AN APPROACH TO IMPROVE PROTEIN QUALITY OF GLANDLESS COTTONSEED FLOUR BY AMINO ACID(S) SUPPLEMENTATION

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CHAPTER I

INTRODUCTION

During the past few years much attention has been given to cottonseed flour as a new protein source to meet the needs of malnourished people. The advantage of using cottonseed flour is that it is one of the cheapest and most available sources of concentrated protein (1,2). The composition and nutritional quality of cottonseed meals vary greatly with the variety of cotton, the time of harvest, and the industrial processes during which oil is extracted (3). Deglanded cottonseed by liquid cyclone process (LCP) and glandless cottonseed by plant breeding technique are the two recent developmental achievements which reduced the toxic component, gossypol, in cottonseed (4-6). Gossypol is physiologically detrimental to monogastric animals (1,4,5).

Texas Woman's University (TWU) is one of the institutes where research on cottonseed flour has been and is being conducted actively. There have been a number of studies investigating protein value of cottonseed flour, using both humans and animals as test subjects. The results are well summarized in a professional paper (7).

From these results it is concluded that cottonseed protein is acceptable and has a potential for human consumption. However, its nutritional quality is still lower than casein, that is, the protein efficiency ratio (PER) of cottonseed protein ranges from 1.21 to 2.36 compared to 2.50 of casein (7,8). The reason for the low protein efficiency ratio seems to be deficiencies of some essential amino acids in cottonseed protein. Cottonseed protein is shown to be low in lysine, methionine, and isoleucine in comparison to casein (9,10). The amino acid compositions of glandless cottonseed protein and casein published in the literature are included in Appendix A.

Amino acid deficiencies in a protein can be corrected by three possible approaches (11,12): The first one is amino acid fortification--by adding synthetic amino acid(s) quantitatively to achieve the appropriate level; the second one is protein supplementation--by adding small amount of protein which is rich in the deficient amino acid(s) of the deficient protein; and the third one is protein complementation--by combining two protein sources which can mutually balance each other's deficiencies or excesses. Defatted cottonseed flour is relatively high in protein content (about 50-60%) (1,2,4,6,12) but deficient in some amino acids. To correct amino acid deficiencies

in cottonseed flour, emphasis needs to be placed on quality rather than quantity. The first approach of fortification would be a reasonable one in improving the quality of cottonseed protein.

Few studies are available on amino acid fortification of cottonseed protein. Braham et al. (13) reported that adding lysine alone did not improve the quality of cottonseed meal. Howe et al. (8) suggested that lysine and threonine appeared to be equally limiting in cottonseed meal. These two studies analyzed the results by only determining protein efficiency ratio, which might result in somewhat insensitive conclusions. It may thus be advantageous to determine protein quality by using a more accurate method such as the determination of biological value (BV).

The purpose of this study is to determine the most effective method of fortifying amino acids to raw defatted glandless cottonseed protein to improve its quality by using protein efficiency ratio (PER) and biological value (BV) as criteria.

CHAPTER II

REVIEW OF LITERATURE

Studies on amino acid fortification of cereals and legumes were reviewed in detail by Jansen (14,15). Results of clinical studies on amino acid(s) fortification of cereals and legumes from various countries were also presented by many investigators (16). These studies showed the possibility of improving the nutritive quality of cereals and legumes by fortification of amino acids such as lysine, tryptophan, methionine, and possibly isoleucine and threonine. However, amino acid fortification seems to be still an area of controversy. Jansen and Howe (17) and Howe et al. (18) pointed out that protein shortage among those who live mainly on cereal grains is primarily due to quality rather than quantity. They suggested that the obvious means of improving protein quality of such a group was by amino acid fortification. Hegsted (19) criticized that amino acid fortification might not be effective in the practical point of view, since the cost would be too high to the undernourished people. Jansen (15) suggested in 1974 that the cost for lysine fortification was inexpensive and the high cost for

tryptophan and threonine fortification would be lowered greatly when they became commercially available.

The research on amino acid fortification of cottonseed protein is limited. Heywang and Bird (20) compared the hatchability of chickens fed 30 percent cottonseed (CS) meal, with or without the addition of DL-lysine or dried manure. They found that a diet of 30 percent CS meal fortified with 5 percent dried manure and 0.8 percent DL-lysine showed the most improved hatchability in comparison to the other diets which were fortified with either lysine or dried manure alone. Braham et al. (13) fortified both low and high quality cottonseed meals with L-lysine to achieve 0.6 percent of available lysine and fed them to rats. Protein effeciency ratio of fortified low quality cottonseed meal was still lower than that of fortified high quality cottonseed meal. Fortification of lysine alone did not affect the protein efficiency ratio. Studies by Howe et al. (8) on the supplementary effects on cottonseed meals with lysine, methionine or threonine alone, or in combination showed that fortification of 0.1 or 0.2 percent L-lysine-HCl plus 0.2 percent DL-threonine resulted in the best supplementary effect. These data suggested that lysine and threonine were equally deficient in cottonseed meals. Elias and Bressani (21) obtained

highest protein efficiency ratio in defatted cottonseed flour fortified with 0.20 percent L-lysine-HCl, 0.05 percent DL-methionine, and 0.10 percent DL-threonine. They concluded that lysine was the first limiting amino acid followed by threonine and methionine. Reber et al. (22) observed that protein efficiency ratio of roasted cottonseed protein was significantly lower than that of cooked cottonseed protein. However, when roasted cottonseed protein was fortified with 0.4 percent L-lysine, the protein efficiency ratio was comparable to that of the cooked one (23,24).

Plasma amino acid levels are affected by various dietary, physiological, and pathological factors in both animals and humans (25,26). Plasma amino acid concentrations reflect the balance between the entry of amino acids into the blood from digestion of food protein and from breakdown of tissue protein, and the exit of amino acids from the blood to the tissues for protein synthesis (27). Plasma amino acid levels, therefore, represent a significant portion of the metabolic pool of free amino acids in the body (28). This pool becomes low when animals are depleted (28). Swendseid and Kopple (29) suggested that the plasma amino acid concentration during postabsorption was fairly constant in normal subjects ingesting adequate

protein diets, but altered in subjects receiving protein-deficient diets. Thus the level of fasting plasma amino acid could be a sensitive indicator to the protein nutritional status of a subject (29).

Kim (30) determined fasting plasma amino acid concentration of 12 college women fed a diet containing defatted liquid cyclone process (LCP) cottonseed protein as protein source ranging from zero to 100 percent for 35 days. RI-5 (a reference protein) was used as a substitute to maintain the same protein content in the diet as the level of LCP cottonseed protein decreased. The results showed that a ratio of total essential to total nonessential amino acids was lowest with 100 percent LCP cottonseed protein. Plasma lysine concentration was considerably higher compared to other diets when RI-5 was the sole source of the protein.

Sneed (31) and Sneed et al. (32) fed a diet containing 99 percent of the protein from glandless cottonseed protein to 6 college women. After consuming such a diet for a week, significant decrease in threonine, proline, isoleucine, tyrosine, and lysine concentrations were found in fasting plasma. No further decrease in the concentrations of these amino acids was found when protein in the diet was reduced to 17.6 mg/kcal during the

following 5 weeks. Sutton (33) conducted a similar study in which glandless cottonseed protein was fortified with 17 mg of L-lysine-HCl per gram of protein. Fasting plasma lysine level was increased after a week of consuming the diet. Fortification of lysine in the diet appeared to prevent the decrease of plasma lysine concentration.

Urea plays an important role in metabolic and physiological aspects in mammals (34,35). Blood urea level is affected by the protein quality of the diet. Numerous studies showed an inverse relationship between the quality of dietary protein and plasma urea level in pigs (36,37), in rats (23,38-40), and in humans (41,42).

Early human studies by Rose et al. (43,44) showed an increase in urinary nitrogen when an experimental diet of complete protein was changed to a valine or methionine deficient one. It was identified that increased urinary urea was responsible for the increase in the nitrogen excretion. Taylor et al. (41) observed a negative correlation between urinary urea nitrogen level and net protein utilization in humans. A negative correlation between the quality of dietary protein and urinary urea level was also observed in pigs (37) and in rats (45-47).

CHAPTER III

MATERIALS AND METHODS

This study consists of two parts: (I) the identification of amino acid(s) in low quantity and the determination of the amount of available lysine in raw defatted glandless cottonseed protein; and (II) the fortification of amino acid(s) identified as being low in part (I) and the testing of the quality of fortified cottonseed protein by bioassay and biochemical methods.

Part I

A. Analysis of Amino Acid Composition

Amino acid compositions of cottonseed protein[1] and casein[2] after acid hydrolysis were determined by Beckman 121M Amino Acid Analyzer. The hydrolysates were prepared according to the procedure outlined by Beckman Instruments,

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[1]Raw defatted glandless cottonseed flour obtained from Texas A & M University, College Station, Texas. This cottonseed flour contained 0.0062% free gossypol.

[2]ANRC (Animal Nutrition Research Council) casein, Sheffield Chemical, Lyndhurst, New Jersey.

Inc. (48). The detailed procedure is in Appendix B. By comparing the amino acid compositions of the two proteins, the deficient amino acids in the cottonseed protein were identified. The amount of lysine determined by this procedure is the total lysine.

B. Analysis of Available Lysine

Test proteins treated with 1-fluoro-2,4-dinitrobenzene (FDNB) according to the procedure of Blom et al. (49) were acid hydrolyzed, and analyzed for the content of amino acids as previously described. The detailed procedure of FDNB treatment is in Appendix C. The amount of lysine obtained by this method is the unavailable lysine. The amount of available lysine was obtained by subtracting the amount of unavailable lysine from the total lysine (50).

Part II

A. Fortification of Lysine

Lysine was identified as the most deficient amino acid in part I. Twenty-two days old male Holtzman[1] rats upon arrival were housed individually in suspended wire mesh cages under uniform conditions of light (12-hour

[1]Holtzman Co., Madison, Wisconsin.

light/12-hour dark) and temperature (22 ± 1 °C), and were fed Purina Laboratory Chow[1] and water ad libitum. After 5 days of acclimation, 74 rats within a body weight range of 12 grams were selected for testing.

1. Protein efficiency ratio (PER) test. Fifty of the 74 rats were randomly assigned to 5 groups for PER test. The procedure was according to the method of the Association of Official Analytical Chemists (AOAC) (51). One group of rats was sacrificed before feeding was initiated to determine concentrations of fasting serum amino acids. The remaining 4 groups of rats were fed one of 4 diets presented in Table 1. The dietary compositions (10% protein) are presented in Table 2. The rats were provided food and water ad libitum. Body weight and feed consumption were measured weekly. After 28 days of feeding, PER of each rat was calculated by the following formula:

$$\text{PER} = \frac{\text{weight gain (g)}}{\text{protein consumed (g)}} \qquad (51,52).$$

An adjusted group PER was calculated by the following formula (51,52).

$$\text{Adjusted PER} = \text{mean PER of test protein} \times \frac{2.50}{\text{mean PER of reference ANRC casein}}$$

Overnight fasting blood samples were obtained from these

[1]Ralston Purina Co., St. Louis, Missouri.

TABLE 1

LYSINE FORTIFICATION OF DEFATTED GLANDLESS COTTONSEED PROTEIN

Group (Diet)	Protein source		L-lysine-HCl
			%
1	Casein[a]		-
2	Cottonseed protein[b]		-
3	Cottonseed protein	+	0.50[c]
4	Cottonseed protein	+	0.55[d]

[a]ANRC casein, Sheffield Chemical, Lyndhurst, New Jersey.

[b]Raw defatted glandless cottonseed protein obtained from Texas A & M University, College Station, Texas.

[c]Level to achieve the same amount of total lysine as in Group 1.

[d]Level to achieve the same amount of available lysine as in Group 1.

TABLE 2

COMPOSITION OF DIET (%)

Ingredient	Diet				0%-Protein[f] diet	Casein diet[f] (20% protein)
	1	2	3	4		
Casein (ANRC)[a]	11.40	-	-	-	-	22.79
Cottonseed flour[b]	-	17.33	17.33	17.33	-	-
L-Lysine-HCl	-	-	0.50	0.55	-	-
Sucrose	69.21	64.26	63.76	63.71	65	58.44
Corn starch[c]	-	-	-	-	15	-
Corn oil[d]	7.99	7.90	7.90	7.90	8	7.98
Salt mixture (USP XVII)	5	5	5	5	5	5
Vitamin mix (AOAC)	1	1	1	1	1	1
Non-nutritive fiber[e]	1	1	1	1	1	1
Water	4.40	3.51	3.51	3.51	5	3.79

[a]Protein (N x 6.25), 87.75%; lipid, 0.1%; and moisture, 5.3%.

[b]Protein (N x 6.25), 57.71%; lipid, 0.6%; and moisture, 8.6%.

[c]ARGO corn starch, Englewood Cliff, New Jersey.

[d]Mazola corn oil, Englewood Cliff, New Jersey.

[e]Cellulose type, Teklad Test Diets, Madison, Wisconsin.

[f]Used for nitrogen balance study.

rats to determine serum urea level and serum amino acid concentration.

2. Nitrogen balance. The remaining 24 rats were transferred to individual metabolic cages after acclimation and fed a casein diet (20% protein) for 4 days. The rats were then randomly assigned to 4 groups and fed one of 4 diets (Diets 1-4) shown in Table 1. After being maintained on their respective diets for 3 days, urine excretion and fecal excreta were collected for three consecutive collection periods of 4 days. Rats were then switched to a diet containing zero percent protein for 7 days. Urine and feces were collected during the last 4 days. The rats were provided food and water ad libitum while they were in the metabolic cages. Body weight and feed consumption were measured every 4 days. Fecal and urinary samples were used for nitrogen, urea, and creatinine determinations. Nitrogen balance (NB) and biological value (BV) were calculated for each rat by using the following formulas (52,53).

$$NB = N \text{ intake} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N})$$

$$BV = \frac{N \text{ intake} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N})}{N \text{ intake} - (\text{fecal N} - \text{metabolic N})}$$

B. Fortification of Amino Acids

Twenty-two days old male Holtzman rats were used. The environmental condition was the same as previously described. After 6 days of acclimation, 48 rats within a body weight range of 12 grams were selected for testing.

1. Protein efficiency ratio (PER) test. Thirty of the 48 rats were randomly assigned to 3 groups and fed one of 3 diets (Diets 5-7) presented in Table 3. The dietary compositions of the 3 diets (10% protein) are presented in Table 4. The experimental procedures were identical to the procedure described on page 11.

2. Nitrogen balance. The remaining 18 rats were transferred to individual metabolic cages after acclimation and fed a casein diet (20% protein) for 4 days. The rats were then randomly assigned to 3 groups and fed one of 3 diets shown in Table 3. The experimental procedures were identical to that described on page 14.

Preparation of Specimens

Serum. Rats were anesthetized with ether after overnight fasting, and blood was drawn from the vena cava by using unheparinized syringes. Blood samples were allowed to stand for spontaneous clotting for 30 minutes at room temperature, and centrifuged at 4,000 r.p.m. for 30 minutes. Serum samples were immediately deproteinized by

TABLE 3

AMINO ACIDS FORTIFICATION OF DEFATTED GLANDLESS COTTONSEED PROTEIN

Group (Diet)	Protein source	L-Lysine-HCl	L-Methionine	L-Isoleucine
		%	%	%
5	Casein[a]	-	-	-
6	Cottonseed protein[b]	+ 0.50[c]	+ 0.13[d]	-
7	Cottonseed protein	+ 0.50	+ 0.13	+ 0.20[e]

[a,b]Refer to Table 1 for vendors of the protein source.

[c]This level of fortification was chosen from experiment of lysine fortification.

[d]Level to achieve the same amount of methionine as in Group 5.

[e]Level to achieve the same amount of isoleucine as in Group 5.

TABLE 4

COMPOSITIION OF DIET (%)

Ingredient	Diet		
	5	6	7
Casein (ANRC)[a]	11.40	-	-
Cottonseed flour[b]	-	17.33	17.33
L-Lysine-HCl	-	0.50	0.50
L-Methionine	-	0.13	0.13
L-Isoleucine	-	-	0.20
Sucrose	69.21	63.63	63.43
Corn oil[c]	7.99	7.90	7.90
Salt mixture (USP XVII)	5	5	5
Vitamin mix (AOAC)	1	1	1
Non-nutritive fiber[d]	1	1	1
Water	4.40	3.51	3.51

[a,b]Refer to Table 2 for vendors of the nutrient analysis.

[c]Mazola corn oil, Englewood Cliff, New Jersey.

[d]Celluose type, Teklad Test Diets, Madison, Wisconsin.

adding 45 mg of sulfosalicylic acid per ml of serum. The precipitated protein was removed by centrifugation at 4,000 r.p.m. for 30 minutes. The deproteinized serum samples from two rats were pooled with equal volumes. The pooled serum sample was then diluted with equal volume of lithium citrate buffer (pH 2.2). The diluted sample was filtered through a 0.45 μm pore size filter, and 20 μl of the filtrate was applied to the Beckman 121M Amino Acid Analyzer for amino acid analysis.

Urine. Urine was preserved with toluene and a few drops of 10 percent hydrochloric acid. A pooled 4-day urine was diluted to a total volume of 200 ml, and filtered. One ml was used to determine nitrogen, and 200 μl was used for determination of creatinine. The urine was further diluted (1:5), and 20 μl was used for determination of urea nitrogen.

Feces. Fecal excreta were kept in 10 percent sulfuric acid. A 4-day pooled collection of feces was homogenized with water to make a final total volume of 100 ml. Two ml was used for the determination of nitrogen.

Analytical Procedures

Urinary and fecal nitrogen contents were determined by a modified micro-Kjeldahl method (54,55). Serum and

urinary urea nitrogens and urinary creatinine were determined by the methods of Giorgio (56). Detailed procedures are in Appendix D to F.

Statistical Analysis

Data were analyzed by one-way analysis of variance for each variable examined. When a significant effect of diet was detected, further comparisons among groups were performed by using the Newman-Keuls test (57).

CHAPTER IV

RESULTS AND DISCUSSION

Amino Acid Composition and Available Lysine

Amino acid compositions and available lysine contents of raw defatted glandless cottonseed protein and ANRC (Animal Nutrition Research Council) casein are presented in Table 5. The amount of each essential amino acid in cottonseed protein was expressed as percentage of that in casein. Lysine, methionine, and isoleucine appeared to be the three lowest amino acids in descending order. Data on the amino acid compositions of glandless cottonseed protein and casein published in the literature also showed that lysine, methionine, and isoleucine were the three lowest amino acids in descending order (Appendix A). Tryptophan content of cottonseed protein was not shown to be low. Swaminathan (58) reported that cottonseed protein was limiting in lysine and methionine. However, Howe et al. (8) and Elias and Bressani (21) demonstrated in animal feeding studies that lysine, threonine, and methionine appeared to be limiting amino acids.

Amounts of the three lowest amino acids added to

TABLE 5

AMINO ACID COMPOSITIONS AND AVAILABLE LYSINE CONTENTS OF DEFATTED GLANDLESS COTTONSEED PROTEIN AND ANRC CASEIN

Amino acid	Cottonseed (CS) protein	Casein	$\frac{\text{CS protein}}{\text{casein}} \times 100$
	g/16 g N		
Lysine	4.35	8.36	52.03
Histidine	2.63	2.98	88.26
Arginine	12.80	4.26	300.47
Threonine	3.74	4.41	84.81
Valine	3.77	6.26	60.22
Methionine	1.74	3.01	57.81
Isoleucine	2.85	4.84	58.88
Leucine	6.29	9.84	63.92
Phenylalanine	5.69	5.13	110.92
Aspartic acid	9.59	7.36	
Serine	4.47	5.84	
Glutamic acid	20.64	21.45	
Proline	3.75	11.24	
Glycine	4.30	1.76	
Alanine	3.88	3.32	
Cystine	2.15	1.01	
Tyrosine	2.92	5.78	
Available lysine	3.99	8.36	47.73

the cottonseed protein diet were aimed to achieve the same amounts of those amino acids as in casein diet. Level of lysine added was expressed as either to achieve the same total lysine content or to achieve the same available lysine content as in the casein diet. These levels were subsequently used for animal feeding studies to test the effect of lysine fortification. L-methionine (0.13%) or/and L-isoleucine (0.20%) were further added with L-lysine-HCl (0.50 or 0.55%) for experimentation of amino acids fortification. The dietary regimen is summarized in Table 6.

Fortification of Lysine

Data on body weight change in rats from lysine fortification study are presented in Table 7. Rats fed casein diet (Group 1) showed significantly higher weight gain than that of cottonseed protein diet with or without lysine fortification (Groups 2-4). No significant difference in weight gain was found in rats fed cottonseed protein diet with or without lysine fortification (Groups 2-4). Protein intakes did not significantly differ among the rats fed the 4 different diets (Groups 1-4) (Table 8). Boctor and Harper (59) reported that the addition of lysine to a basal diet containing 25 percent

TABLE 6

DIETARY REGIMEN

Group (Diet)	Protein source	Amino acid fortification (%)					
		L-Lysine-HCl		L-Methionine		L-Isoleucine	
1	Casein	-		-		-	
2	Cottonseed protein	-		-		-	
3	Cottonseed protein	+	0.50	-		-	
4	Cottonseed protein	+	0.55	-		-	
5	Casein	-		-		-	
6	Cottonseed protein	+	0.50	+	0.13	-	
7	Cottonseed protein	+	0.50	+	0.13	+	0.20

TABLE 7

BODY WEIGHT CHANGE OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 28 DAYS

Group[a]	Initial body wt.	Final body wt.	Weight gain
	g	g	g
1	94.6 ± 2.23[b]	253.2 ± 28.11	158.6 ± 28.71
2	96.0 ± 1.40	211.5 ± 11.79**	115.5 ± 12.66**
3	93.3 ± 2.25	224.6 ± 24.32*	131.3 ± 24.79*
4	93.6 ± 2.70	217.9 ± 26.90**	124.0 ± 27.11**

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 10 rats.

*Significant at $p < 0.05$ compared to Group 1 (Control).

**Significant at $p < 0.01$ compared to Group 1 (Control).

TABLE 8

PROTEIN EFFICIENCY RATIO (PER) OF DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 28 DAYS

Group[a]	Protein consumed	PER	Adjusted PER	Serum urea nitrogen
	g			mg/100 ml
1	47.6 ± 7.04[b]	3.32 ± 0.13	2.50	12.1 ± 1.74
2	46.3 ± 3.97	2.50 ± 0.17*	1.88	13.0 ± 3.91
3	48.3 ± 5.45	2.69 ± 0.27*	2.03	13.8 ± 4.37
4	45.8 ± 5.94	2.69 ± 0.26*	2.03	13.6 ± 3.26

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 10 rats.

*Significant at $p < 0.01$ compared to Group 1 (Control).

wheat gluten stimulated food intake. Such an effect was not seen in the present study. Mean protein efficiency ratio (PER) of each dietary group is shown in Table 8. The PER of casein group (Group 1) was significantly higher than those of cottonseed protein groups (Groups 2-4). Differences of the PER among the cottonseed protein groups (Groups 2-4) were not statistically significant. Other investigators also had shown that the PER of cottonseed protein was not significantly improved by lysine fortification alone at the levels of 0.01 to 0.39 percent L-lysine-HCl (8,21).

Fasting serum urea nitrogen levels are also included in Table 8. All 4 groups of rats had similar serum urea nitrogen levels. In studies (39,40) in which a negative correlation between the quality of dietary protein and plasma urea level was observed, blood samples of 3-4 hour postprandial time were used. In the present study overnight fasting blood samples were used. This may contribute to the fact that no correlation was found between serum urea nitrogen level and the type of dietary source.

Data from nitrogen balance study of rats fed cottonseed protein diet with or without lysine fortification are presented in Tables 9 to 13. Data were expressed

over the 12-day period. While nitrogen intakes, urinary nitrogen levels, and endogenous and metabolic nitrogen levels were similar among the 4 groups of rats, significantly ($p < 0.01$) more nitrogen was excreted in feces of rats fed cottonseed protein diet (Groups 2-4) than that of the casein diet (Table 9). Nitrogen balance data expressed as either per 12-day period or per 100 gram body weight are shown in Table 10. No significant difference in nitrogen balance was found among rats fed the 4 different diets. In human studies, it was also found that fortification of lysine alone to glandless cottonseed protein did not improve nitrogen balance of subjects (33, 60-62). Since cottonseed protein is low in all 3 amino acids (lysine, methionine, and isoleucine), the fortification of lysine alone might have caused greater imbalance in the amino acid content of cottonseed protein. Thus it produced no beneficial effect on nitrogen balance of the subject.

Significantly less ($p < 0.01$) nitrogen excretion in feces of rats fed casein diet (Group 1) was observed. This was the result of higher ($p < 0.01$) digestibility of casein (Table 11). Lysine fortification did not influence the digestibility of cottonseed protein diet (Groups 2-4). Biological value and net protein utilization derived from

TABLE 9

NITROGEN INTAKE AND EXCRETION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 12 DAYS

Group[a]	Nitrogen intake	Urinary nitrogen	Endogenous nitrogen	Fecal nitrogen	Metabolic nitrogen
	g	g	g	g	g
1	3.06 ± 0.34[b]	1.02 ± 0.05	0.36 ± 0.04	0.20 ± 0.03	0.13 ± 0.01
2	3.22 ± 0.45	1.20 ± 0.11	0.33 ± 0.04	0.36 ± 0.05*	0.12 ± 0.01
3	2.96 ± 0.45	1.22 ± 0.21	0.37 ± 0.05	0.32 ± 0.03*	0.14 ± 0.01
4	2.70 ± 0.73	1.15 ± 0.22	0.35 ± 0.03	0.32 ± 0.04*	0.13 ± 0.02

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

*Significant at $p < 0.01$ compared to Group 1 (Control).

TABLE 10

NITROGEN BALANCE OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 12 DAYS

Group[a]	Body weight	Nitrogen balance (NB)	NB/100 g b. wt.
	g	g	g
1	163.7 ± 7.02[b]	2.32 ± 0.30	1.42 ± 0.14
2	156.8 ± 13.53	2.11 ± 0.33	1.34 ± 0.13
3	155.2 ± 7.47	1.93 ± 0.30	1.24 ± 0.17
4	144.5 ± 16.57*	1.72 ± 0.52	1.17 ± 0.25

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

*Significant at $p < 0.05$ compared to Group 1 (Control).

TABLE 11

BIOLOGICAL VALUE OF DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION

Group[a]	Digestibility	Biological value	Net protein utilization
	%	%	%
1	97.7 ± 0.74[b]	77.6 ± 2.01	75.9 ± 2.27
2	92.7 ± 0.86*	70.7 ± 2.77*	65.5 ± 2.62*
3	93.7 ± 1.78*	69.6 ± 1.85*	65.2 ± 1.78*
4	92.8 ± 1.62*	68.0 ± 3.64*	63.1 ± 3.84*

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

*Significant at $p < 0.01$ compared to Group 1 (Control).

nitrogen balance data are presented in Table 11. Both biological value and net protein utilization of group 1 were significantly greater than those of the other groups. Lysine fortification (Groups 3 & 4) did not improve either the biological value or the net protein utilization of cottonseed protein diet.

Certain urinary nitrogen metabolites were determined and the results are presented in Table 12. Differences in urinary urea nitrogen level among 4 groups of rats were not statistically significant. Creatinine excretions did not differ significantly among the 4 groups of rats. Since creatinine excretion in adult animals and men has been recognized to be essentially constant and not to be influenced by dietary protein manipulation (63), the result on creatinine excretion does not come as a surprise. A Pearson correlation coefficient was performed on the biological value of dietary proteins and urinary urea nitrogen. Negative correlations were found (Table 13). Higher correlation was found when urea nitrogen was expressed either as mg/100 g b. wt./day or as a ratio of urea nitrogen to creatinine. Urinary urea levels have been found to be negatively correlated to quality of dietary proteins (37,41,46,47).

Fasting serum amino acid concentrations of rats are

TABLE 12

URINARY NITROGEN METABOLITES OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 12 DAYS

Group[a]	Body weight (g)	Urea N (mg/100 g b. wt./day)	Urea N (mg/day)	Creatinine	Urea N / creatinine	Creatinine (mg/100 g b. wt./day)
1	163.7[b] (7.02)	40.1 (2.61)	65.5 (3.30)	4.2 (0.25)	15.8 (0.81)	2.6 (0.18)
2	156.8 (13.53)	50.1 (8.92)	78.0 (10.60)	4.3 (0.64)	18.8 (4.81)	2.7 (0.22)
3	155.2 (7.47)	50.3 (10.07)	78.2 (16.85)	4.3 (0.46)	18.0 (2.52)	2.8 (0.30)
4	144.5* (16.57)	49.7 (6.06)	71.9 (13.51)	3.8 (0.50)	18.9 (2.00)	2.6 (0.12)

[a]Refer to Table 6 for diet designation.

[b]Mean (SD) of 6 rats.

*Significant at $p < 0.05$ compared to Group 1 (Control).

TABLE 13

CORRELATION BETWEEN BIOLOGICAL VALUE OF DIETARY PROTEINS AND URINARY NITROGEN METABOLITES IN RATS

	Urea nitrogen	Urea nitrogen	$\frac{\text{Urea N}}{\text{creatinine}}$
	mg/day	mg/100 g b. wt./day	
Biological value	-0.4094[a] ($p < 0.05$)	-0.6841 ($p < 0.001$)	-0.5971 ($p < 0.001$)

[a]Pearson correlation coefficient of 24 rats.

shown in Table 14 and 15. Lysine, methionine, and isoleucine in fasting serum of rats fed cottonseed protein diet (Group 2) for 28 days were not lower than those of the casein group, although these amino acids were low in quantity in cottonseed protein. Lysine fortification at 0.55 percent in the cottonseed protein diet produced the highest serum lysine concentration and it was significant at $p < 0.05$ when it was compared to that of rats in group 1. Since arginine content of cottonseed protein diet was about three times as much as that of casein diet, the fasting serum of rats fed cottonseed protein diet with or without lysine fortification (Groups 2-4) showed significantly higher ($p < 0.05$) level of arginine than that of rats fed casein diet (Group 1).

It is interesting to note that regardless of lysine fortification, fasting serum threonine concentration of rats fed cottonseed protein diet (Groups 2-4) was about half of that of the control group (Group 1). It is possible that in rats serum threonine concentration is affected most sensitively when slight deficiency of such an amino acid is present in the diet. The concentrations of valine, methionine, isoleucine, leucine, and phenylalanine in the fasting serum of group B fed Purina Laboratory Chow were significantly higher than those of

TABLE 14

FASTING SERUM ESSENTIAL AMINO ACID CONCENTRATION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 28 DAYS (micromole/liter serum)

Amino acid	Group[a]				
	B[b]	1	2	3	4
Lysine	544.9[c] (46.0)	611.5 (38.8)	593.6 (65.4)	583.3 (69.5)	733.8** (81.5)
Histidine	68.5 (7.9)	76.8 (13.4)	67.3 (10.5)	65.1 (11.2)	66.6 (7.1)
Arginine	185.9 (22.4)	212.0 (29.2)	255.5*,+ (62.2)	269.6*,+ (49.5)	259.3*,+ (30.3)
Threonine	282.5 (81.6)	422.6** (21.7)	212.2 (27.3)	222.8 (43.1)	221.5 (35.0)
Valine	257.7** (38.8)	166.5 (23.3)	117.5* (12.4)	136.2 (16.5)	140.3 (17.6)
Methionine	74.1** (8.8)	61.6 (4.2)	60.6 (4.3)	57.4 (4.6)	61.9 (3.2)
Isoleucine	118.8** (11.5)	90.9 (12.5)	88.8 (8.9)	91.8 (8.6)	98.1 (4.7)
Leucine	186.2** (19.9)	133.9 (19.4)	129.4 (12.4)	126.8 (10.8)	141.8 (4.9)
Phenylalanine	70.6** (10.7)	53.1 (9.7)	49.8 (3.6)	50.0 (6.1)	52.4 (3.6)
Total	1,789.2 (204.3)	1,829.1 (133.8)	1,574.6 (139.1)	1,603.0 (179.2)	1,775.6 (130.8)

[a] Refer to Table 6 for diet designation.

[b] Baseline prior to experimental feeding.

[c] Mean (SD) of 5 pooled samples.

* Significant at $p < 0.05$ compared to Group 1 (Control).

+ Significant at $p < 0.05$ compared to Group B (Baseline).

** Significant at $p < 0.05$ compared to all groups.

TABLE 15

FASTING SERUM NONESSENTIAL AMINO ACID CONCENTRATION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH OR WITHOUT LYSINE FORTIFICATION FOR 28 DAYS (micromole/liter serum)

Amino acid	Group[a]				
	B[b]	1	2	3	4
Aspartic acid	15.3[c,**] (2.3)	36.8 (4.7)	45.8 (12.4)	39.9 (1.8)	51.6[*,o] (7.4)
Serine	327.9[**] (47.6)	634.8 (41.1)	595.2 (63.0)	632.0 (59.4)	671.3 (97.3)
Glutamine	476.5 (81.5)	427.5 (47.8)	616.7[*,+] (46.5)	622.5[*,+] (51.8)	628.2[*,+] (64.9)
Glutamic acid	77.5[**] (17.0)	180.8 (29.9)	227.9[*] (33.1)	222.7[*] (22.5)	238.2[*] (22.0)
Glycine	479.0 (57.7)	456.8 (47.6)	475.2 (70.1)	409.8 (33.7)	402.8 (39.5)
Alanine	420.6 (62.7)	366.0 (84.7)	347.5 (46.0)	326.8 (35.2)	360.3 (53.4)
Cystine	35.6 (9.0)	28.3 (4.8)	37.6 (6.7)	46.2[*] (7.9)	27.1[o] (4.6)
Tyrosine	73.3 (13.2)	85.3 (9.4)	75.6[o] (7.7)	98.4[+] (11.6)	74.2[o] (9.6)
Taurine	290.7 (68.0)	152.3[**] (16.5)	341.7 (30.2)	270.5[++] (43.7)	276.0[++] (8.5)
Citrulline	81.8 (7.5)	68.9 (13.2)	66.9 (7.9)	73.3 (15.1)	86.9 (9.0)
Ornithine	75.8 (20.2)	106.0 (17.5)	97.5 (23.0)	96.7 (19.2)	95.1 (8.8)
Total	2,354.1 (151.3)	2,543.5 (248.6)	2,928.2[*,+] (200.4)	2,838.8[*,+] (70.5)	2,911.6[*,+] (119.8)

[a,b,c,*,+,**] Refer to Table 14.

[++] Significant at $p < 0.05$ compared to Group 2.

[o] Significant at $p < 0.05$ compared to Group 3.

the other groups. Since Purina Laboratory Chow contained approximately 22 percent protein while all other test diets contained 10 percent protein, it is possible that the quantity of dietary protein have an effect on the concentrations of serum amino acids. Taurine concentration in fasting serum of rats fed casein diet (Group 1) was significantly lower ($p < 0.05$) than those of the other groups (Groups B & 2-4). This may be due to the fact that only a small amount of cystine is present in casein and taurine is a cystine metabolite. The ratio of total essential to total nonessential amino acids in fasting serum was lower in rats fed diets with cottonseed as protein source (Groups 2-4) than that of rats fed either casein diet or Purina Laboratory Chow (Groups 1 & B).

In human study, Sneed (31) and Sneed et al. (32) observed decreases in fasting plasma levels of threonine, proline, isoleucine, tyrosine, and lysine one week after consuming a diet containing cottonseed protein. If cottonseed protein diet fortified with 17 mg of L-lysine-HCl per gram of protein was consumed for one week, there was an increase in fasting plasma lysine level (33). This suggests that lysine fortification could prevent the decrease of plasma lysine concentration. Since the level of protein in the diet, the length of feeding period, and

the species used as test subjects are different between the above human studies and the present animal study, comparison on the effect of lysine fortification will not be valid.

In summary, lysine fortification of cottonseed protein diet did not increase either the protein efficiency ratio or the biological value. Fasting serum amino acid concentration of rats was affected neither by lysine fortification of cottonseed protein diet nor by the amino acid composition of the diet. The overall effects of two levels of lysine fortification to cottonseed protein diet could not be differentiated. The effect of adding lysine to achieve the same total lysine or the same available lysine content as in casein diet was similar. The lower level of fortification (0.50% L-lysine-HCl) was thus chosen for the following experiment of additional amino acid fortification.

Fortification of Amino Acids

Body weight changes of rats for lysine, methionine, and isoleucine fortification on PER study are presented in Table 16. Weight gain of rats in group 7 was significantly less ($p < 0.05$) than that of the control group (Group 5). Protein intakes were similar among the 3 groups (Table 17). Table 17 shows the mean protein efficiency ratio (PER) of

TABLE 16

BODY WEIGHT CHANGE OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 28 DAYS

Group[a]	Initial body wt.	Final body wt.	Weight gain
	g	g	g
5	88.1 ± 1.93[b]	254.2 ± 23.76	166.1 ± 22.97
6	89.3 ± 2.45	234.0 ± 27.11	144.8 ± 27.36
7	89.3 ± 2.29	223.4 ± 26.07*	133.1 ± 25.51*

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 10 rats.

*Significant at $p < 0.05$ compared to Group 5 (Control).

TABLE 17

PROTEIN EFFICIENCY RATIO (PER) OF DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 28 DAYS

Group[a]	Protein consumed	PER	Adjusted PER	Serum urea nitrogen
	g			mg/100 ml
5	49.9 ± 6.57[b]	3.33 ± 0.11	2.50	12.1 ± 1.65
6	49.6 ± 6.49	2.90 ± 0.19*	2.18	10.7 ± 2.00
7	46.4 ± 6.32	2.87 ± 0.16*	2.16	11.4 ± 3.52

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 10 rats.

*Significant at $p < 0.01$ compared to Group 5 (Control).

each dietary group. The PER of cottonseed protein diet fortified with lysine and methionine or lysine, methionine, and isoleucine (Groups 6 & 7) were not improved to the same extent as that of the casein diet. Studies on fortification of the same amino acids in combination to cottonseed protein up to the level of 0.20 percent had showed no improvement in the PER (21). In the present study, the levels of amino acids fortification were increased, but there was no observable improvement in the PER values. No difference in fasting serum urea nitrogen levels was found among the 3 groups of rats (Table 17).

Tables 18 to 22 show data from nitrogen balance study of rats fed cottonseed protein diet fortified with several amino acids. Results on nitrogen intake, urinary nitrogen excretion, and endogenous and metabolic nitrogen excretions were similar in the 3 groups of rats (Table 18). Rats in casein group (Group 5) excreted significantly less nitrogen in feces ($p < 0.01$) than rats in cottonseed protein groups. Nitrogen balance data are presented in Table 19. No difference was found in nitrogen balance expressed as either total or per 100 gram body weight among the 3 groups. Rats in this nitrogen balance study appeared to have greater nitrogen balance than rats in the previous nitrogen balance study (Tables 10 & 19). Comparing the

TABLE 18

NITROGEN INTAKE AND EXCRETION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 12 DAYS

Group[a]	Nitrogen intake	Urinary nitrogen	Endogenous nitrogen	Fecal nitrogen	Metabolic nitrogen
	g	g	g	g	g
5	3.78 ± 0.39[b]	1.30 ± 0.24	0.41 ± 0.07	0.22 ± 0.02	0.15 ± 0.01
6	3.88 ± 0.19	1.48 ± 0.08	0.45 ± 0.03	0.37 ± 0.04*	0.14 ± 0.03
7	3.93 ± 0.46	1.42 ± 0.10	0.41 ± 0.03	0.36 ± 0.04*	0.15 ± 0.04

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

*Significant at $p < 0.01$ compared to Group 5 (Control).

TABLE 19

NITROGEN BALANCE OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 12 DAYS

Group[a]	Body weight	Nitrogen balance (NB)	NB/100 g b. wt.
	g	g	g
5	181.2 ± 9.91[b]	2.82 ± 0.25	1.56 ± 0.06
6	169.7 ± 5.25	2.62 ± 0.14	1.54 ± 0.09
7	168.7 ± 11.45	2.72 ± 0.37	1.61 ± 0.13

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

results of this nitrogen balance study to those of the previous one, the additional fortification of methionine or/and isoleucine in addition to lysine to cottonseed protein diet (Groups 6 & 7) appeared to have overcome the amino acid imbalance caused by lysine fortification alone (Groups 3 & 4) (Tables 10 & 19).

Digestibility (D), biological value (BV), and net protein utilization (NPU) of the 3 dietary proteins are shown in Table 20. Digestibility of casein group (Group 5) was significantly higher ($p < 0.01$) than those of cottonseed protein groups (Groups 6 & 7). Although cottonseed protein was fortified with lysine, methionine, and isoleucine, casein still showed significantly better BV ($p < 0.05$ or $p < 0.01$) and NPU ($p < 0.01$) than those of the fortified cottonseed protein. Differences of the BV and the NPU between groups 6 and 7 were not statistically significant.

Data on urinary nitrogen metabolites are presented in Table 21. Less urea nitrogen excretion was found in rats fed casein diet. No difference was found in creatinine excretions. Significant negative correlation was found between BV and urea nitrogen level expressed as mg/day or mg/100 g b. wt./day or as the ratio of urea nitrogen to creatinine (Table 22).

TABLE 20

BIOLOGICAL VALUE OF DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION

Group[a]	Digestibility	Biological value	Net protein utilization
	%	%	%
5	98.2 ± 0.55[b]	76.3 ± 2.71	74.9 ± 2.83
6	93.9 ± 0.68**	71.8 ± 1.18**	67.5 ± 1.20**
7	94.7 ± 1.11**	73.1 ± 1.43*	69.3 ± 1.99**

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 6 rats.

*Significant at $p < 0.05$ compared to Group 5 (Control).

**Significant at $p < 0.01$ compared to Group 5 (Control).

TABLE 21

URINARY NITROGEN METABOLITES OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 12 DAYS

Group[a]	Body weight (g)	Urea N (mg/100 g b. wt./day)	Urea N (mg/day)	Creatinine (mg/day)	Urea N / creatinine	Creatinine (mg/100 g b. wt./day)
5	181.2[b] (9.91)	45.8 (7.61)	83.3 (17.00)	5.5 (0.48)	15.2 (2.53)	3.0 (0.16)
6	169.7 (5.25)	56.0** (1.72)	94.9 (5.18)	5.3 (0.33)	18.1* (0.60)	3.1 (0.12)
7	168.7 (11.45)	54.7** (2.51)	92.3 (8.39)	5.1 (0.41)	18.0* (1.18)	3.0 (0.20)

[a] Refer to Table 6 for diet designation.

[b] Mean (SD) of 6 rats.

* Significant at $p < 0.05$ compared to Group 5 (Control).

** Significant at $p < 0.01$ compared to Group 5 (Control).

TABLE 22

CORRELATION BETWEEN BIOLOGICAL VALUE OF DIETARY PROTEINS AND URINARY NITROGEN METABOLITES IN RATS

	Urea nitrogen	Urea nitrogen	$\frac{\text{Urea N}}{\text{creatinine}}$
	mg/day	mg/100 g b. wt./day	
Biological value	-0.6667[a] ($p < 0.001$)	-0.8612 ($p < 0.001$)	-0.8261 ($p < 0.001$)

[a]Pearson correlation coefficient of 18 rats.

Fasting serum amino acid concentrations of rats fed cottonseed protein diet with amino acids fortification are presented in Tables 23 and 24. Among the 3 amino acids used for fortification purpose only the concentration of lysine in the fasting serum of rats fed the fortified diets was increased ($p < 0.05$). Rats in casein group (Group 5) again showed significantly higher ($p < 0.05$) fasting serum concentration of threonine than those in groups 6 and 7. Concentrations of histidine, valine, phenylalanine, glutamine, and citrulline were significantly lower ($p < 0.05$) in the fasting serum of rats in groups 6 and 7 than those in group 5. It is not clear if such an effect occurred by further fortification of methionine or/and isoleucine in addition to lysine to cottonseed protein diet or was simply due to experimental variations.

TABLE 23

FASTING SERUM ESSENTIAL AMINO ACID CONCENTRATION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 28 DAYS

Amino acid	Group[a] 5	6	7
	micromole/liter serum		
Lysine	499.7 ± 72.0[b]	624.5 ± 45.3*	624.7 ± 90.8*
Histidine	63.7 ± 6.1	45.9 ± 3.9*	46.5 ± 3.3*
Arginine	176.0 ± 45.2	197.8 ± 27.9	223.9 ± 72.5
Threonine	441.6 ± 47.7	187.6 ± 23.3*	197.9 ± 18.9*
Valine	170.2 ± 31.4	114.9 ± 18.9*	119.5 ± 11.3*
Methionine	63.2 ± 9.6	56.8 ± 4.9	60.0 ± 6.4
Isoleucine	98.6 ± 14.9	81.5 ± 9.8	95.3 ± 10.9
Leucine	136.0 ± 24.9	113.3 ± 13.6	132.3 ± 26.9
Phenylalanine	58.6 ± 7.8	44.7 ± 10.2*	47.2 ± 4.6*
Total	1,707.5 ± 247.2	1,467.1 ± 76.2	1,547.4 ± 211.8

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 5 pooled samples.

*Significant at $p < 0.05$ compared to Group 5 (Control).

TABLE 24

FASTING SERUM NONESSENTIAL AMINO ACID CONCENTRATION OF RATS FED DEFATTED GLANDLESS COTTONSEED PROTEIN WITH AMINO ACIDS FORTIFICATION FOR 28 DAYS

Amino acid	Group[a] 5	6	7
	micromole/liter serum		
Aspartic acid	57.7 ± 4.4[b]	56.4 ± 7.3	52.7 ± 6.3
Serine	661.1 ± 66.1	526.7 ± 80.3*	564.9 ± 78.3
Glutamine	473.6 ± 20.6	581.1 ± 69.4*	569.2 ± 54.7*
Glutamic acid	223.5 ± 16.9	218.8 ± 22.3	217.1 ± 18.9
Glycine	481.1 ± 84.4	363.0 ± 67.4*	403.3 ± 38.2
Alanine	428.4 ± 46.9	406.2 ± 47.6	384.5 ± 25.6
Cystine	19.7 ± 2.3	21.0 ± 2.5	24.7 ± 7.0
Tyrosine	93.0 ± 13.3	59.3 ± 10.5*	68.5 ± 9.2*
Taurine	191.3 ± 43.8	462.4 ± 26.8*	400.4 ± 28.3*,+
Citrulline	80.8 ± 9.9	50.5 ± 3.8*	53.9 ± 4.2*
Ornithine	136.1 ± 24.1	150.2 ± 42.2	145.9 ± 68.7
Total	2,846.5 ± 196.7	2,895.5 ± 245.7	2,885.2 ± 203.7

[a]Refer to Table 6 for diet designation.

[b]Mean ± SD of 5 pooled samples.

*Significant at $p < 0.05$ compared to Group 5 (Control).

+Significant at $p < 0.05$ compared to Group 6.

CHAPTER V

SUMMARY AND CONCLUSION

The present study was designed to investigate the most effective way of fortifying amino acids to raw defatted glandless cottonseed flour to improve the protein quality. The study consisted of two parts: (I) to analyze the composition of amino acid and the amount of available lysine in raw defatted glandless cottonseed protein, and (II) to fortify amino acid(s) identified as being low in part (I) to cottonseed protein, and to test the quality of fortified cottonseed protein by bioassays and biochemical methods. The bioassays included protein efficiency ratio (PER) study, and nitrogen balance (NB) study, from which biological values (BV) of the test proteins were derived. The biochemical analyses included the determinations of fasting serum amino acid concentration, fasting serum urea nitrogen, urinary urea nitrogen, and urinary creatinine. Statistical evaluation included one-way analysis of variance and Newman-Keuls test.

Lysine, methionine, and isoleucine were shown to be the three lowest amino acids in raw defatted glandless cottonseed protein. Fortification of 0.50 or 0.55 percent

L-lysine-HCl to cottonseed protein did not improve PER value significantly in comparison to that of the cottonseed protein without lysine fortification. The PER values of the lysine fortified groups were still significantly lower than that of the casein group. Nitrogen balance data did not show improvement in the lysine fortified groups. Digestibility (D), biological value (BV), and net protein utilization (NPU) were not improved either. Casein diet showed significantly higher D, BV, and NPU than those of cottonseed protein diet with or without lysine fortification. From this set of data, fortification of 0.50 percent L-lysine-HCl was chosen for subsequent studies since no difference in either PER or BV was found between the two levels of lysine fortification of cottonseed protein. In the practical sense, lower level of fortification could subside the cost of a fortification program.

Further fortification with 0.13 percent L-methionine or 0.13 percent L-methionine plus 0.20 percent L-isoleucine in addition to 0.50 percent L-lysine-HCl to cottonseed protein did not improve the PER to the same extent as that of the casein diet. The adjusted PER was slightly improved by fortification of lysine and methionine, but remained level with fortification of lysine, methionine, and isoleucine. Biological value did not show appreciable

improvement by fortification of lysine and methionine or lysine, methionine, and isoleucine. Urinary urea nitrogen level was not significantly lower in rats fed casein diet than those fed cottonseed protein diets. Significant negative correlations were found between the biological values and urea nitrogen excretions. No appreciable effect of amino acid(s) fortification was found on fasting serum concentrations of amino acids. Regardless of amino acid(s) fortification, concentration of threonine was lower in the fasting serum of rats fed cottonseed protein diets than that of rats fed casein diet. In contrast, taurine was higher in concentration in rats fed cottonseed protein diets than that of rats fed casein diet. Fasting serum urea nitrogen levels were not differentiated by either dietary protein sources or fortification of different amino acids.

In conclusion, the data from the two PER studies showed slight improvement of the adjusted PER when lysine and methionine were added to cottonseed protein. However, such an effect was not found in the biological value. The fortification of raw defatted glandless cottonseed flour diet with 0.50 percent L-lysine-HCl and 0.13 percent L-methionine appeared to be most effective in improving the quality of cottonseed protein.

APPENDICES

APPENDIX A

AMINO ACID COMPOSITIONS OF GLANDLESS COTTONSEED PROTEIN AND CASEIN (g/16 g N)

Amino acid	Glandless cottonseed protein[a]		Casein[b]
	Range	Average	
Lysine	4.3- 4.6	4.5	8.4
Histidine	2.6- 2.9	2.7	3.0
Arginine	11.2-13.2	12.1	3.8
Tryptophan	1.0- 1.3	1.2	1.6
Cystine	2.2- 2.6	2.4	0.5
Aspartic acid	8.6- 9.3	9.1	7.3
Threonine	2.8- 3.2	3.0	4.8
Serine	3.9- 4.4	4.2	6.2
Glutamic acid	19.9-22.4	21.6	22.5
Proline	3.1- 3.7	3.4	11.8
Glycine	3.7- 4.6	4.1	2.0
Alanine	3.6- 4.2	3.9	3.1
Valine	4.1- 4.8	4.4	6.9
Methionine	1.2- 1.7	1.4	2.6
Isoleucine	2.8- 3.2	3.0	5.5
Leucine	5.3- 6.1	5.7	9.7
Tyrosine	1.6- 3.6	2.9	5.9
Phenylalanine	5.0- 6.2	5.4	5.3

[a]Lawhon, J. T., Cater, C. M. & Mattil, K. F. (1977) Evaluation of the food use potential of sixteen varieties of cottonseed. J. Am. Oil Chem. Soc. 54, 75-80.

[b]Food and Agriculture Organization (FAO) (1970) Amino Acid Content of Foods and Biological Data, p. 132, FAO, Rome, Italy.

APPENDIX B

PREPARATION OF HYDROLYSATE OF DIETARY PROTEIN

1. Approximately 50 mg of the samples were placed in hydrolysate tubes.
2. Add 2 ml of 6 N HCl into the samples.
3. Evacuate the samples for 4-5 hours under dry ice/ methanol.
4. Hydrolyze the samples at 100 ^{o}C for 24 hours.
5. Evaporate HCl in a vacuum desiccator with NaOH in it.
6. Add 2 ml of lithium citrate buffer (pH 2.2) into the dried samples, and mix.
7. Dilute the samples to have proper concentrations (about 1:50 dilution).
8. Filter the diluted samples through 0.45 um pore size filters.
9. The filtered samples are ready to be run by the Beckman 121M Amino Acid Analyzer.
10. Amount of lysine obtained by this procedure is the total lysine.

APPENDIX C

DETERMINATION OF AVAILABLE LYSINE

1. Weigh out samples (about 25 mg) and put into hydrolysate tubes.
2. Add 2 ml of saturated $NaHCO_3$ solution into the samples, mix, and allow to stand for 1 min.
3. Add 75 μl of FDNB (1-fluoro-2,4-dinitrobenzene, pure grade) dissolved in 3 ml of 96% ethyl alcohol.
4. Heat the sample tubes to 40 °C for 5 hours and shake occasionally.
5. Evaporate the alcohol.
6. Extract the dried samples 8 times with 15 ml of diethyl ether and remove the ether by decantation.
7. Add 2 ml of 6 N HCl into the dried sample residues.
8. Evacuate the samples for 4-5 hours under dry ice/ methanol.
9. Hydrolyze the samples at 100 °C for 24 hours.
10. Extract the hydrolyzed sample solutions 3 times with 2 ml of diethyl ether.
11. Evaporate HCl in a vacuum desiccator with NaOH in it.
12. Add 2 ml of lithium citrate buffer (pH 2.2) into the dried samples, and mix.
13. Dilute the samples to have proper concentrations (about 1:5 dilution).
14. Filter the diluted samples through 0.45 μm pore size filters.
15. The filtered samples are ready to be run by the Beckman 121M Amino Acid Analyzer.
16. Lysine obtained by this procedure is the unavailable lysine.
17. The available lysine can be calculated by subtracting the unavailable lysine from the total lysine obtained by the procedure included in Appendix B.

APPENDIX D

DETERMINATION OF NITROGEN BY MICRO-KJELDAHL METHOD

Reagents: Digestion mixture (40 g of K_2SO_4/250 ml of H_2O + 250 ml of conc-H_2SO_4 + 20 ml of 1 M $CuSO_4 \cdot 5H_2O$)

4% Boric acid

40% NaOH

1% Methyl red

0.05 N HCl

Nitrogen standard (1.2380 g of $(NH_4)_2SO_4$/250 ml of H_2O)--2 ml of N std contains 2.1 mg of N.

Procedure:

1. Digestion--Put samples into 30 ml micro-Kjeldahl flasks: N std, 2 ml; urine, 1 ml of the 4-day pooled (200 ml); feces, 2 ml of the 4-day pooled and homogenized (100 ml)

   Add 2 ml of digestion mexture into the samples.

   Digest the samples until color of solutions turns clear green.

   Stop digestion and cool the sample solutions.

2. Distillation--Add a digested sample solution into the inner chamber, rinse a Kjeldahl flask with about 5 ml of H_2O, and add it into the inner chamber.

   When the digested sample solution in the inner chamber begins to boil, add 10 ml of NaOH into the sample solution very slowly while the condenser tip is immersed in 5 ml of boric acid plus 1-2 drops of methyl red, and close the stopcock as soon as NaOH is added.

   Distill for 7-8 min, and rinse the condenser tip with H_2O.

   Titrate with 0.05 N HCl.

Calculation:

$$\text{mg N} = 14 \times \frac{\text{ml acid}}{1000} \times 0.05 \times 1000 = \text{ml acid} \times 0.7$$

APPENDIX E

DETERMINATION OF UREA NITROGEN

Reagent: Urea nitrogen reagent--

Add 44 ml of conc-H_2SO_4 and 66 ml of 85% o-phosphoric acid into about 100 ml of H_2O, mix it, and cool it. Then add the following, dissolving each successively:

50 mg of thiosemicarbazide
2.0 g of cadmium sulfate octahydrate
10 ml urea solution (2.6 mg/100 ml)

Mix the solution, dilute to 1 liter with H_2O, and keep it in an amber bottle.

2% Diacetyl monoxime--keep it in an amber bottle.

Urea N standards (30 and 60 mg urea N/100 ml)

Procedure: Put 5.0 ml of urea N reagent, and 20 µl of two standards, serum samples (deproteinized), or urine samples (diluted to 1:5) into test tubes, and mix.

Place the test tubes into boiling water.

Remove the test tubes after 12 min, immerse them in cool water for 5 min, and mix.

Read absorbances of two standards and the unknown samples against reagent blank at 540 nm.

Construct a two-point calibration curve and determine the concentrations of the unknown samples.

APPENDIX F

DETERMINATION OF URINARY CREATININE

Reagents: 0.036 M Picric acid

1.4 N HaOH

Creatinine standard (13.2 mg/100 ml)

Procedure: Put 0.1 ml of creatinine standard or 0.2 ml of urine samples into test tubes, and dilute to a total volume of 3 ml with H_2O

Add 1.0 ml of picric acid into the test tubes and mix.

Add 0.5 ml of 1.4 N NaOH into the first test tube, set a timer for 15 min, and mix the test tube.

Add 0.5 ml of 1.4 N NaOH into the remaining test tubes at 30 sec intervals.

After exactly 15 min after adding NaOH, read absorbances of the standard and the unknown samples against reagent blank at 500 nm at 30 sec intervals.

Calculation:

$$\text{mg creatinine/0.2 ml urine} = \frac{Ax}{As} \times 0.132 \times 0.1$$

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