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Limiting amino acids in raw and processed amaranth grain protein from biological tests

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Abstract. Amino acid supplementation studies with young rats were carried out using raw and processed amaranth grain (*A. cruentus*) of dark- and cream- or light-colored seeds. The results of various studies repeatedly indicated that threonine is the most limiting amino acid in raw and processed, dark and cream-colored grain. Protein quality as measured either as NPR or PER was improved by threonine addition alone or with other amino acids and decreased liver fat to values similar to those measured with casein. This finding contradicts the reports that state that leucine, determined by chemical score, is the most limiting amino acid. Leucine addition alone or with other amino acids did not improve protein quality. The study confirmed cream-colored grain to be nutritionally superior to dark grain and that properly processed grain, light- or dark-colored, has higher protein quality than raw grain.

Introduction

Amaranth is a relatively new cereal-like grain, although it was extensively consumed by Mayan, Aztec and Incan civilizations of the past [13, 17, 19]. Today, people consume young leaves more often than the grain itself [18]; however, some societies produce amaranth grain for direct consumption or as special foods such as Alegría in Mexico [17]. Efforts are now being made throughout the world to introduce this crop into production systems and to develop procedures to use it for consumption [13].

Several amaranth grain composition studies have been reported [2, 4, 19, 20]. These show protein content to be around 15%, with lipid values of approximately 7%. Levels reported probably depend on plants' genetic makeup and on environmental factors [2, 6, 10]. The relatively high content of these two nutrients, as well as the very attractive essential amino acid

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protein pattern make amaranth nutritionally superior to cereal grains, and in fact, protein quality of well-processed grain approaches protein quality of casein [5, 12]. Comparison of amaranth grain essential amino acid pattern with FAO/WHO reference pattern [21] shows amaranth protein to be a good source of lysine, superior to levels found in most cereal grains [8]. This same comparison shows leucine to be the first limiting amino acid in amaranth protein as concluded by several workers [2, 4, 14, 19, 20]. However, biological evidence in this respect has not been provided. This is considered important since several authors [10, 14] have suggested other amino acids besides leucine limit the amaranth grain protein quality. The purpose of the studies to be reported was therefore to establish biologically the limiting essential amino acids in amaranth grain protein. Since processing, when carried out under controlled conditions, increases amaranth protein quality [4, 5, 9, 12, 15] amino acid supplementation was done on both, raw and processed samples.

Material and methods

Grain amaranth of *A. cruentus* of dark- and light-colored seeds was used. All grains were produced at INCAP's experimental farm facilities, and in all cases the grain used represented a pool of cultivars from the same species and color.

Previous studies indicated small differences in protein quality, not statistically significant, between samples of cultivars of the same species processed by atmospheric or pressure cooking [5, 6, 9, 10].

Processed amaranth was obtained by cooking the grain in the autoclave

Table 1. Amino acid content of amaranth grain (mg g N)

Amino acid	Content[a]	FAO reference	Score (%)	Rat requirement	Score (%)
Lysine	351	340	103	360	97
Threonine	237	250	95	260	91
Total S.A.A.	248	220	113	312	*79*
Valine	256	310	83	312	82
Isoleucine	226	250	90	260	87
Leucine	356	440	*81*	391	91
Total aromatic A.A.	490	380	124	417	117
Tryptophan	76	60	127	78	97

[a] Average of A.A. content in *A. caudatus*, *A. hypochondriacus*, *A. cruentus* and *A. edulis*.

at 15 lb pressure for 10–12 min using a grain-to-water ratio of 1:3. After cooking, the grains were placed in trays in a layer not thicker than 1 cm and dried overnight at 60 °C. Dried, it was ground with a hammer mill to pass a 20-mesh screen. All processed samples were analyzed for nitrogen content by the Kjeldahl Method [1] to calculate protein level to be used in the experimental diets.

The levels of amino acids added were calculated on the basis of the average amino acid content of various samples [2, 4, 14, 19] and compared to the FAO/WHO amino acid pattern. Table 1 summarizes the average essential amino acid content derived from values from four species. The Table also presents the FAO reference amino acid pattern and the rat's requirements for growth. Based on the two patterns, the amino acids which showed consistent limitations are: threonine, valine, isoleucine and leucine. Total sulfur amino acid limitations were found only using the rat amino acid requirements, while lysine was only marginally deficient. Difference between the analysis and the pattern was used as the level to add. When L-amino acids* were not available, the DL-form was used doubling the amount. In one study, at the end of the 28-day experimental period, the rats were sacrificed and their livers removed. These were weighed and then dried. The dried livers and carcases were analyzed for fat content.

Experimental diets were calculated to contain variable levels of protein depending on the study, that determined the amount of amaranth flour to be used. Protein levels of the diets are given in the results and discussion section. The diets were supplemented with 5% vegetable oil, 1% cod liver oil, 4% mineral mixture [7],** and corn starch to adjust to 100%. All diets were supplemented with 5 ml/100 g of diet of a complete B-vitamin mixture [11].***

In all experiments eight weanling white rats, 22–23 days of age, of the Wistar strain of the INCAP colony were used per diet. The animals placed in individual all-wire screen cages were distributed by weight among experimental groups so as to have the same initial weight ± 1 g in the group. The room had a constant temperature of 22 °C and a 12-hr light cycle.

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*** Grams liter. Thiamine, 0.600; Riboflavin 0.600; Pyridoxine, 0.600; Cyanocobalamine, 0.600; Calcium panthotenate, 2.000; Niacin, 1.000; Folic acid, 0.004; Inositol, 8.000; Para-Amino Benzoic Acid, 6.000; Vitamin K, 0.200; Choline Chloride, 30.000. The mixture is dissolved in a 50% ethyl alcohol solution.

The animals were provided with food and water *ad libitum* and food intake and weight changes were measured every 7 days, for either a total of 14 or 28 days, depending on the assay. A sample of the experimental diets was taken for nitrogen analysis with the purpose of calculating NPR at 14 days [3] and PER [1] at 28 days. For NPR assays a protein free diet was fed to 8 animals as indicated above. The weight loss in 14 days was used to correct for maintenance requirements.

Results and discussion

Cooked dark- and cream-colored amaranth at two levels of protein in the diet

Experiment 1

Results of amino acid supplementation to cooked dark- and cream- or light-colored amaranth grain are shown in Table 2, at two levels of dietary protein. With diets containing around 8.0% protein, dark-colored amaranth showed an improved quality when supplemented with 0.2% L-threonine using the unsupplemented diet at point of reference. On this basis, some response was also observed when valine, lysine and methionine were added alone, but the response with these amino acids was not greater than that observed with threonine supplementation. It is of interest to underline that leucine addition, the amino acid that other reports claim to be the first limiting amino acid compared to essential amino acid patterns [2, 4, 14, 19, 20], did not show any effect. On the contrary, NPR was slightly lower than the point of reference, the unsupplemented diet. Only the simultaneous addition of leucine, valine, threonine, isoleucine, lysine and methionine gave a higher response than that recorded with threonine addition alone. With respect to the cream- or light-colored amaranth, threonine addition alone gave a higher NPR but it was equal to that obtained with methionine supplementation alone. For this sample, responses higher than the reference unsupplemented diet, but lower than that of threonine and methionine, not statistically significant were observed for the addition of leucine, valine, isoleucine, and lysine. All amino acids added together resulted in higher quality than that observed with threonine, as was observed with the dark-colored grain. In all cases the cream-colored grain gave higher weight gains and higher NPR values than the dark grain. No explanation can be offered for this observation, although in studies with chickens the metabolizable energy of the dark grain was lower than that of the light-colored grain.

Table 2. Effect of amino acid supplementation of cooked amaranth grain of dark and light color

Amino acid added	Addition in diet (%)	Dark color		Light color	
		Ave. wt gain, g ($\bar{x}$ ± SD)	NPR[a] ($\bar{x}$ ± SD)	Ave. wt gain, g ($\bar{x}$ ± SD)	NPR[a] ($\bar{x}$ ± SD)
Diets with 8% protein content					
None	–	17 ± 7	2.59 ± 0.37	44 ± 9	3.32 ± 0.48
+ L-leucine	0.25	17 ± 11	2.34 ± 0.51	51 ± 10	3.48 ± 0.33
+ L-valine	0.20	24 ± 12	2.78 ± 0.53	50 ± 10	3.46 ± 0.52
+ L-threonine	0.20	36 ± 4	3.01 ± 0.21	43 ± 5	3.64 ± 0.20
+ L-isoleucine	0.20	15 ± 3	2.18 ± 0.29	47 ± 11	3.34 ± 0.38
+ L-lysine	0.15	28 ± 10	2.83 ± 0.44	49 ± 6	3.42 ± 0.31
+ L-methionine	0.20	27 ± 7	2.88 ± 0.42	46 ± 10	3.60 ± 0.45
+ L-leu + L-val + L-thr + L-ileu + L-lys + L-met	As above	43 ± 7	3.40 ± 0.22	56 ± 5	3.68 ± 0.34
Casein		46 ± 9	3.53 ± 0.36	46 ± 9	3.53 ± 0.36
Diets with 10% protein content					
None	–	35 ± 12	2.82 ± 0.37	62 ± 8	3.38 ± 0.29
+ L-leucine	0.25	29 ± 7	2.48 ± 0.22	62 ± 5	3.28 ± 0.16
+ L-valine	0.20	32 ± 10	2.51 ± 0.40	53 ± 5	3.31 ± 0.21
+ L-threonine	0.20	41 ± 12	2.92 ± 0.34	41 ± 8	3.44 ± 0.16
+ L-isoleucine	0.20	40 ± 7	2.58 ± 0.34	42 ± 7	2.91 ± 0.24
+ L-lysine	0.15	34 ± 12	2.59 ± 0.50	45 ± 9	2.80 ± 0.44
+ L-methionine	0.20	36 ± 8	2.74 ± 0.22	57 ± 7	3.34 ± 0.38
+ L-leu + L-val + L-thr + L-ileu + L-lys + L-met	As above	52 ± 9	3.23 ± 0.24	60 ± 8	3.41 ± 0.42
Casein		56 ± 9	3.83 ± 0.31	56 ± 9	3.83 ± 0.31

[a] 14-day assay.

Likewise, tannin content was higher in dark-colored grain as compared to the light-colored grain [22].

At the higher level of dietary protein for the dark-colored grain, threonine again gave the highest response. At this protein level, around 10.0%, none of the amino acids added alone, resulted in a significantly better quality than the unsupplemented diet. As in previous cases, all amino acids added together resulted in an improvement in quality superior to that with threonine addition alone. Threonine addition alone also gave the best protein quality when added to the cream-colored grain at the high level of protein.

In this case, some response was observed for valine and methionine addition alone. However, all amino acids did not result in a better quality than that recorded for threonine addition alone. In these experiments it was observed that some diets induced higher weight gains than those resulting with diets containing threonine, but did not result in a corresponding higher NPR. These effects were due to higher diet intake which not necessarily imply higher protein quality, since NPR is a ratio of weight gain corrected for maintenance needs over protein intake. As in the lower level of dietary protein, animal weight gain and NPR were higher for the cream-colored grain as compared to the dark grain.

These results then confirm that cream-colored grains have higher protein quality than the dark seeds, and strongly suggest that threonine, rather than leucine, is the first limiting amino acid in amaranth protein. Leucine levels in amaranth protein were low, and when compared to the essential amino acid pattern, it is the first limiting amino acid [2, 14, 15]. On the other hand, threonine is present in adequate amounts so the effects resulting from its addition are possibly due to low availability as is the case for rice protein [8, 16].

As would be expected, weight gain for the dark and the cream-colored grain was higher at the higher protein level in the diet, while NPR values do not show a similar trend. For the dark-colored grain NPR values at the high value of dietary protein increased for the diets with no amino acid addition and for diets supplemented with leucine and isoleucine. For the cream-colored grain only the NPR of the unsupplemented group increased slightly but certainly not statistically significant. These results were to be expected since sensitivity to addition of amino acids, as measured by assays such as those used in this study, decrease as protein intake increases, even though weight gain may be higher.

Experiment 2

Effect of amino acid addition to dark-colored amaranth grain, raw and processed, is shown in Table 3. The data show improved weight gain and protein quality for the cooked grains, as reported before [4, 5, 15], although some investigators did not find significant changes in a study with 25 selections of *A. caudatus* [5, 9].

The results with raw grain again clearly show the improvement in protein quality with threonine addition alone or single amino acid addition. The last two columns of Table 3 present the percent change from raw to cooked for weight gain and NPR.

It can be observed that for both parameters, threonine addition alone induced the least percentage change due to cooking. Leucine addition alone

Table 3. Effect of amino acid supplementation on the performance of rats fed raw and cooked dark-colored amaranth

Amino acid added	In diet (%)	Raw		Cooked		Percent change	
		Ave. wt gain, g ($\bar{x} \pm$ SD)	NPR[a] ($\bar{x} \pm$ SD)	Ave. wt gain, g ($\bar{x} \pm$ SD)	NPR[a] ($\bar{x} \pm$ SD)	wt	NPR[a]
None	–	14 ± 9	2.06 ± 0.38[b]	34 ± 11	2.94 ± 0.42	143	43
+ L-leucine	0.25	14 ± 8	2.18 ± 0.52	29 ± 7	2.48 ± 0.22	107	14
+ L-valine	0.20	12 ± 2	2.00 ± 0.22	32 ± 10	2.51 ± 0.40	166	26
+ L-threonine	0.20	26 ± 3	2.77 ± 0.26	41 ± 12	2.92 ± 0.34	58	5
+ L-isoleucine	0.20	16 ± 4	2.14 ± 0.21	40 ± 7	2.58 ± 0.34	150	21
+ L-lysine	0.15	11 ± 8	1.99 ± 0.60	34 ± 12	3.58 ± 0.50	209	80
+ L-methionine	0.20	16 ± 8	2.16 ± 0.34	36 ± 8	2.74 ± 0.22	125	27
L-leu + L-val + L-thr + L-ileu + L-lys + L-meth	As above	40 ± 10	2.87 ± 0.21	51 ± 9	3.23 ± 0.24	28	13
Casein		56 ± 7	3.84 ± 0.31	56 ± 7	3.84 ± 0.31		

[a] 14-day assay.
[b] SD of group average.

to both raw and processed grain followed threonine in inducing smaller changes in weight gain and NPR, while percentage change for the other amino acids or for the unsupplemented diet were the highest. This information was interpreted to mean that threonine was first limiting, improving the protein quality of both raw and processed amaranth to almost the same extent. The lower percentage increments in weight gain and NPR observed upon processing when the raw and cooked amaranth were supplemented with threonine suggest that this is the first limiting amino acid in the protein. If processing were responsible for increasing its availability, and that of leucine, no response should have been observed upon addition of the amino acids in the processed sample. The second limiting amino acid was leucine, while the percentage change for the other diets is due more to processing rather than to effects of amino acid.

Experiment 3

Further evidence for the effects of threonine is shown in Table 4. In this study, PER from threonine addition alone was higher than that observed with leucine added alone and similar to the PER when both amino acids were added together. Statistical analyses showed differences between groups in carcass water and fat to be significant. Highest body fat deposition took place on the diet supplemented with leucine and significantly less in the diets supplemented with threonine. When supplemented with threonine, liver fat was the lowest for the amaranth diets and only slightly higher than for casein. The data are similar to those reported for lysine and threonine supplementation of rice [8, 16] and they suggest again threonine to be the first limiting amino acid in amaranth protein.

The difficulty to obtain clearer results is probably due to the well-balanced amino acid content in amaranth grain protein; however, the consistency observed from the effect of threonine addition, even though not statistically significant, must be interpreted to mean that this is the first limiting amino acid in amaranth grain protein.

Experiment 4

In this and other studies [9] some response has been observed with the addition of methionine. This response may be due to dietary protein level, or more likely to level of supplementation. This was tested and the results are shown in Table 5. Addition of 0.2% DL-threonine showed a response on the basis of the unsupplemented diet, which was not improved by adding two or three times as much. On the contrary the higher levels reduced weight gain and protein quality, a common observation in amino acid supplementation studies [8]. The addition of methionine at levels of 0.1 to 0.3% did not result in a PER value above that observed with threonine.

Table 4. Effects of threonine and leucine added alone or together to cooked amaranth grain on weight gain, PER and liver fat contents of rats

Diet	Ave. weight gain ($\bar{x}$ ± SD)	PER[a] ($\bar{x}$ ± SD)	Carcass		Liver		
			Water (%) ($\bar{x}$ ± SD)	Fat wt (%) ($\bar{x}$ ± SD)	Fresh wt (%) ($\bar{x}$ ± SD)	Dried wt (%) ($\bar{x}$ ± SD)	Fat (%) ($\bar{x}$ ± SD)
Amaranth alone	91 ± 4	2.80 ± 0.21	64.2 ± 2.4	33.0 ± 2.6	6.86 ± 0.72	2.11 ± 0.31	16.14 ± 4.17
+0.25% L-leu	97 ± 7	2.85 ± 0.20	64.4 ± 2.3	38.1 ± 4.8	6.92 ± 1.23	2.11 ± 0.41	13.97 ± 1.84
+0.20% L-thr	93 ± 7	3.01 ± 0.21	68.0 ± 2.2	30.1 ± 5.0	5.93 ± 0.44	1.83 ± 0.31	10.91 ± 1.69
+0.25% L-leu +0.20% L-thr	105 ± 4	2.97 ± 0.11	64.7 ± 0.8	36.8 ± 2.9	6.53 ± 0.65	2.00 ± 0.22	11.85 ± 1.18
Casein	135 ± 5	3.06 ± 0.19	65.6 ± 3.7	34.3 ± 4.6	7.65 ± 0.88	2.29 ± 0.24	9.98 ± 0.79

[a] 28-day assay.

Experiment 5

A second study on amino acid levels was conducted and results are shown in Table 6. In this case, lower levels of addition of amino acids were tested. The addition of 0.1% DL-threonine gave some response which was not improved by adding twice as much. Four combinations between two levels of threonine and two of methionine did not improve PER above that observed with 0.1% DL-threonine added alone. The same conclusion is evident with supplementation of threonine and leucine, and with threonine, leucine and methionine. It is of interest to point out that food intake was higher when two or three amino acids were added, which may explain the higher weight gain measured.

The results of the studies showed in Table 5 and 6 again were interpreted to indicate that threonine is the first limiting amino acid, possibly followed by leucine and methionine.

Imeri *et al.* [10] from chemical amino acid data on 25 *A. caudatus* selections, using chemical score, indicated that 5 selections were low in sulfur amino acids, 1 in lysine, 4 in threonine, 12 in leucine and 3 had no amino acid deficiencies.

Pedersen *et al.* [14] suggested, using chemical score, that the limiting amino acids in amaranth grain protein were leucine, valine or threonine; however, from feeding studies, Imeri *et al.* [10] suggested that at 8% dietary protein, threonine was deficient; at 9.5% methionine, and at 11.0% leucine on *A. caudatus*. These results show the difficulty in establishing the limiting amino acid in amaranth grain protein. Although amino acid analysis by acid

Table 5. Effect of threonine and methionine supplementation of cooked grain amaranth flour

Amino acid added	In diet (%)	Ave. wt gain, g ($\bar{x} \pm$ SD)	Food intake, g ($x \pm$ SD)	PER[a] ($\bar{x} \pm$ SD)	Ave. PER same amino acid ($\bar{x} \pm$ SD)
None	-	87 ± 7	353 ± 23	2.4 ± 0.08	2.44 ± 0.08
DL-threonine	0.2	101 ± 10	361 ± 22	2.6 ± 0.10	2.50 ± 0.13[b]
DL-threonine	0.4	96 ± 15	365 ± 26	2.5 ± 0.10	
DL-threonine	0.6	88 ± 8	346 ± 17	2.4 ± 0.20	
DL-methionine	0.1	88 ± 13	345 ± 30	2.5 ± 0.20	2.47 ± 0.21[b]
DL-methionine	0.2	95 ± 13	371 ± 11	2.5 ± 0.25	
DL-methionine	0.3	90 ± 15	366 ± 38	2.4 ± 0.18	
Casein	-	112 ± 13	416 ± 37	2.6 ± 0.13	

Average initial weight = 47 g.
Protein content in diets = 10.8%.
[a] 28-day assay.
[b] Average of the three levels of amino acid.

Table 6. Effect of threonine, methionine and leucine supplementation on the protein quality of cooked amaranth grain flour

Amino acid added	Level (%)	Ave. wt gain, g ($\bar{x} \pm$ SD)	Food intake, g ($\bar{x} \pm$ SD)	PER[a] ($\bar{x} \pm$ SD)
None	-	101 ± 11	396 ± 26	2.29 ± 0.20
DL-threonine	0.1	107 ± 16	402 ± 26	2.49 ± 0.22
DL-threonine	0.2	110 ± 10	392 ± 33	2.52 ± 0.19
DL-threonine + L-methionine	0.1 + 0.1	118 ± 16	428 ± 42	2.50 ± 0.12
DL-threonine + L-methionine	0.1 + 0.2	113 ± 14	426 ± 37	2.43 ± 0.16
DL-threonine + L-methionine	0.2 + 0.1	108 ± 13	395 ± 29	2.46 ± 0.17
DL-threonine + L-methionine	0.2 + 0.2	114 ± 11	410 ± 25	2.50 ± 0.12
DL-threonine + L-leucine	0.2 + 0.1	120 ± 16	426 ± 34	2.57 ± 0.19
DL-threonine + L-methionine + L-leucine	0.2 + 0.2 + 0.1	123 ± 16	442 ± 40	2.48 ± 0.21
Casein	-	121 ± 18	419 ± 50	2.66 ± 0.39

Average initial weight = 47 g.
Protein content in diets = 11.0%.
[a] 28-day assay.

hydrolysis is known to decrease amino acid levels, it is doubtful that it would destroy leucine.

The information presented demonstrates that threonine is the first limiting amino acid in amaranth grain protein possibly followed by leucine, valine and methionine.

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