Carbodiimide-Mediated Covalent Attachment of Lysine to Wheat Gluten and Its Apparent Digestibility by Penaeid Shrimp

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Carbodiimide-mediated covalent attachment of lysine to wheat gluten was evaluated with respect to its availability to penaeid shrimp. Normality of acid hydrolysis and carbodiimide reaction criteria (levels of carbodiimide and L-lysine hydrochloride) were optimized, and the resultant lysine-enriched samples were evaluated for extent of isopeptide bonding. *In vivo* apparent digestibilities of both enriched and unenriched glutens to shrimp were compared. Large-scale lysine enrichment of wheat gluten yielded a 590% increase in lysine content of wheat gluten. Only an additional 6% of the lysine present was attached by isopeptide bonding. *In vivo* apparent dry matter digestibility, protein digestibility, and lysine availability of both the enriched and unreacted glutens were similar, confirming suitability of reaction criteria. The ability of shrimp to hydrolyze isopeptide-bound lysine was indicated by similarities in apparent digestibility of lysine in enriched and unenriched glutens.

Keywords: *Lysine; carbodiimide; gluten; shrimp*

INTRODUCTION

Although the shrimp farming industry in the United States has an annual crop value of $18 million (Lawrence, personal communication), fundamental nutritional information such as requirements for indispensable amino acids (IAA) has not been determined. Requirements for IAA for terrestrial animals and some finfish have been determined due to (1) an ability to use crystalline amino acids (CAA) in such diets (Robinson, 1991) and (2) minimal manipulation of feed pellets during consumption in these species. The use of CAA in determination of IAA for shrimp has been limited due to problems associated with pellet stability (leaching of CAA; Provasoli and D'Agostino, 1969; Deshimaru and Kuroki, 1974a,b) and atypical absorption kinetics (Deshimaru, 1976; Cowey and Lucquet, 1983).

The semipurified protein wheat gluten is first-limiting in lysine when fed to juvenile penaeid shrimp (*Penaeus vannamei*; Fox et al., 1994) and, in small amounts, can be substantially enriched with lysine via covalent attachment methodology (Li-Chan et al., 1979; Li-Chan and Nakai, 1980; Ikura et al., 1985; Iwami et al., 1986). Prior to using covalently bound lysine in a requirement diet, its nutritional availability to shrimp must be determined. For example, improper reaction and subsequent drying of lysine-enriched wheat gluten can result in reduced availability of lysine due to Maillard-type reactions (Friedman and Finot, 1990).

The objective of this research was to evaluate lysine enrichment of wheat gluten via carbodiimide-mediated reaction: (1) extent of large-scale lysine enrichment possible via covalent attachment; (2) comparison of ε-free lysine content in enriched and unenriched gluten species; and (3) comparison of *in vivo* apparent dry matter, protein, and lysine digestibility of covalently lysine-enriched gluten to other intact and crystalline lysine sources.

EXPERIMENTAL PROCEDURES

Materials. Vital wheat gluten, alginate, vitamin premix, and mineral premix were obtained commercially from ICN Biomedicals, Inc., Costa Mesa, CA. All crystalline amino acids, α-cellulose, 1-ethyl-3-[3-(di-methylamino)propyl]carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were from Sigma Chemical Co., St. Louis, MO. Menhaden fish meal and menhaden oil were obtained from Zapata Haynie Co., Dulac, LA. Chromic oxide and sodium hexametaphosphate were obtained from Fisher Chemical Co., Houston, TX. Wheat starch, diatomaceous earth, lecithin, and cholesterol were obtained from the United States Biochemical Corp., Cleveland, OH. L-Ascorbyl 2-polyphosphate was obtained from Rangen, Inc., Buhl, ID. Dietary vitamin and mineral premixes were obtained from Rangen Co., Boise, ID. Shrimp, *P. vannamei*, were obtained as postlarvae from Harlingen Shrimp Farm, TX.

Optimization of Reaction Criteria. Reaction criteria for large-scale lysine enrichment of wheat gluten were first determined by solubilizing 500 mg samples of vital wheat gluten in 0.05, 0.10, and 0.50 N hydrochloric acid as described by Wu et al. (1976). The three resultant enriched samples plus one unhydrolyzed sample were then reacted with L-lysine hydrochloride and EDC at the following levels: (1) no addition; (2) 100 mg of L-lysine hydrochloride and 100 mg of EDC; (3) 400 mg of L-lysine hydrochloride HCl and 150 mg of EDC; (4) 700 mg of L-lysine hydrochloride and 190 mg of EDC. To each of these 16 mixtures was added 50 mg of NHS to reduce the possibility of racemization and formation of N-acylureas. Reactions were terminated by addition of glacial acetic acid to 1 N final concentration. Samples were purified by successive dialysis against 1 N acetic acid and water. Purified samples were freeze-dried and analyzed for amino acid concentration according to the procedure of Cohen et al. (1991).

Large-scale lysine enrichment of wheat gluten was under reaction conditions determined by the previously described methodology with only small modifications. Vital wheat gluten (400 g) was solubilized in 8.0 L of 0.1 N HCl, subdivided into four equal volumes, and autoclaved at 121 °C for 15 min. After cooling, protein was precipitated by isoelectric flocculation at pH 4.5 using 10 N NaOH. Flocculated protein was centrifuged at 1100g (3000 rpm) for 10 min at 7 °C (Sorvall Model RC2-B refrigerated centrifuge) and washed twice with pH 4.5 distilled water. Protein pellets were resuspended in 3500 mL of water, the pH was adjusted to

Table 1. Indispensable Amino Acid (IAA) Composition (Percent As-Fed) of Diets Used for Apparent Digestibility Determinations[a]

IAA	CAA-Lys[b]	COV-Lys[b]	WG[b]
lysine	8.26	8.26	1.00
arginine	2.20	2.36	2.44
histidine	1.02	1.05	1.13
methionine	0.77	0.64	0.85
threonine	1.51	1.56	1.67
valine	2.39	2.53	2.64
isoleucine	2.13	2.51	2.36
leucine	4.21	4.60	4.66
phenylalanine	3.12	3.50	3.45

[a] Values calculated from ingredient amino acid composition. [b] Abbreviations of diets are as follows: CAA-Lys, wheat gluten + L-lysine hydrochloride; COV-Lys, covalently lysine-enriched wheat gluten; WG, wheat gluten (unenriched).

6.0, and the pellets were divided into two 1750 mL samples. Samples were further diluted in an equal volume of distilled water and, while mixing, the following ingredients were added: (1) 76 g of EDC, (2) 20 g of NHS, and (3) 280 g of L-lysine hydrochloride. The reaction mixture was adjusted to pH 6 and stirred at room temperature for 4 h. Reaction was terminated by addition of glacial acetic acid to pH 2.5. The mixture was then centrifuged under similar conditions as above and protein precipitated with 10 N NaOH (pH range 8.0–9.0). Flocculated protein was centrifuged as before, and the protein precipitates were pooled and resuspended in distilled water to achieve a total volume of about 800 mL. The sample was then dialyzed using 6000–8000 molecular weight membranes for 1.5 days against running water (4 °C) prior to freezing and freeze-drying. Enriched freeze-dried samples were then analyzed for amino acid concentration.

Characterization of Lysine Bonding. Large-scale lysine-enriched wheat gluten, unenriched wheat gluten, and 0.1 N HCl hydrolyzed wheat gluten samples were subjected to fluorometric analysis of amino groups according to procedures of Goodno et al. (1980) to determine the relative proportion of isopeptide bonding of lysine. Fluorescence was read on a Sequoia-Turner Model 450 fluorometer. The excitation maximum was at 360 nm, and the emission maximum was at 450 nm. The equation of Goodno et al. (1980) was used to estimate predicted relative fluorescence. Actual relative fluorescence values of samples were compared to predicted relative fluorescence values.

Evaluation of Apparent Digestibility. Diets similar to those previously developed at the Texas A&M Shrimp Mariculture Project (Akiyama, 1989) were used to investigate the apparent digestibility of covalently lysine-enriched wheat gluten by penaeid shrimp. Shrimp were fed three different dry feeds varying with respect to method of lysine supplementation or enrichment: wheat gluten and L-lysine hydrochloride (CAA-Lys), covalently lysine-enriched wheat gluten (COV-Lys), and wheat gluten (WG). Single protein source feeds (WG, COV-Lys) each contained 94% of their respective protein source. The CAA-Lys feed contained a mixture of wheat gluten (84.81%) and was supplemented with 9.19% L-lysine hydrochloride to achieve a lysine concentration similar to that of the COV-Lys feed. All feeds contained 3% binder (2% alginate and 1% sodium hexametaphosphate), 2.00% attractant (fish solubles), and 1% chromic oxide. Indispensable amino acid (IAA) composition of feeds is shown in Table 1. All feeds, except for the COV-Lys feed, were cold-extruded via a Hobart A-200 extruder and dried at 60 °C until a moisture content of

8% was achieved. Dry pellets were ground to achieve diameters suitable for feeding to shrimp. Due to limited availability, the COV-Lys diet was extruded using a confection syringe (3.175 mm aperture) and subsequently ground to an appropriate size. All dry feeds were also tested for pH, water stability, sinkability, and attractability to *P. vannamei*. Feed pH was determined by grinding feed pellets (no. 35 sieve) with subsequent suspension and reading in deionized water at a 1:1 w/v ratio. For pellet water stability testing, 1.0 g of feed from each diet was first submerged and dry matter weight loss was determined at 30, 60, 90, and 120 min. For sinkability, similarly sized feed particles were broadcasted into seawater and sinking time was recorded. Attractability of pellets was determined by measuring the time required for shrimp to grasp and consume pellets once immersed.

Juvenile shrimp (*P. vannamei* Boone, 11 g) were stocked into 200 L tanks (0.34 m^2 bottom area) at densities of 10 shrimp/tank. Level of replication was four tanks per dietary treatment. Seawater exchange rate was 270% of the total tank volume per hour. Temperature, salinity, and dissolved oxygen concentration in tanks were maintained at acceptable levels for the duration of the digestability feeding trial (29 ± 2 °C, 30 ± 2 ppt, and 7.3 ± 0.2 mg ppm, respectively). Shrimp were preconditioned to culture system conditions for a period of 3 days during which they were fed a commercial feed (45% crude protein).

Amino Acid Determinations. Precolumn derivatization reversed-phase high-pressure liquid chromatography (Pico-Tag HPLC) was undertaken according to methods described in Cohen et al. (1991).

Determination of Apparent Digestibility. Feeding of shrimp, collection of feces, and sample preparation were according to methods described in Akiyama (1989). Apparent digestibility of feeds was evaluated as apparent dry matter digestibility (ADMD), apparent protein digestibility (APD), and apparent lysine availability (AA-Lys) using the equations

$$ADMD = 100 \times (1 - Cr_{diet}/Cr_{feces}) \qquad (1)$$

where ADMD is the apparent dry matter digestibility and Cr is the chromic oxide concentration and

$$AND = 100 [1 - (N_{feces}/N_{diet}) (Cr_{diet}/Cr_{feces})] \quad (2)$$

where AND is the apparent nutrient (protein or lysine) digestibility and N is the nutrient concentration. Samples were analyzed for chromic oxide (McGinnis and Kasting, 1964) and Kjeldahl nitrogen (Ma and Zuazago, 1942). Optical density of prepared samples were determined on a Bausch and Lombe Spectronic 100 spectrophotometer.

Statistical Analyses. All data were statistically analyzed using Statistical Analytical System, Inc. (SAS), procedures (SAS, 1988). Comparison of the amino acid profile of lysine-enriched gluten (large scale) to that of wheat gluten was undertaken by two-way ANOVA. Two-way analysis of variance and Student–Neumann–Keuls test were used to compare means among diets for ADMD, APD, and AA-Lys. All statistical analyses were undertaken using a rejection level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Optimization of Reaction Criteria. Results from small-scale enrichment of wheat gluten with lysine

Attachment of Lysine to Wheat Gluten

J. Agric. Food Chem., Vol. 43, No. 3, 1995 **735**

Table 2. Small-Scale Lysine Enrichment of Wheat Gluten[a]

reaction criteria	acid hydrolysis level (normality of HCl)			
	none	0.05	0.10	0.50
no EDC, no Lys	1.35 ± 0.01	1.62 ± 0.09	1.68 ± 0.26	1.58 ± 0.11
100 mg of EDC + 100 mg of Lys	1.73 ± 0.21	2.14 ± 0.29	3.27 ± 0.10	4.64 ± 0.09
150 mg of EDC + 400 mg of Lys	2.00 ± 0.02	4.15 ± 0.04	4.94 ± 0.49	6.31[b] ± 0.87
190 mg of EDC + 700 mg of Lys	2.27 ± 0.13	4.28 ± 0.39	5.94 ± 0.10	6.26[b] ± 0.07

[a] Values represent mean percentage lysine ± standard deviation of three replicates. [b] Enrichment values are all significantly different with respect to reaction criteria. Values are also significantly different within acid hydrolysis level, except those indicated by this superscript within the 0.50 N HCl group.

Table 3. Amino Acid Composition (Grams per 100 g of Gluten) of Wheat Gluten and Lysine-Enriched Gluten[a]

amino acid	wheat gluten	lysine-enriched gluten	*F* value	*P* > *F*
Asp	1.99 ± 0.01	1.89 ± 0.04	14.79	0.0310
Glu	24.61 ± 0.22	22.42 ± 0.55	0.27	0.6268
Ser	3.15 ± 0.01	2.82 ± 0.05	131.56	0.0003
Gly	2.32 ± 0.02	1.69 ± 0.02	1488.38	0.0001
Ala	1.72 ± 0.04	1.67 ± 0.02	3.75	0.1249
Pro	8.90 ± 0.04	8.60 ± 0.37	2.57	0.1839
Tyr	2.58 ± 0.05	2.14 ± 0.07	82.37	0.0008
NEAA[b]	45.27 ± 0.39	41.23 ± 1.07	1723.69	
His	1.20 ± 0.05	1.12 ± 0.05	4.60	0.0987
Arg	2.60 ± 0.03	2.52 ± 0.04	7.10	0.0561
Thr	1.78 ± 0.02	1.66 ± 0.06	10.51	0.0316
Val	2.81 ± 0.13	2.69 ± 0.05	2.05	0.2256
Met	0.91 ± 0.12	0.68 ± 0.05	10.40	0.0322
Ile	2.51 ± 0.05	2.67 ± 0.06	15.16	0.0176
Leu	4.96 ± 0.18	4.89 ± 0.12	0.35	0.5841
Phe	3.67 ± 0.18	3.72 ± 0.10	0.12	0.7241
Lys	1.28 ± 0.02	8.79 ± 0.19	4635.54	0.0001
EAA[b]	21.72 ± 0.78	28.74 ± 0.72	4682.83	
EAA − Lys	20.44	19.95		
EAA/NEAA	0.48	0.70		

[a] Values represent means ± standard deivation of three replicates from one prepared sample. [b] NEAA, nonessential amino acids; EAA, essential amino acids.

hydrochloride via covalent attachment methodology are shown in Table 2. Lysine content increased with normality of HCl hydrolysis and level of reaction constituents, with highest enrichment in 0.1 and 0.5 N HCl-hydrolyzed samples at the two highest reaction levels of EDC and lysine hydrochloride. A 4.7-fold lysine enrichment maximum was achieved as a result of 0.5 N HCl hydrolysis of 500 mg of gluten followed by reaction with 400 mg of lysine hydrochloride, 50 mg of NHS, and 150 mg of EDC. However, all samples solubilized with 0.5 N HCl showed brown discoloration, indicative of Maillard reaction. The 0.1 N HCl-hydrolyzed gluten sample reacted with 700 mg of L-lysine hydrochloride, 50 mg of NHS, and 190 mg of EDC was only slightly less enriched (4.4-fold increase). These criteria were chosen for subsequent large-scale enrichment.

Large-scale lysine enrichment of wheat gluten yielded a significant 6.87-fold increase in lysine content (Table 3), which accounted for most of the difference in amino acid concentration. There was also a significant increase in isoleucine (6.4%) in the lysine-enriched gluten; however, this change was small compared to that of lysine. Significant decreases in glycine, serine, tyrosine, and aspartic acid were found in the lysine-enriched gluten. There were also significant decreases in some IAA, particularly methionine and threonine, but not to the extent as shown for the previously mentioned DAA.

Enrichment of wheat gluten with lysine via covalent attachment indicated that a 6-fold increase in lysine

content was possible using gluten solubilized under mild hydrolysis conditions (0.1 N HCl). Hydrolysis under more acidic conditions, although facilitating slightly higher levels of enrichment, resulted in apparent Maillard interaction. Proteins that have undergone these types of reactions are often rendered less digestible due to formation of cross-linked amino acids (Friedman and Finot, 1990). Other sources of lysine (e.g., ε-amino blocked derivative forms such as N^ϵ-benzylidenelysine and N^ϵ-acetyllysine) appear to be less susceptible to Maillard reaction and result in lower frequency of isopeptide bonding (Li-Chan et al., 1979); however, their use is limited by cost or additional effort with respect to synthesis.

Large-scale production of covalently lysine-enriched gluten also indicated an enhancement of the environment process over that of small-scale preliminary efforts. This might be explained by the following observations: (1) mixing of reagents took place in larger vessels which were capable of being mechanically stirred (enhanced reaction conditions); (2) separation of precipitated protein was more easily accomplished; and (3) purification of the enriched gluten (i.e., removal of reagents) was more efficient.

A slight decrease in the concentration of other amino acids would be expected in the enriched gluten due to attachment of lysine via the carbodiimide reaction. Excluding lysine, most of the variance in amino acid profile between enriched and unenriched glutens occurred in the DAA, particularly with respect to aspartic acid, glycine, serine, and tyrosine (Table 3). There are various possible reasons for this change in content of DAA of the lysine-enriched wheat gluten. Mild acid hydrolysis by autoclaving in the presence of 0.1 N HCl was used as a pretreatment for solubilization of gluten prior to the carbodiimide-mediated reaction for lysine attachment. Solubilization by acid treatment was reported to result in not only deamination of glutamine and asparagine residues but also rupture of a few peptide linkages in gluten molecules (Wu et al., 1976). Subsequent loss of smaller molecular weight fragments produced by such peptide bond hydrolysis could occur during dialysis and would be reflected in changes in amino acid profile. Acid treatment could also catalyze changes in some of the amino acid residues. Wu et al. (1976) reported that 0.5 N HCl solubilization causes a loss of 40% in glycine and tryptophan and a 20% loss in aspartic acid, alanine, lysine, and arginine.

The changes in glycine, serine, and tyrosine reported in the present study did not agree with previous results reported by Li-Chan et al. (1979) for other enriched forms of gluten; however, total AA-N levels of the enriched and nonenriched samples were similar. Differences in amino profile of the enriched gluten samples in the present study and reported by Li-Chan et al.

Table 4. Observed Relative Fluorescence of Various Gluten Samples

protein source	rel fluorescence obsd[a]	rel fluorescence predicted[b]	ε-amino free lysine[c] (%)
wheat gluten	0.90 ± 0.04	1.00	90
hydrolyzed gluten	0.91 ± 0.04	1.00	91
lysine-enriched	3.27 ± 0.11	3.91	84

[a] Values represent means ± standard deviations of four replicates. [b] Values taken or calculated from Goodno et al. (1980). [c] (Observed relative fluorescence)/(predicted relative fluorescence) × 100.

(1979) may be attributed to differences in preattachment hydrolysis conditions (pepsin rather than acid hydrolysis for gluten solubilization) as well as different lysine attachment reaction criteria.

Evaluation of Lysine Bonding. Actual observed relative fluorescence of wheat gluten, 0.1 N HCl-solubilized wheat gluten, and covalently lysine-enriched gluten samples versus predicted values is shown in Table 4. The higher value for the enriched gluten sample was indicative of lysine enrichment. Comparison of actual to predicted fluorescence values indicated that 84% of the lysine attached to wheat gluten was via normal peptide bonding and 16% was via isopeptide bonding. The orientation of isopeptide bonding was not determined (i.e., α–ε, γ–α, or γ–ε).

Previous covalent attachment studies have emphasized the role of amino acid enrichment in the nutritional improvement of semipurified proteins such as wheat gluten (Li-Chan et al., 1979; Li-Chan and Nakai, 1980, 1981a,b) and soy protein (Voutsinas and Nakai, 1979; Matheis et al., 1985). These studies also evaluated biological availability of the enriched protein/attached amino acids. In the present study, about 90% of the lysine present in the unenriched gluten samples was peptide-bound (ε-amino lysine free). The difference between observed relative fluorescence and that predicted by Goodno et al. (1980) indicated that ε-amino free lysine content of the enriched samples was 84%. Thus, a 6.87-fold increase in lysine content of the enriched samples resulted only in a 6% increase in isopeptide-bound lysine as a percentage of the total lysine. Apparently, covalent attachment of lysine via the proposed reaction criteria described for large-scale enrichment can be used to avoid excessive isopeptide bonding.

In a previous study (Li-Chan et al., 1979), addition of lysine hydrochloride to 0.5 N HCl-solubilized wheat gluten via carbodiimide reaction resulted in only 48% ε-amino free total lysine versus the 84% shown in the present study. They also achieved a lower level of lysine enrichment (3.7-fold increase) compared to that of the large-scale enrichment in this study (6.87-fold increase). This discrepancy could be attributed to (1) use of 0.1 versus 0.5 N HCl for acid solubilization, (2) differences in concentration of reactants, and (3) improvements in the attachment procedure related to the scale of reaction. The present study indicates that derivatized forms of lysine may not be required for covalent lysine enrichment of wheat gluten via the carbodiimide reaction.

Evaluation of Digestibility. There was no significant dry matter weight loss observed among diet samples for the submergence period tested. Thus, it was concluded that diets possessed adequate binding characteristics. All feed particles sank to the bottom of culture tanks within 1 min of submergence, and all feeds were equally attractive to separate groups of

Table 5. Apparent Dry Matter Digestibility (ADMD), Apparent Protein Digestibility (APD), and Apparent Lysine Digestibility (AA-Lys) of Various Diets by Shrimp[a]

diet	ADMD (%)	APD (%)	AA-Lys (%)
CAA-Lys	89.80 ± 0.23[a]	96.79 ± 0.58[a]	96.79 ± 0.58[a]
COV-Lys	78.16 ± 1.78[b]	82.94 ± 1.07[b]	91.97 ± 0.30[b]
WG	78.75 ± 2.04[b]	81.71 ± 0.36[b]	91.81 ± 0.45[b]

[a] Values represent means ± standard deviation of four replicates. Vertical values with similar superscripts are not statistically different ($P \leq 0.05$).

shrimp. Feed pH values were 6.51, 7.08, and 6.60 for the CAA-Lys, COV-Lys, and WG diets, respectively.

There was no significant difference between ADMD values for shrimp fed the COV-Lys feed and the WG feed (78.16 and 78.75%, respectively); however, the CAA-Lys feed showed significantly higher ADMD (89.80%) than either of the other two diets (Table 5).

Relationships among diets in terms of mean apparent protein digestibility (APD) were similar to those shown for ADMD, and there was a general increase in APD values over ADMD values (Table 5). APD values for feeds increased with degree of refinement of protein (amino acid) source. Ranking of feeds in terms of APD was as follows: CAA-Lys > COV-Lys = WG. The CAA-Lys feed had the highest APD (96.79%) and, again, there was no significant difference in APD between shrimp fed the COV-Lys diet and the WG diet.

As with ADMD and APD comparisons, apparent lysine availability values (AA-Lys) for shrimp fed the various feeds increased significantly with increased level of protein refinement of feeds (Table 5). AA-Lys for shrimp fed the various feeds ranged from 96.79% for the CAA-Lys feed to 91.97 and 91.81% for the COV-Lys and WG feeds, respectively. Again, there was no significant difference in lysine availability of the COV-Lys and WG feeds to shrimp.

The major emphasis of this part of the study was to compare apparent availability of covalently attached lysine to that of crystalline lysine and "naturally bound" lysine (WG diet). Apparent availability of lysine in the CAA-Lys feed was higher than that other feeds largely due to supplementation with 8.66% L-lysine hydrochloride to achieve a lysine content similar to that of the COV-Lys feed. Thus, although similar in lysine content, a larger proportion of the lysine was in crystalline form. Deshimaru (1976), working with Penaeus japonicus, showed that total absorption of CAA was similar to that of amino acids derived from intact protein but occurred more rapidly.

As mentioned, there was no difference in either ADMD, APD, or AA-Lys between the COV-Lys feed and the WG feed. Because APD and AA-Lys were similar for COV-Lys and WG diets, it may be concluded that they can be used as protein sources for diets in the estimation of requirement for lysine.

Although isopeptide-bound lysine is not considered available to terrestrial animals (Li-Chan et al., 1979), the present study indicated that shrimp might have some capacity for utilization of isopeptide-bound lysine. There was a slight increase in the level of isopeptide-bound lysine in the covalently lysine-enriched wheat gluten, yet no difference was shown in lysine availability. For isopeptide-bound lysine to be digested by penaeid shrimp, a specific activity with respect to enzymes capable of hydrolyzing the isopeptide bond of ε-N-glutamyllysine is required. Puigserver et al. (1979) have shown that the isopeptide bond resulting from

Attachment of Lysine to Wheat Gluten

J. Agric. Food Chem., Vol. 43, No. 3, 1995 **737**

covalent methionine enrichment of casein (ϵ-N-methionyllysine) was very efficiently hydrolyzed by intestinal membrane-bound aminopeptidase in rats. Lee (personal communication) has suggested that hydrolysis of isopeptide bonds involving glutamic acid and lysine (e.g., ϵ-N-glutamyllysine) could be effected by an endopeptidase such as nonspecific carboxylesterase due to its small molecular weight (12 000) and specificity for this bond.

In conclusion, large-scale production of lysine-enriched wheat gluten via carbodiimide reaction methodology appears not only to be feasible but also resulted in more efficient overall enrichment. The similarity between apparent availability of lysine from covalently enriched wheat gluten to unenriched wheat gluten indicates its potential for use in diets used for estimation of requirement for lysine by penaeid shrimp. The significance of this research is that a methodology for determination of the IAA requirements of a large group of economically important crustaceans is finally available. Further research is warranted with respect to enrichment and bioavailability testing of other IAA-deficient proteins.

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