either by mild acid hydrolysis using 0.5 N HCl according to Wu et al. (1976) or by pepsin hydrolysis of a gluten dispersion (generally 2%) at pH 1.6, 37 °C, for the specified time period, using a pepsin/gluten ratio of 1:100 (w/w). After pH adjustment to neutrality, the solubilized gluten was freeze-dried.

Carbodiimide Reaction. Figure 1 illustrates the scheme for the carbodiimide reaction for lysine attachment. Freshly prepared aqueous solutions of lysine or lysine derivative and EDC were added to a solution containing 500 mg of solubilized gluten. *N*-Hydroxysuccinimide (NHS) was added to reduce the possibilities of racemization and formation of *N*-acylureas (Weygand et al., 1966). The pH and volume were adjusted. After incubation, the reaction was stopped by the addition of glacial acetic acid to 1 N concentration, followed by dialysis against first 1 N acetic acid and then against water. After

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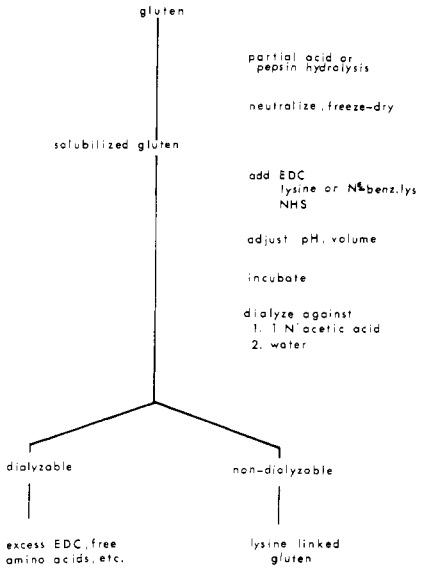

Figure 1. Carbodiimide reaction scheme for lysine attachment to gluten. EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; N^{ϵ}-benz-lys, N^{ϵ}-benzylidenelysine.

pH adjustment to neutrality, the sample was freeze-dried.

Optimization Techniques. To avoid the need to execute a large number of experiments which increases exponentially as the number of potentially influential factors increases in full factorial experimental designs, two-level and three-level fractional factorial designs (Taguchi, 1957; Box and Hunter, 1961) were used to select influential factors and interactions.

Simplex optimization of Morgan and Deming (1974) has been used for optimization of analytical procedure (Dols and Armbrecht, 1976). This technique was also used in this study to find the best condition for linking lysine to gluten after a slight modification of the algorithm.

Determination of Lysine and Other Amino Acids. After first blocking cysteine residues by reaction with 4-vinylpyridine as described by Cavins et al. (1972), acid hydrolysis for amino acid analysis was carried out with *p*-toluenesulfonic acid for 24 h at 110 °C 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 model M6800 amino acid analyzer.

Lysine content was also measured by the dinitrobenzenesulfonate (DNBS) spectrophotometric method according to Concon (1975).

Digestibility of samples was determined by amino acid analysis after an in vitro pepsin pancreatin digestion by the method of Stahmann and Woldegiorgis (1975), with the following modification: after digestion, to 5.0 mL of the digestion mixture was added 5.0 mL of 14% sulfosalicylic acid; the mixture was then shaken for 15–30 min, pH adjusted to 2.2 with 6 N NaOH, and volume adjusted to 15 mL with pH 2.2 citrate buffer. The sample was then centrifuged (1000*g*, 30 min), filtered through Whatman No. 1 filter paper and then through a sintered glass ultrafine filter, and stored frozen until analyzed.

Unless otherwise specified, lysine content of samples was generally expressed as relative lysine increase, defined here as the ratio of lysine content in the sample to lysine content in untreated vital gluten.

Nitrogen content was analyzed on a Technicon Auto Analyzer II system, after prior digestion of samples by the microKjeldahl digestion method of Concon and Soltess (1973). A factor of 5.7 was used as the conversion factor for protein content of gluten samples.

Table I. Factor Levels for Three-Level Design for Reaction between Acid-Solubilized Gluten and Lysine

factor	level 1	level 2	level 3
polarity	0	5% ethanol	5% ether
ionic strength (% NaCl)	0	1	5
pH	5	7.5	10
temperature, °C	10	25	40
reaction time, h	0.5	6	24
activation time, min	0	15	60
HCl concn, N	0.05	0.10	0.50

Table II. Analysis of Variance of Results from Three-Level Design for Reaction between Acid-Solubilized Gluten and Lysine

factor[a]	SS	df	mean square (MS)	*F* ratio[b]
polarity	0.0044	2	0.0022	4.89
pH	0.0175	2	0.0088	19.44**
temperature	0.0056	2	0.0028	6.22*
HCl concn	0.0024	2	0.0012	2.67
polarity × ionic strength	0.0022	4	0.0006	1.23
polarity × pH	0.0064	4	0.0016	3.56
ionic strength × pH	0.0068	4	0.0017	3.78
error	0.0027	6	0.00045	
total	0.0480	26		

[a] The sum of squares (SS) values for time of activation, reaction time, and ionic strength were low and were incorporated into the error term. [b] $F(0.01)2, 6 = 10.43$**; $F(0.05)2, 6 = 5.14$*; $F(0.01)4, 6 = 9.15$**; $F(0.05)4, 6 = 4.53$*.

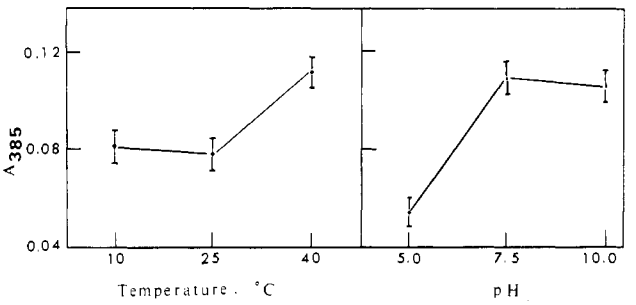

Figure 2. Effects of temperature and pH on the increase in lysine content by binding of lysine to acid-solubilized gluten, measured at A_{385} by the DNBS reaction. (Confidence limits calculated as $\pm$ [($t_{0.05}$ at df_e)/(no. of data averaged)]$\sqrt{S_e}/\sqrt{df_e}$, where df_e is the degrees of freedom for error and S_e is the sum of squares for error).

RESULTS AND DISCUSSION

Optimization of Reaction Conditions. *a. Binding of Lysine to Acid-Solubilized Gluten.* Preliminary selection of influential factors in the carbodiimide reaction for lysine attachment was based on the results of fractional factorial analysis of variance. A three-level design was used to study the reaction between acid-solubilized gluten and lysine. The seven factors under consideration were polarity, ionic strength, pH, temperature, reaction time, activation time with EDC, and HCl concentration for gluten solubilization. The levels chosen are shown in Table I. As Table II indicates, only pH ($P < 0.01$) and temperature ($P < 0.05$) were found to be significantly influential on the amount of lysine increase. Figure 2 shows that within the chosen levels of the factors, pH 7.5 and 40 °C were found to be most favorable for lysine attachment.

Table III. Factor Levels for Two-Level Design for Reaction between Acid-Solubilized Gluten and Lysine

factor	level 1	level 2
EDC amount, mg	100	190
lysine amount, mg	100	700
HCl concn, N	0.05	0.5
gluten amount, mg	100	500

Table IV. Analysis of Variance of Results from Two-Level Design for Reaction between Acid-Solubilized Gluten and Lysine

factor[a]	SS	df	MS	F^b
lysine amount	0.0500	1	0.0500	23.25*
HCl concn	0.0191	1	0.0191	8.88
gluten amount	0.0019	1	0.0019	4.23
EDC × lysine	0.0328	1	0.0328	15.26
EDC × HCl concn	0.0424	1	0.0424	19.72*
error	0.0043	2	0.00215	
total	0.1577	7		

[a] The sum of squares (SS) values for EDC amount and the other interactions were small and were incorporated into the error term. [b] $F(0.05)1, 2 = 18.513$*; $F(0.01)1, 2 = 98.503$**.

A two-level experimental design was also used to study the effects of EDC, gluten, and lysine amounts and HCl concentration for gluten solubilization on the reaction between lysine and acid-solubilized gluten (Table III). The most influential factor was the lysine amount ($P < 0.05$, Table IV), the increase in lysine in the product being higher with an increase in the amount of lysine used during the reaction.

In these experiments, a range of 3–20-fold increase in lysine could be attained. However, the lysine–gluten link resulting from the reaction between free lysine and acid-solubilized gluten, which contains many glutamic acid side chains, could be α–α peptide bonds, α–ϵ, γ–α, and/or γ–ϵ isopeptide bonds, formed by condensation of either α-(C-terminal amino acid or added lysine) or γ-(glutamic acid residue) carboxyl groups with either α-(N-terminal amino acid or added lysine) or ϵ-(lysine residue or added lysine) amino groups. The isopeptide bonds may not all be readily digestible.

b. Selection of Lysine Derivatives for ε-Amino Protection. Trifluoroacetyl, formyl, benzylidene, and *tert*-butyloxycarboxyl groups were studied as possible protecting groups for the ϵ-amino group of lysine. Of these, only the N^{ϵ}-benzylidenelysine was incorporated during the carbodiimide reaction. No significant lysine increase was observed when any of the other three derivatives were used. Further optimization studies were therefore carried out using pepsin-hydrolyzed gluten and N^{ϵ}-benzylidenelysine, which should result primarily in α–α peptide bonds.

c. Binding of N^{ϵ}-Benzylidenelysine to Pepsin-Solubilized Gluten. A two-level experimental design was used to select influential factors in the reaction between pepsin-hydrolyzed gluten and N^{ϵ}-benzylidenelysine. The seven factors considered are shown in Table V. As indicated in Table VI, within the chosen range of levels of these factors, only pH and pepsin hydrolysis time were significantly influential ($P < 0.05$) on the extent of lysine increase, the conditions of lower pH and longer pepsin hydrolysis time being more effective.

d. Effect of Pepsin Hydrolysis Duration. Although a longer pepsin hydrolysis time favored lysine increase, it resulted in significantly lower product yield (Table VII). Average yields of 42 and 30% were obtained for the 2-h and 16-h hydrolyzed samples, respectively. Table VIII shows the results of amino acid analysis of a 38-h pepsin hydrolyzed gluten sample before and after dialysis, and the corresponding lysine-enriched sample obtained by the carbodiimide method, which involves dialysis to remove excess reagents and side reaction products. The dialyzed pepsin-hydrolyzed gluten and lysine-enriched sample both show lower contents of several amino acids, such as alanine, valine, isoleucine, leucine, and phenylalanine. These results imply that the low yield of product obtained when a long hydrolysis time is used for solubilization of gluten may be due to the production of small peptides or free amino acids which are lost during dialysis. Yang and McCalla's work (1968) on the action of proteolytic enzymes on gluten indicated increasing amounts of nonprotein nitrogen and terminal amino nitrogen with increasing hydrolysis time from 2 to 48 h, with either 1 or 2% pepsin as the enzyme. However, free amino acid nitrogen was not detected until 12 h of hydrolysis, when free phenylalanine, leucine, valine, and tyrosine were present. Proteolysis of glutenin by pepsin was shown by Oka et al. (1965) to rapidly decrease the viscosity of the dispersion and increase the water-soluble fraction. These workers suggested that these early changes were results of cleavage of a few

Table V. Factor Levels for Two-Level Design for the Reaction between Pepsin-Hydrolyzed Gluten and N^{ϵ}-Benzylidenelysine

factor	level 1	level 2
gluten concn, %	1	5
reaction time, h	6	21
reaction temp, °C	40	65
pH	6.4	8.1
pepsin hydrolysis time, h	2	16
EDC, mg	200	270
N^{ϵ}-benzylidenelysine, mg	200	468

Table VI. Analysis of Variance of Results of Two-Level Design for Reaction between Pepsin-Hydrolyzed Gluten and N^{ϵ}-Benzylidenelysine (Dependent Variable = Lysine Increase)

factor	SS	df	MS	F^a
pepsin hydrolysis time	4.931	1	4.931	9.726*
pH	4.794	1	4.794	9.456*
temperature	0.019	1	0.019	0.037
N^{ϵ}-benzylidenelysine amount	0.088	1	0.088	0.174
EDC amount	0.003	1	0.003	0.006
time	0.645	1	0.645	1.272
gluten concn	0.675	1	0.675	1.331
time × gluten concn	0.025	1	0.025	0.049
pH × temp	0.003	1	0.003	0.006
error	3.040	6	0.507	
total	14.223	15		

[a] $F(0.01)1,6 = 13.74$**; $F(0.05)1,6 = 5.99$*.

Table VII. Analysis of Variance of Results of Two-Level Design for the Reaction between Pepsin-Hydrolyzed Gluten and N^{ϵ}-Benzylidenelysine (Dependent Variable = Product Yield)

factor[a]	SS	df	MS	F^b
pepsin hydrolysis time	0.0124	1	0.0124	75.78**
pH	0.0002	1	0.0002	1.22
N^{ϵ}-benzylidenelysine amount	0.0004	1	0.0004	2.44
gluten concn	0.0165	1	0.0165	100.83**
error	0.0018	11	0.00016	
total	0.0313	15		

[a] The sum of squares (SS) values for temperature, EDC amount, and time were small and were incorporated into the error term. [b] $F(0.01)1,11 = 9.65$**; $F(0.05)1,11 = 4.84$*.

Table VIII. Amino Acid Composition (g/100 g) of Gluten, Solubilized Gluten, and Lysine Enriched Gluten Samples[a]

	gluten	38-h pepsin gluten	dialyzed 38-h pepsin gluten	N^{ϵ}-benzylidenelysine 38-h pepsin gluten	N^{ϵ}-benzylidenelysine 1-h pepsin gluten
Asp	2.81	3.04	2.64	3.19	2.85
Thr	2.23	2.50	2.62	3.11	2.30
Ser	4.48	4.61	3.83	3.89	4.14
Glu	37.45	37.07	39.51	33.58	36.89
Pro	12.67	12.23	14.82	15.44	12.61
Gly	2.88	3.06	2.98	2.29	2.55
Ala	2.17	2.36	1.67	1.36	1.83
Val	2.96	3.04	2.45	2.30	2.65
Met	1.16	1.04	0.88	1.21	1.17
Ile	3.15	3.32	2.46	2.29	2.37
Leu	6.50	6.57	5.35	5.14	5.15
Tyr	3.09	3.14	3.00	3.15	3.42
Phe	4.86	4.86	4.21	4.35	4.81
Lys	1.33	1.45	1.29	*5.73*	*5.93*
His	1.78	1.63	2.10	2.36	1.43
Arg	3.00	2.90	2.88	3.03	2.91
Trp	0.79	0.69	0.65	0.89	0.77
Cys	1.68	1.50	1.65	1.70	1.60

[a] Experimental conditions: N^{ϵ}-benzylidenelysine 38-h pepsin gluten-pepsin hydrolysis time for solubilization, 38 h; gluten concentration, 0.8% (500 mg in 62.5 mL); EDC, 174 mg; N^{ϵ}-benzylidenelysine, 250 mg; NHS, 46 mg; pH 7.2; reaction temp, 54 °C; reaction time, 19 h. N^{ϵ}-benzylidenelysine 1-h pepsin gluten-pepsin hydrolysis time for solubilization, 1 h; gluten concentration, 2.5% (500 mg in 20 mL); EDC, 289 mg; N^{ϵ}-benzylidenelysine, 188 mg; NHS, 50 mg; pH 5.7; reaction temp, 40 °C; reaction time, 6 h.

Table IX. Results of Simplex Search for Reaction between N^{ϵ}-Benzylidenelysine and 1-h Pepsin Hydrolysate of Gluten

vertex no.	simplex no.	vertices retained	factor levels[a] pH	N^{ϵ}-benz·lys, mg	EDC, mg	rel Lys increase
1	I		4.50	100	100	1.30
2			9.70	188	144	2.40
3			5.70	477	144	3.10
4			5.70	188	289	4.40
5	II	2, 3, 4	9.56	468	285	1.70
6		2, 3, 4	8.30	376	238	2.30
7	III	2, 3, 4	5.77	193	147	3.50
8	IV	3, 4, 7	1.74	384	240	1.00[b]
9		3, 4, 7	7.71	237	168	4.10
10	V	4, 7, 9	7.08	−65	260	1.00[b]
11		4, 7, 9	6.05	341	172	3.90
4′			5.70	188	289	4.33[c]
12	VI	4, 9, 11	7.21	317	273	3.70
13		4, 9, 11	6.13	224	178.5	4.10
14	VII	4, 9, 13	6.97	91	252	1.00[b]
15		4, 9, 13	6.74	153.5	232	3.83

[a] The boundary conditions were as follows: pH, 4.5-10.0; N^{ϵ}-benzylidenelysine, 100-500 mg; EDC, 100-300 mg. [b] Violated boundary condition; a response of 1.00 was arbitrarily assigned to force the factor back inside the boundary by a contraction. [c] The response for vertex 4 was checked after it had occurred in $(k + 1)$ or four successive simplexes.

peptide bonds in glutenin; further pepsin action would cause a substantial increase in small peptide fragments.

A shorter hydrolysis time of 1 h was then attempted for solubilization of gluten prior to lysine attachment. Dispersions (0.5, 2.0, and 5.0%) of gluten all showed visible increase in dispersibility and decrease in viscosity within 15 min of addition of pepsin at a pepsin/gluten ratio of 1:100 (w/w) at pH 2, 37 °C. After 1 h, samples were neutralized to pH 7. The 2 and 5% dispersions showed some tendency for sedimentation after neutralization while the 0.5% sample remained well dispersed. Therefore, for further studies, 0.5% dispersions of gluten were solubilized using 0.005% pepsin at pH 2, 37 °C, for 1 h, followed by neutralization to pH 7 and freeze-drying.

Table VIII indicates that the lysine-enriched sample obtained using the 1-h hydrolyzed gluten had an amino acid composition more similar to that of untreated gluten than the enriched 38-h hydrolyzed gluten except for lower isoleucine and leucine contents. The yield of product remained relatively low, usually between 45 and 60%, but was still a significant improvement over the samples prepared after long pepsin hydrolysis times.

e. Binding of N^{ϵ}-Benzylidenelysine to 1-h Pepsin-Hydrolyzed Gluten. Optimization was attempted by the simplex method using a relatively short (1 h) pepsin hydrolysis time for gluten solubilization. The variable factors were pH, N^{ϵ}-benzylidenelysine amount, and EDC amount. The reaction conditions which were held constant were pepsin-hydrolyzed gluten concentration (2.5%), NHS amount (50 mg), reaction temperature (40 °C), and reaction time (6 h). The factor levels at each vertex and results of the simplex search are shown in Table IX. Figure 3 shows the relative increase in lysine content as a function of the simplex search. The leveling off in response at about fourfold increase in lysine content indicates that the search has homed in on the optimum conditions for the reaction. The values for the centroid of the last complete simplex are pH 6.39, 248 mg of N^{ϵ}-benzylidenelysine, and 202 mg of EDC. The lysine increase

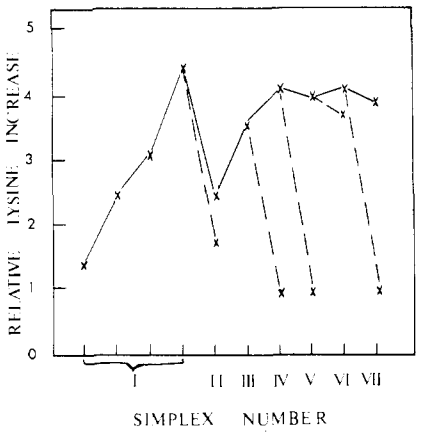

Figure 3. Relative increase in lysine content by the carbodiimide reaction as a function of simplex number. (See Table IX for factor levels at each vertex. Dashed lines indicate vertices generated at failed steps.)

obtained under these experimental conditions was 3.80.

f. Multiple Regression Analysis to Confirm the Obtained Optimum. Multiple regression analysis of the combined data of the two-level factorial design in Table V and an initial simplex containing eight vertices was also used to obtain an approximation of the optimum reaction conditions for maximal lysine increase. The boundary conditions for the simplex were pepsin hydrolysis time (3–48 h), pepsin hydrolyzed gluten concentration (1–5%), reaction time (1–24 h), reaction temperature (25–60 °C), pH (4.5–10.0), EDC amount (50–200 mg), N^{ϵ}-benzylidenelysine amount (50–500 mg). Multiple regression analysis of the 24 data values obtained from the two-level design and simplex, followed by solving of the simultaneous partial derivative equations yielded the following values for the reaction conditions: pepsin hydrolysis time, 30 h; solubilized gluten concentration, 1%; N^{ϵ}-benzylidenelysine amount, 140 mg; EDC amount, 130 mg; pH, 6.48; reaction time, 2.5 h; reaction temperature, 55 °C. The estimated optimal and experimental values for the lysine increase resulting from the carbodiimide reaction under the above reaction conditions were 3.99 and 3.89, respectively. This result confirms that within the ranges of current experimental conditions approximately fourfold increase in lysine content is near the optimum unless new factors are introduced, e.g., use of trypsin instead of pepsin.

Availability of the Attached Lysine Residues. Amino acid analysis after acid hydrolysis allows determination of the "total" amino acid composition of a protein. However, it cannot indicate the nutritional availability of the amino acids.

The DNBS reaction was used to measure the content of "free" ϵ-amino groups in the lysine-linked gluten samples, while an in vitro pepsin pancreatin digestion test was carried out to indicate the relative ease of enzymatic hydrolysis of the attached lysine residues. These results are compared with the total lysine content measured after *p*-toluenesulfonic acid hydrolysis, as shown in Table X.

The percentage of lysine residues in lysine-linked acid gluten measured by the DNBS reaction is low (48%), implying that the ϵ-amino groups of the lysine residues may have been blocked, perhaps in the form of α–ϵ or γ–ϵ bonds as a result of condensation with α-(C-terminal amino acid) or γ-(glutamic acid residue) carboxyl group of acid-solubilized gluten or even with α-carboxyl group of the added lysine. In contrast, in the case of acid-solubilized gluten reacted with $N\epsilon$-benzylidenelysine, followed by removal of the N^{ϵ}-benzylidene group, the proportion of DNBS-available lysine ϵ-amino groups was high (91%), indicating

Table X. Comparison of Lysine Contents Measured after Acid Hydrolysis, Dinitrobenzenesulfonate (DNBS) Reaction, and in Vitro Pepsin Pancreatin (PP) Digestion

samples[b]	Lys content, g/100 g[a]		
	acid	DNBS	PP digest
gluten	1.4	1.2 (89)	1.0 (75)
acid-solubilized gluten	1.5	1.3 (87)	0.61 (41)
Lys acid gluten	5.5	2.6 (48)	2.3 (41)
N^{ϵ}-benzylidenelysine acid gluten[c]	3.1	2.8 (91)	1.3 (41)
pepsin (38 h) gluten	1.5	*d*	1.0 (69)
N^{ϵ}-benzylidenelysine pepsin (38 h) gluten[c]	5.9	5.2 (89)	3.8 (65)
pepsin (1 h) gluten	1.4	*d*	1.0 (75)
N^{ϵ}-benzylidenelysine pepsin (1 h) gluten[c]	5.9	5.3 (90)	3.9 (65)
egg (whole)	6.2	5.4 (87)	1.9 (31)
casein (isoelectric)	7.3	7.3 (100)	3.3 (45)

[a] Lysine contents measured by DNBS and PP digestion were corrected for nitrogen content of each sample. Numbers in parentheses indicate the content of DNBS-available or PP-digestible lysine as a percentage of the total lysine content measured after acid hydrolysis. [b] Experimental conditions: lysine acid gluten–0.5 N HCl solubilized gluten concentration, 2.1%; EDC, 50 mg; L-lysine hydrochloride, 50 mg; pH 5.0; reaction temperature, 22 °C; reaction time, 2 h. N^{ϵ}-benzylidenelysine acid gluten–0.5 N HCl solubilized gluten concentration, 2.3%; EDC, 100 mg; activation time, 15 min; N^{ϵ}-benzylidenelysine, 100 mg; pH 8.5; reaction temperature, 60 °C; reaction time, 5.5 h. N^{ϵ}-benzylidenelysine pepsin (38 h) and (1 h) gluten preparation was described in the footnote to Table VIII. [c] N^{ϵ}-benzylidene group was removed after the carbodiimide reaction, prior to lysine determination. [d] Not determined.

the effectiveness of the protective group during the carbodiimide reaction and of the unblocking procedure after the reaction. However, pepsin pancreatin digestion showed lower percentages (41%) of pepsin pancreatin digestible lysine for the acid-solubilized gluten as well as the corresponding lysine and N^{ϵ}-benzylidenelysine enriched samples, when compared to the value obtained for untreated gluten (75%). This may have been due to the presence of γ–α bonds or β–α bonds (β-carboxyl group of aspartic acid residue). Alternatively, it may reflect a reduction in overall digestibility of gluten after acid solubilization. When compared to untreated gluten, the acid-solubilized gluten samples showed decreased enzymatic release of several of the amino acids, especially aspartic acid, threonine, and serine.

In contrast to the acid-solubilized samples, the lysine-enriched samples prepared from N^{ϵ}-benzylidenelysine reaction with pepsin-hydrolyzed gluten showed high proportions of both DNBS-available and pepsin pancreatin digestible lysine. The digestion profiles showed similar release of amino acids to that from untreated gluten, with the exception of slightly lower values of isoleucine, which may be explained by the lower total content of this amino acid after pepsin treatment of gluten (Table VIII).

It should be noted that hydrolysis by the pepsin pancreatin digestion assay is not complete (Stahmann and Woldegiorgis, 1975). As shown in Table X, 31 and 45% of the total lysine were released under the conditions of this assay for whole egg and isoelectric casein, respectively. Gluten showed 75% release of its lysine. Comparable values were obtained for the total amino acids released by the three proteins, ranging from 18 to 24 g of amino acids released per 100 g of protein.

Despite the lack of complete hydrolysis by this method, the amount of the limiting amino acid released by enzymatic hydrolysis is a good indicator of amino acid avail-

ability (Stahmann and Woldegiorgis, 1975). The high values of pepsin pancreatin released lysine for the pepsin-hydrolyzed gluten samples enriched using N^{ϵ}-benzylidenelysine are comparable to or higher than the values for the whole egg and isoelectric casein standards. These results offer potential use of these high lysine gluten samples as good quality proteins.

These preliminary tests thus indicate good digestibility and availability of the lysine-enriched gluten samples, particularly those prepared with pepsin hydrolyzed gluten and N^{ϵ}-benzylidenelysine. It is possible that isopeptide links, which were not measured by these in vitro analyses, may be nutritionally available in the body. For example, ϵ-(γ-glutamyl)-L-lysine and ϵ-(α-glutamyl)-L-lysine have both been found to be available as lysine sources for the rat (Mauron, 1972). N^{ϵ}-Benzylidenelysine has also been shown to be virtually 100% utilized as a lysine source during growth trials on rats (Finot et al., 1977a). Finot et al. (1977b) tested various N-substituted lysine derivatives for their growth-promoting effect in rat assays. ϵ-N-(α-L-aminoacyl), α-N-ϵ-N-di-L-aminoacyl, and Schiff base type derivatives were all utilized efficiently as lysine sources for the rat. None of the α-N-acyl- and ϵ-N-acylglycyl derivatives were utilized at all. ϵ-N-Acyl and ϵ-N-(ω-L-aminoacyl) derivatives were generally not utilized, with the exception of ϵ-N-(γ-L-glutamyl)lysine and ϵ-N-formyl- and ϵ-N-acetyllysine. Kornguth et al. (1963) observed that papain, chymotrypsin, trypsin, leucine amino peptidase, and pronase did not attack the ϵ-(γ-glutamyl)lysine. However, this dipeptide is probably absorbed into the intestinal wall and hydrolyzed in the kidney since it can be found in the plasma of rats and chicks fed it in their diet (Waibel and Carpenter, 1972), and an ϵ-lysine acylase has been suggested to function in the mammalian kidney (Leclerc and Benoiton, 1968).

In vivo digestibility tests and animal feeding studies are recommended to check nutritional availability and to establish the absence of any toxic substances in the lysine-linked gluten. Investigation is also warranted on the feasibility and benefits of using the carbodiimide reaction for analogous attachment of threonine for further nutritional improvement of wheat gluten.

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Reactions of Hymenoxon: Base Conversion to Psilotropin and Greenein and Formation of "Michael Adduct" with Cysteine

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Studies on the reaction of hymenoxon in alkaline solution indicated that at pH 10–12 hymenoxon was converted to intermediates that formed psilotropin and greenein when the reaction medium was adjusted to pH 1. Hymenoxon was shown to react with the sulfhydryl group of cysteine. The second-order rate constant for the reaction was 504 L mol^{-1} min^{-1}.

Hymenoxon is a toxic α-methylene sesquiterpene lactone that has been identified as a component of *Hymenoxys odorata* (Kim et al., 1975; Ivie et al., 1975) and *Helenium hoopsii* and *Baileye multiradiata* (Hill et al., 1977). Under basic conditions, hymenoxon can be converted to isomeric dilactones, psilotropin, and greenein. This reaction has been used to confirm the structure of hymenoxon (Kim et al., 1975) and to produce derivatives that aid in the rapid screening of plants for the presence of hymenoxon (Hill

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