Guanidination of Lysine in Cottonseed Protein

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The conversion of lysine residues to homoarginine was determined for 11 feed protein sources by incubating the proteins in 0.4 M O-methylisourea (OMIU) solution at pH 10.5 and at room temperature. The conversion efficiency in these protein sources was found to vary from 36.1 to 74.5%. Proteins from soybean meal, lupin, and fish meal had moderately high conversions (69.0–74.5%), while the conversion of lysine in cottonseed protein was very low (36.1%). For this reason, studies were undertaken to determine the optimum conditions for the maximum guanidination of cottonseed protein. Effects of different incubation times (24 to 144 h), lysine:OMIU ratios (1:8 to 1:32), and pH (9.5 to 13.0) on the guanidination of lysine in cottonseed protein were studied in separate experiments. On the basis of this monofactorial approach, optimum conditions for the maximum guanidination of cottonseed protein were determined to be as follows: lysine:OMIU ratio, 1:12; pH, 12.5; and incubation time, 72 h. Potential protein racemization during guanidination under strong alkaline conditions can be avoided by incubating at 4 °C, but this will be associated with significant losses in conversion efficiency.

Keywords: *Cottonseed protein; guanidination; optimum conditions; homoarginine*

INTRODUCTION

A novel technique using chemical labeling of lysine in dietary proteins to distinguish between exogenous and endogenous amino acids in the ileum of monogastric animals was proposed by Hagemeister and Erbersdobler (1985). Labeling is achieved by the guanidination reaction wherein lysine residues in dietary protein are transformed to homoarginine (2-amino-6-guanidinohexanoic acid) by treatment with O-methylisourea (OMIU) under alkaline conditions (Mauron and Bujard, 1964). After the labeled protein is fed, endogenous losses of amino acids are determined by comparing amino acid: homoarginine ratios in the diet and ileal digesta. The basic principle is that homoarginine is not used for protein synthesis and therefore does not appear in endogenous proteins. Many of the limitations associated with the conventional methods, such as feeding a nitrogen-free diet, regression analysis to zero nitrogen intake, and fasting of animals (Sibbald, 1987), can be overcome by the homoarginine technique. Siriwan et al. (1994) reported that values for ileal endogenous amino acid secretions obtained by the use of guanidinated casein were about 2-fold greater than those estimated either by feeding a nitrogen-free diet or by extrapolation to zero nitrogen intake.

Most studies using guanidinated proteins to measure endogenous amino acid losses have utilized isolated proteins, such as casein and gelatin, as sources of homoarginine (Siriwan et al., 1989; Moughan and Rutherfurd, 1990; Schmitz et al., 1991; Barth et al., 1993; Roos et al., 1994; Siriwan et al., 1994) mainly because of the high degree of guanidination achieved with isolated proteins. Use of this technique, however, is not dependent on the degree of conversion of lysine residues to homoarginine provided that the conversion is at random. Nevertheless, as pointed out by Rutherfurd and Moughan (1990) and Siriwan (1990), a high degree of conversion may be necessary to ensure random

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distribution of homoarginine within the protein and to improve precision of the determination of endogenous amino acids. Preliminary work conducted in our laboratory and those of Siriwan et al. (1994) showed that only moderate levels of guanidination are achieved in proteins from common feed ingredients. In the case of cottonseed protein, conversion efficiency was found to be low, ranging between 32 and 37%; this clearly limits the application of homoarginine technique to estimate endogenous amino acid output when cottonseed protein is fed to animals. It is known that the process of guanidination is influenced by several factors including temperature, incubation time, pH of the medium, and the concentration of OMIU (Maga, 1981; Rutherfurd and Moughan, 1990), but optimal reaction conditions for different dietary proteins have not been fully characterized. In the present study, this question was addressed in relation to cottonseed protein by examining the influence of lysine:OMIU ratio, incubation time, and pH on the efficiency of guanidination.

MATERIALS AND METHODS

Materials. Feed ingredients were purchased from commercial sources in Sydney. The ingredients were ground in a laboratory mill to pass through a 1-mm screen and stored in airtight plastic containers until used. Casein (lactic acid casein) was obtained from Cottee Corporation Pty. Ltd. (Chatswood, New South Wales, Australia), and isolated soybean protein (isolated soy protein, Supro 500 E) was obtained from Protein Technologies International (Leper, Belgium). O-Methylisourea hydrogen sulfate (99% purity) was purchased from Aldrich-Chemie (Steinherm, Germany) and $Ba(OH)_2 \cdot 8H_2O$ and NaOH was purchased from Merck Pty. Ltd. (Kilsyth, Victoria, Australia). All chemicals and reagents used were of analytical grade.

Preparation of 0.4 M O-Methylisourea. The 0.4 M OMIU (free base) was prepared using a procedure modified from the methods reported by Rutherfurd and Moughan (1990), Siriwan (1990), and Schmitz et al. (1991). To prepare 1 L of 0.4 M OMIU, 69 g of OMIU hydrogen sulfate was weighed into a beaker and dissolved in approximately 600 mL of distilled water. A sample of 65.55 g of $Ba(OH)_2 \cdot 8H_2O$ was weighed and added directly to the OMIU hydrogen sulfate solution. The solution was left to stand for 1 h, and the barium

Table 1. Crude Protein and Lysine Contents (g/100 g of Air-Dry Weight) and Percentage Lysine Converted to Homoarginine in Different Protein Materials under Standard Guanidination Conditions (Experiment 1)[a]

ingredient	crude protein[b]	lysine	% conv of lysine to homoarginine
casein	87.6	7.90	97.2 ± 0.9
isolated soy protein	84.2	5.66	98.8 ± 1.0
soybean meal	48.3	3.06	74.5 ± 1.1
sunflower meal	34.5	1.19	49.0 ± 0.9
cottonseed meal	39.5	1.58	36.1 ± 1.1
lupin	34.0	1.71	70.8 ± 0.7
meat and bone meal	55.7	2.85	50.9 ± 0.3
fish meal	63.8	4.12	69.0 ± 0.5
blood meal	91.3	8.54	59.8 ± 0.8
feather meal	87.1	1.70	60.7 ± 0.3
corn	8.1	0.26	57.5 ± 0.5
wheat	9.7	0.31	61.1 ± 0.4
sorghum	11.6	0.27	61.6 ± 0.7

[a] 0.4 M OMIU, 1:12 lysine:OMIU ratio; pH 10.5, incubation at room temperature (22 °C) for 24 h, average and standard error of four individually reacted samples. [b] N × 6.25.

sulfate precipitate was then separated by centrifugation at 1600g for 15 min at room temperature. The clear supernatant was transferred to a beaker, and the volume was brought to about 950 mL. At this stage, the pH of free OMIU solution was between 9.0 and 9.5. This was increased to pH 10.5 by the gradual addition of 2 M NaOH and the volume was made up to 1 L.

Experimental Procedures. In recent studies carried out in our laboratory, casein has been successfully guanidinated by treatment with 0.4 M OMIU solution at pH 10.5 and at room temperature for 24 h, with a high degree of conversion of lysine to homoarginine (Angkanaporn et al., 1994; Imbeah et al., 1994). The extent of guanidination of 2 isolated proteins and 11 feed proteins (Table 1) under these reaction conditions were investigated in experiment 1. Quadruplicate batches of feed ingredients (ca. 10 g of protein) were weighed into beakers and 0.4 M OMIU solution (freshly prepared and adjusted to pH 10.5) was added to each beaker. The suspensions were incubated at pH 10.5 for 24 h at room temperature (22 ± 2 °C) with occasional stirring. The pH was checked at 3 h intervals during the first 12 h and adjusted as necessary. After 24 h, the pH was gradually reduced to 3.0 using 4 N HCl to precipitate the protein. The solution was allowed to stand for 3 h and then centrifuged at 1600g for 15 min to separate the precipitate. The precipitate was washed with distilled water at pH 3.0 and recentrifuged; this step was repeated twice. The precipitate (guanidinated protein) was then lyophilized, ground in a laboratory grinder, and stored in airtight plastic containers at −4 °C until analyzed for amino acids.

The objective of experiment 2 was to investigate the effects of lysine:OMIU ratios (molar basis) on the guanidination rate of cottonseed protein incubated in 0.4 M OMIU at pH 10.5 and room temperature (20 ± 1 °C) for 24 h. Duplicate batches of cottonseed meal (ca. 10 g of protein) were incubated in different volumes of 0.4 M OMIU solutions to give six different lysine:OMIU ratios ranging from 1:8 to 1:32. After 24 h, the guanidinated proteins were removed and processed as described above.

Experiments 3 and 4 were conducted to study the effects of incubation time and pH, respectively, on guanidination rates of cottonseed protein. Incubation times evaluated ranged from 24 to 144 h, with samplings at 12 h intervals up to 72 h and at 24 h intervals thereafter. pH ranges examined were from 9.5 to 13.0. Standard conditions, except for the variables tested, were employed in these two experiments. Both experiments were conducted at room temperatures. Average room temperatures during experiments 3 and 4 were 17 ± 2 °C and 20 ± 1 °C, respectively. pH readjustment, separation of guanidinated protein, and sample preparation for amino acid analysis were carried out as described above.

Amino Acid Analysis. The lysine and homoarginine concentrations of guanidinated samples were determined by high-performance liquid chromatography (HPLC). Sample preparation for HPLC analysis involved hydrolysis of meal samples and freeze-dried guanidinated protein samples according to the procedure described by Siriwan et al. (1993). Briefly, the samples were hydrolyzed under nitrogen with 8 N HCl containing phenol (3 g/L) for 16 h at 120 °C. DL-Norleucine was added to the hydrolysate as an internal standard. Each hydrolysate was then diluted and adjusted to pH 2.2−2.3, the same as the amino acid standard (Standard H, Pierce Chemical Co., Rockford, IL). The hydrolysates were passed through a 0.20-μm Nylon 66 membrane filter (Alltech, Baulkham Hills, New South Wales, Australia). Aliquots of the hydrolysates were then subjected to ion-exchange column chromatography, using a Shimadzu amino acid analysis system (Shimadzu Corp., Kyoto, Japan). Underivatized amino acids were eluted by a gradient of pH 3.20 sodium citrate eluent to pH 10.00 sodium citrate eluent at a flow rate of 0.4 mL/min and a column temperature of 60 °C. *O*-Phthalaldehyde (OPA; Sigma Chemicals Co., St. Louis, MO) was used for postcolumn derivatization of amino acids. All reagents used for HPLC, except OPA, were obtained from BDH Chemicals (BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia). Homoarginine eluted relatively late in the gradient, at 56 min, remote from any other peaks.

Calculation. The extent of guanidination of lysine was calculated from the concentrations of lysine and homoarginine present in the guanidinated samples using the following formula:

$$\text{extent of guanidination (\%)} = \frac{\text{mol of homoarginine}}{\text{mol of homoarginine} + \text{mol of lysine}} \times 100$$

Statistical Analysis. Analysis of variance was conducted using the General Linear Models procedure (Minitab, 1991), and significant differences among the means were determined using the least significant difference (Steel and Torrie, 1982).

RESULTS AND DISCUSSION

Previous studies in our laboratory have utilized guanidinated casein for the estimation of endogenous amino acid losses; since it is a purified protein, we were able to achieve a high degree of conversion of lysine to homoarginine (89−98%) in casein (Angkanaporn et al., 1994; Imbeah et al., 1994). However, it is of practical interest to extend these studies to the guanidination of common ingredients used in animal feed formulations. Guanidination of casein was achieved by reaction with 0.4 M OMIU solution in alkaline conditions (pH 10.5) for 24 h. When various protein materials were guanidinated under these standard conditions, the extent of conversion was found to vary widely (Table 1). As expected, isolated proteins (casein and isolated soy protein) were almost completely guanidinated in contrast to the feed proteins. Among the feed proteins, conversion of lysine residues to homoarginine were moderately high for soybean protein, lupin *(Lupinus angustifolicus)* protein, and fish protein (69−71%); moderate for proteins from blood meal, feather meal, maize, wheat, and sorghum (58−62%); low for proteins from meat and bone meal and sunflower meal (49−51%); and very low for cottonseed meal (36%). The observed variability probably reflects incomplete guanidination and/or the inaccessibility of some lysine residues closely associated with other polymeric components within the ingredient. In view of the variability observed, optimal reaction conditions in terms of incubation time, OMIU concentration, and pH need to be established for each feed protein if maximum conversion rates are to be achieved.

We precipitated and recovered guanidinated proteins at pH 3.0. It should be noted, however, that not all of the guanidinated proteins from different feed materials may be expected to precipitate at pH 3.0. To achieve

Table 2. Effects of Different Lysine:OMIU Ratios on the Formation of Homoarginine in Cottonseed Protein (Experiment 2)[a]

lysine:OMIU ratio	% conv of lysine to homoarginine	lysine:OMIU ratio	% conv of lysine to homoarginine
1:8	31.1 ± 0.5	1:20	31.1 ± 0.6
1:12	33.8 ± 0.9	1:24	30.4 ± 0.3
1:16	33.3 ± 0.3	1:32	32.1 ± 0.1
		lsd[b]	1.24

[a] 0.4 OMIU, pH 10.5, incubation at room temperature (20 °C) for 24 h, average and standard error of two individually reacted samples. [b] Least significant difference (*P* < 0.05).

Table 3. Influence of Guanidination Time on the Formation of Homoarginine in Cottonseed Protein (Experiment 3)[a]

time (h)	% conv of lysine to homoarginine	time (h)	% conv of lysine to homoarginine
24	28.8 ± 0.3	72	45.8 ± 0.3
36	36.5 ± 0.4	96	45.7 ± 0.5
48	42.5 ± 0.2	120	45.9 ± 1.5
60	43.0 ± 0.5	144	47.0 ± 0.2
		lsd[b]	1.66

[a] 0.4 OMIU, pH 10.5, 1:12 lysine:OMIU ratio, incubation at room temperature (17 °C), average and standard error of four individually reacted samples. [b] See Table 2.

Table 4. Influence of Guanidination pH on the Formation of Homoarginine in Cottonseed Protein (Experiment 4)[a]

pH	% conv of lysine to homoarginine	pH	% conv of lysine to homoarginine
9.5	11.1 ± 0.2	11.5	39.0 ± 0.1
10	26.9 ± 0.8	12.0	48.4 ± 1.0
10.5	35.0 ± 0.2	12.5	52.5 ± 1.0
11.0	38.2 ± 0.1	13.0	38.9 ± 0.4
		lsd[b]	2.52

[a] 0.4 OMIU, 1:12 lysine:OMIU ratio; incubation at room temperature (20 °C) for 24 h, average and standard error of four individually reacted samples. [b] See Table 2.

Table 5. Influence of Temperature and Guanidination Time on the Formation of Homoarginine in Cottonseed Protein[a]

temp (°C)	time (h)	% conv of lysine to homoarginine
21	48	63.0 ± 0.6
	72	64.1 ± 0.1
4	48	52.3 ± 0.9
	72	52.4 ± 0.1
	96	55.0 ± 0.2
	120	53.5 ± 1.4
	144	54.7
lsd[b]		2.45

[a] 0.4 OMIU, 1:12 lysine:OMIU ratio, pH 11.5, average and standard error of two individually reacted samples. [b] See Table 2.

maximum recovery, the isoelectric point of each protein needs to be determined and appropriate pH values must be employed. It may be argued that the use of a single pH for isoelectric precipitation may lead to partial recovery of the total protein in heterogeneous feed proteins and that the extent of guanidination of these recovered proteins could be different from other proteins which remain soluble at the particular pH. In the present study, though the protocol used may have resulted in variable recoveries, the recoveries were always over 80% discounting this possible error. In the case of casein, the recovery was almost 100%, and for soybean meal and cottonseed meal, the recoveries are in the range of 92−97%. Therefore the isoelectric precipitation of guanidinated feed proteins at a single pH would have had little effect on the extent of guanidination of the precipitated protein.

Rutherfurd and Moughan (1990) studied the influence of protein:OMIU ratio on the conversion of lysine to homoarginine. However, ascertaining the influence of lysine:OMIU ratio will be more relevant than protein:OMIU ratio since lysine is the substrate for the guanidination reaction. This aspect is of interest because of the high cost of OMIU hydrogen sulfate; establishment of the optimum lysine:OMIU ratio will prevent the excess and wasteful use of the expensive OMIU solution. Ratios lower than 1:8 were not considered based on the results of an earlier study from our laboratory (Imbeah et al., 1996). The influence of different lysine:OMIU ratios on the conversion of lysine to homoarginine is shown in Table 2. A lysine:OMIU ratio of 1:12 was found sufficient for maximum guanidination of cottonseed protein. Conversion of lysine to homoarginine was greater (*P* < 0.05) at this ratio compared to a ratio of 1:8. No further improvements were observed with higher ratios.

The conversion of lysine to homoarginine increased (*P* < 0.05) markedly between 24 (28.8%) and 48 (42.5%) h of incubation (Table 3). The guanidination reaction in cottonseed protein was essentially complete at 72 h (45.8% conversion), with little improvement with longer incubation periods. The results showed that an incubation period of 48 h was sufficient to achieve a high level

of guanidination of cottonseed protein, although 72 h appeared to be necessary for maximum guanidination. The conversion efficiency obtained for 24 h incubation in experiment 3 was lower than that obtained for cottonseed protein in experiment 1 (28.8 vs 36.1%). The lower ambient temperatures experienced during experiment 3 (17 vs 22 °C) are likely to be responsible for this variability. Temperature dependency of the guanidination reaction has been shown by Maga (1981).

The conversion of lysine to homoarginine was low at pH 9.5 and 10.0, and increased (*P* < 0.05) with increasing pH (Table 4). Maximum conversion of 52.5% was observed at pH 12.5. Interestingly, the conversion dropped (*P* < 0.05) sharply to 38.9% when the pH was increased from 12.5 to 13.0. This sharp decline is probably associated with changes in the tertiary structure of cottonseed protein at pH 13.0 and the resultant structural alteration and inaccessibility of some lysine residues for guanidination. It is also possible that some de-guanidination may occur at pH 13.

The maximum conversion efficiency achieved for cottonseed protein using the monofactorial approach used in the present experimental series was 52.5% (Tables 2−4). However, this could be further improved to 64.1% by combining the optimum conditions for different variables, as shown in Table 5. In three subsequent studies involving large-scale guanidination (5−10 kg batches) of cottonseed protein, we have obtained an average of 63.4% conversion of lysine residues to homoarginine by incubating in 0.4 M OMIU (1:12 lysine:OMIU ratio) at pH 11.5 and at room temperature (19 ± 1 °C) for 72 h, indicating that the guanidination procedure can be scaled up to 5−10 kg, without any loss in efficiency.

Although the highest conversion was achieved at pH 12.5 (Table 4), only a pH of 11.5 was employed in subsequent studies owing to certain limitations associated with the use of high pH. The first concern relates to the possible formation of lysinoalanine under alkaline conditions (Maga, 1981), although Raymond (1980) was

of the opinion that lysinoalanine will be a problem only in food systems that have been exposed to elevated temperatures. Another concern is the potential racemization of the amino acid residues to D-forms during guanidination of proteins under alkaline conditions (Liardon and Hurrell, 1983). Proteins containing D-amino acids have been reported to show decreased digestibility in vitro (Bunjapamai et al., 1982). However, as shown recently by Vrese et al. (1994), the problem of protein racemization at pH values above 10.5 can be avoided by guanidinating the proteins at 4 °C rather than at room temperatures. On the other hand, guanidination of proteins at 4 °C would result in lowered efficiency of conversion owing to the temperature dependency of the reaction. Guanidination of cottonseed protein for 48 h at 4 °C instead of at room temperature in our study lowered ($P < 0.05$) the conversion efficiency from 63 to 52% (Table 5). The conversion efficiency increased ($P < 0.05$) slightly when cottonseed protein was incubated for 96 h, with no further improvement for longer incubation periods. Thus, though potential protein racemization can be avoided by guanidination at 4 °C, the present observations suggest that this will be associated with significant losses in conversion efficiency.

The increased guanidination of cottonseed meal from 36.1% (Table 1) to 55.0% (Table 5) by manipulating guanidination conditions is of practical interest in animal trials for two important reasons. Firstly, it increases the chance for random distribution of homoarginine within guanidinated protein. Implicit in the use of guanidinated proteins to measure endogenous amino acid secretions are the assumptions that (1) the lysine in the test protein is homogeneously guanidinated, and (2) homoarginine in guanidinated proteins is released and absorbed at the same rate as other amino acids. These assumptions have been examined elsewhere (Schmitz et al., 1988; Siriwan et al., 1994; Bryden et al., 1995) and found to be valid. A greater degree of guanidination would, therefore, increase the probability that the digestibility of homoarginine will not be different from that of other amino acids. Secondly, a higher degree of guanidination increases the concentration of marker (homoarginine) in the ileal digesta. Both the above considerations will lead to increased precision in the use of guanidinated proteins to determine endogenous amino acid secretions.

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