APPENDIX V-e

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Plasma Free Amino Acids in Children Consuming Lupin Protein With and Without Methionine Supplementation

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ABSTRACT

Plasma free amino acids were measured in the fasting state and three and four hours postprandially in children after nine days consumption of diets in which lupin provided all of 6.4%-6.7% protein Kcal, either with supplementation of 2% (g/g protein) DL-methionine (L+Met, n=9) or with an isonitrogenous amount of urea (L+U, n=10). Fasting concentration of total amino acids (TAA) and of essential amino acids (TEAA) and the TEAA/TAA molar ratio did not differ between diets. Fasting Met concentration (15±4 μmoles/liter) and the Met/TEAA ratio (0.021±0.005) were markedly depressed after nine days of L+U. Supplementation with Met caused an expected increase in Met concentration (25±6 μmoles/liter) and the Met/TEAA molar ratio (0.039±0.007) and a profound decrease of Thr concentration (119±28 to 77±22 μmoles/liter) and Thr/TEAA (0.165±0.027 to 0.124±0.028). Postprandially after L+U, Met and Met/TEAA did not change from their low fasting values after L+Met, Met and Met/TEAA increased significantly relative to fasting values. Thr concentration increased but the Thr/TEAA ratio decreased significantly (0.124±-0.028 to 0.111±0.027). These studies confirm Met as the first-limiting amino acid in lupin protein and suggest that Thr becomes limiting when Met is provided in adequate amounts.

Legumes are important sources of dietary protein, and, in some cases, edible oil, in most parts of the world. Soybeans exemplify this type of legume in the United States. Climatic conditions preclude cultivation of soybeans in some areas of the world, especially at high altitudes. In the highland farm areas of many of the Andean countries, lupins (Lupinus mutabilis) have been cultivated for centuries but are not now a major crop. The German Agency for Technical Cooperation (GTZ) has been working for the past nine years to define the nutritional characteristics of lupins and to promote their use in Peru. Proximate composition and protein quality studies in animals have been reported (1). We undertook studies in humans to confirm and extend the observations in animals. Results of nitrogen balance and fat absorption studies have been published (2). In this paper we report the results of fasting and postprandial plasma free amino acid determinations in children consuming lupin-based diets with or without supplementary methionine. The data from children consuming unsupplemented lupin confirm that methionine is the first-limiting amino acid. In the presence of adequate methionine, threonine was shown to be next limiting.

MATERIALS AND METHODS

Nine Peruvian children ages 8 to 22 months participated in the studies with the informed consent of their parents[1]. The diets used and the protocol followed have been reported in detail (2) and will only be summarized here. The study diets were based on lupin flour, whose protein content was 55.9%. Table 1 shows the contents of

methionine and threonine, estimated to be the first and second-limiting essential amino acids relative to the FAO/WHO reference pattern (3), as well as the PER and NPU of lupin flour with and without supplementary methionine.

Protein (N x 6.25) provided 6.4% of dietary energy. In one lupin diet (L+Met), 2% (g/g protein) DL-methionine was added. In a second lupin diet (L+U) an isonitrogenous amount of urea was added because of nitrogen balance studies carried out concurrently (results abstracted in Table 1). A control diet (C) in which casein provided 6.4% of calories and to which urea was added in an amount isonitrogenous to the L+U diet was used for comparison. Fat, principally from added soybean and cottonseed oil blend (50:50), provided 48.7% of energy. Sucrose and dextrimaltose were added in equal amounts to complete carbohydrate, 44.9% of total calories. Vitamins and minerals were supplemented to meet or exceed U.S. Recommended Dietary Allowances (4).

Dietary periods were nine days in length. Both the L+U and L+Met diets were always preceded and followed by a control dietary period. The order of consumption of the two lupin diets was alternated in successive children.

Plasma samples were obtained at 8 to 9 a.m. after an overnight fast on the ninth day of each lupin dietary period. The child was then fed and two additional samples were obtained, at 3 hours and 4 hours postprandially. Plasma was separated and frozen to -20° within one hour. Samples were shipped in dry ice to our laboratory in Baltimore, where they were stored at -70° until analysis.

Determination of plasma free amino acids was carried out by liquid column chromatography using norleucine as an internal standard (5,6). Tryptophan was analyzed separately using a photofluorometric procedure (7).

Data were analyzed using "t" tests for paired and unpaired data where appropriate (8).

RESULTS AND DISCUSSION

Fasting values for both the L+U and L+Met diets are compared in Table 2 with data from previous studies from our unit on soy proteins with and without methionine supplementation (9) and milk protein or casein (10). Protein (N x 6.25) provided 6.4% to 6.7% of calories in all diets. The fasting concentration of total plasma free amino acids (TAA) while consuming L+Met was lower, but not significantly so, than while consuming L+U. Neither value differed from values for soybean or milk. The ratio of total essential plasma free amino acids plus cystine and tyrosine (TEAA) to TAA was expectedly lower with vegetable proteins than with milk protein and did not differ among L+U, L+Met, and the supplemented and unsupplemented soy protein diets.

Although a racemic mixture of DL-methionine was used for these studies it is unlikely that the D isomer affected the plasma methionine concentrations measured. L-methionine is actively transported across the small intestinal mucosa. D-methionine is poorly absorbed. The renal tubular transport that results in efficient reabsorption of filtered L-methionine is inactive for D-methionine. As a result

the small amounts of D-methionine that are absorbed are rapidly lost in the urine (11).

Based on fasting values for Met and Met/TEAA lupin appears to be more severely limited in methionine than soy protein. Supplementation of lupin protein with 2% DL-methionine increased the amino acid score from 54 to 83 (assuming only L-methionine was actually absorbed and utilized). Although the amino acid score still remained less than 100, this may reflect an artificially high reference value for methionine + cysteine. Supplementation at the same level corrected the PER of lupin to casein control values in the rat (1), whose requirement for the sulfur-containing amino acid is higher than that of the human. The addition of methionine to lupin at this level was not sufficient to cause a change in fasting TAA or the TEAA/TAA ratio. It produced a striking effect, however, not only on plasma Met and Met/TEAA but also on plasma Thr and the Thr/TEAA molar ratio. Met and Met/TEAA values became normal or supranormal, relative to milk, during consumption of the L+Met diet. The effect of supplying methionine in apparently adequate amounts should be to make threonine, whose amino acid score in lupin flour is 90, the first limiting amino acid in the L+Met diet. The threonine value obtained with L+urea of 119±28 μmoles/liter (milk value 119±35 μmoles/liter) was markedly depressed after nine days consumption of L+Met to 77±22 μmoles/liter. A parallel decrement occurred in the Thr molar ratio, from 0.165±0.027 to 0.124±0.028 (milk value 0.156±0.043), indicating that threonine was in short supply relative to the other essential amino acids.

Previous studies have documented the usefulness of postprandial plasma free amino acid determinations in pinpointing the essential amino acid that is truly limiting for protein synthesis. This approach has been used for both cereals (12-14) and legumes (9,15). In the studies cited a decrease in the fasting concentration of the first-limiting EAA was not always apparent. Postprandially, lysine or methionine concentrations were stable or dropped slightly. These changes in concentration were occurring at the same time that an increase in the concentrations of the other EAA was taking place, the net effect being a more profound perturbation of the EAA/TEAA ratio.

Postprandial values for selected amino acids from the present study are shown in Table 3. Following consumption of a meal of L+U there was little net increase in TAA or TEAA, the TEAA/TAA ratio remaining stable. Methionine concentration and the Met/TEAA ratio remained constant. There was a tendency for Thr concentration and the Thr/TEAA ratio to decrease but the decline was not significant. Values for tryptophan, the other EAA in lupin whose amino acid score is below 100, were normal initially and remained so postprandially. The failure to find a significant postprandial decrease in the Met/TEAA ratio with the L+U diet was somewhat surprising, as the amino acid score for methionine in unsupplemented lupin protein is 54. Most likely, the already very low fasting values for both methionine concentration and Met/TEAA militated against a further marked decrease postprandially, particularly since there was not a rapid influx of amino acids into the circulation postprandially, as documented by the small net change of TAA and TEAA.

Following consumption of the L+Met meal there was a significant increase in TAA and TEAA; this may reflect in large part the apparently low fasting concentration of both. The TEAA/TAA ratio remained stable. Both the rat (1) and human (2) studies with lupin have suggested better digestibility of the L+Met diet, for reasons that are not completely clear. The difference in the postprandial plasma amino acid responses is consistent with these observations but not confirmatory.

Methionine concentration and Met/TEAA increased significantly after the L+Met meal. Threonine concentration did not change, but there was a significant decrease in the Thr/TEAA ratio postprandially, suggesting, as did the fasting values, that in the presence of adequate methionine threonine had become first-limiting.

The current studies are useful in two regards. First they provide biological confirmation of the first- and second-limiting amino acids in lupin protein. Second, they suggest that the principal usefulness of postprandial plasma free amino acids in determining the limiting amino acid of a dietary protein may be in those circumstances in which the deficiency is marginal or before prolonged consumption has produced obvious changes in fasting amino acid concentrations. The marked inadequacy of methionine in lupin had already produced a substantial fall in the fasting plasma methionine concentration at the time of these studies. One can speculate that if the postprandial study had been carried out on day 1 of lupin consumption, when fasting plasma methionine was presumably normal, the postprandial decrease of Met/TEAA

documented with soy protein (9,14) would have been seen. On the other hand, a postprandial fall in Thr/TEAA ratio after the L+Met meal occurred despite the low fasting threonine concentration. The reason for the different responses in Met/TEAA and Thr/TEAA is unclear.

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FOOTNOTE

[1] The protocol followed for these studies was approved by the appropriate review committees of the Instituto de Investigacion Nutrícional and the Johns Hopkins Medical Institution.

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Table 1

Summary of results of studies of protein quality of *Lupinus mutabilis*

	BIOCHEMICAL				RAT		HUMAN	
	L Met + Cyst[1]		Thr[1]					
	g/16g N	AA Score[2]	mg/16g N	AA Score[2]	PER[1]	NPU[1]	Apparent N Absorption[3] %	Apparent N Retention[3] %
Lupin	1.9	54	3.6	90	1.24	34.9	81.8±3.2	15.6±5.8
Lupin + 2% DL-Met[4]	2.9	83	3.6	90	2.18	61.2	84.3±2.7	22.2±6.9

1 Determined by Schoeneberger *et al* (1). PER is corrected to a value of 2.5 for casein.

2 Based on the FAO/WHO reference pattern (3). In calculation of the amino acid score DL-methionine was assumed to provide equal amounts of the D & L isomer. Only L-methionine has been used in the calculation.

3 Lopez de Romaña *et al* (2).

4 DL-Methionine supplemented to 2% (W/W) of protein.

Table 2

Selected values for fasting plasma free amino acid concentrations after 9 days consumption of *Lupinus mutabilis* with or without DL-methionine supplementation. Values for soybean with or without methionine and casein or milk are presented for comparison

	Lupin + Urea (n=10)	Lupin + Met (n=9)	Soy Protein[1] (n=20)	Soy Protein[1] + Met (n=23)	Milk or Casein[2] (n=81)
TAA[3] - µmoles/L	2696 ±400	2396 ±242	2541 ±379	2575 ±460	2633 ±439
TEAA/TAA[4]	0.265 ±0.015	0.258 ±0.017	0.275 ±0.025	0.264 ±0.043	0.287 ±0.030
Met - µmoles/L	15 ±4	25 ±6	19 ±5	22 ±6	21 ±7
Met/TEAA[5]	0.021 ±0.005	0.039 ±0.007	0.027 ±0.007	0.034 ±0.011	0.028 ±0.006
Thr - µmoles/L	119 ±28	77 ±22	--	--	119 ±35
Thr/TEAA[5]	0.165 ±0.027	0.124 ±0.028	--	--	0.156 ±0.043

[1] Data from reference 15. The majority of children consumed only soy protein isolate as protein source. In some instances soy protein was supplemented with manioc flour or small amounts of milk protein. Methionine supplementation provided 12-20 mg/kg body weight as DL-methionine.

[2] Data from reference 10.

[3] TAA = total plasma free amino acids.

[4] TEAA = total plasma free essential amino acids (+ Cys and Tyr).

[5] Individual essential amino acid as the molar fraction of TEAA.

Table 3

Selected postprandial (p.c.) plasma free amino acids after nine days' consumption of lupin protein with or without DL-methionine supplementation

	TAA	TEAA	TEAA/TAA	Met	Met/TEAA	Thr	Thr/TEAA
	µmoles/L	µmoles/L		µmoles/L		µmoles/L	
Lupin+Urea n = 10							
Fasting	2696±400	715±111	0.265±0.015	15± 4†	0.021±0.005†	119±28∞	0.165±0.027∞
3 hours p.c.	2896±388	760± 89	0.263±0.012	16± 6†	0.020±0.006†	113±25∞	0.149±0.028c,∞
4 hours p.c.	2777±422	726±116	0.261±0.012	15± 4†	0.021±0.004†	110±29∞	0.151±0.028a,∞
Lupin+Methionine							
Fasting	2396±242	619± 71	0.258±0.017	25± 6†	0.039±0.007†	77±22∞	0.124±0.028∞
3 hours p.c.	2826±447a	721± 98a	0.256±0.014	35± 9d,†	0.049±0.013c,†	80±22∞	0.111±0.027a,∞
4 hours p.c.	2765±447a	735±128b	0.266±0.016	38±11c,†	0.051±0.009c,†	84±26	0.114±0.024∞

a Differs from corresponding fasting value, P<0.05

b Differs from corresponding fasting value, P<0.02

c Differs from corresponding fasting value, P<0.01

d Differs from corresponding fasting value, P<0.001

* Corresponding values for lupin+urea and lupin+methionine differ significantly, P<0.05

∞ Corresponding values for lupin+urea and lupin+methionine differ significantly, P<0.01

† Corresponding values for lupin+urea and lupin+methionine differ significantly, P<0.001