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Liver and Muscle Proteolytic Activity in Field Bean (*Vicia faba* L.) Fed Birds. Effect of Vitamin E

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S. SANTIDRIAN, M. L. RODRIGUEZ and J. LARRALDE. *Liver and Muscle Proteolytic Activity in Field Bean (*Vicia faba *L.) Fed Birds. Effect of Vitamin E.* Rev. esp. Fisiol., 43 (3), 375-380, 1987.

Liver and muscle proteolytic activities (cathepsin A and D) were measured in growing male chickens fed *ad libitum* over periods of 30 and 60 days on 20% protein diets containing either heated soybean (HSB, control) or raw field bean (RFB, *Vicia faba* L.) as the main sources of protein. Vitamin E (250 mg/100 g diet) was added or not to the RFB diet. It has been found that in comparison to control HSB-fed animals, RFB-fed birds showed a significant reduction in the rate of growth, together with a significant increase in the activities of both cathepsins A and D in liver and muscle. The addition of vitamin E to the RFB diet had no significant effect on either weight gain or liver and muscle proteolytic activities. The possible nature of these effects is discussed.

Key words: Antinutritive substances in raw legumes, Field bean (*Vicia faba* L.), Cathepsin A, Cathepsin D, Vitamin E, Liver, Muscle, Growth.

Legumes are widely used as protein sources both in human and animal nutrition. In the Mediterranean area, as well as in many other countries, these plants are extensively harvested in order to obtain relatively inexpensive protein supplies for domestic animals and humans (11). However, the utilization of raw legumes in animal nutrition produces a number of antinutritive effects which are basically reflected by growth inhibition (16, 17, 25), negative nitrogen balances (21) and an increasing activity of several amino-acid-degrading enzymes in rats and chickens (6). In addition, BELLO *et al.* (3), and later SANTIDRIÁN (21), showed that, in comparison to well nourished animals, those fed the raw legume field bean (*Vicia faba* L.), exhibited drastic changes in the composition of the skeletal musculature; these changes were characterized by a significant reduction in the sarcoplasmic nitrogenous fraction, together with a

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marked increase in the non-protein nitrogenous fraction, whereas the myofibrillar nitrogenous fraction remained unchanged. Both, antinutritive factors (13) and sulphur amino acids deficiency (15, 27) are thought to be the main causes of such effects.

Since lyososomal proteolytic activity plays a decisive role in protein metabolism, we have undertaken the present investigation to further elucidate the catabolic action of raw field bean on chicken protein metabolism by studying the effect of the legume on the activity of both cathepsin A and D in liver as well as in muscle. Furthermore, it is well known taht α-tocopherol (vitamin E) has a specific effect on the architecture of the membrane phospholipids by controlling the profiles of their unsaturated fatty acid components, thereby contributing to the stability of the lysosomal membrane (10). On the other hand, vitamin E is a potent antioxidant that protects membranes from lipid peroxidation (8). For these reasons, we have also investigated the effect on the activities of liver and muscle cathepsin A and D in growing chickens by adding vitamin E to the legume diet.

Materials and Methods

One-day old white male leghorn chickens, weighing about 40 to 50 g were randomly assigned into three dietary groups of 30 animals each and housed in battery brooders with raised floors. Groups were arranged as follows: one was fed a diet containing raw field bean as the main source of protein, and the other was fed the same diet to which vitamin E (dl α-tocopherol acetate), 250 mg/100 g diet, was added. In addition, a group of 30 chickens similar to those described above were fed a standard control diet and used as control. Both water and food were supplied *ad libitum*. The total protein content of each diet (N × 6.25) was 20% - 21%. Diets were prepared according to the criteria previously reported (6). Diet composition is given in table I. Body weight changes were individually recorded every three days. The amount of food intake was determined for each animal group as a whole.

Within each experimental group, ten chickens were killed by decapitation at three different age-intervals: one when they were one-day old (before experimental diets were applied), and the other when they were 30- and 60-days old respectively. Immediately after bleeding, livers were removed and weighed. A piece of pectoral muscle was excised and prepared for enzymatic analysis. Liver and muscle protein content was assayed by the method of LOWRY *et al.* (14). Free liver and muscle cathepsin D activities were measured both in control and legume-treated birds according to the method of GIANETTO and DeDUVE (9): briefly, samples of liver and muscle were homogenized in saccharose 0.25 M which contained EDTA 10^{-3} M, and then centrifuged at 800 g. Proteolytic activities were assayed in the supernatant using hemoglobin as substrate. Samples of both liver and muscle were incubated for 30 min at 37°C in acetic acid-acetate buffer (pH 3.8). As in previous papers (6, 22), activities of cathepsin D are given in units/mg of liver or muscle protein. One unit is defined as the amount of enzyme that catalyzes the liberation of 1 μmol of tyrosine (measured by the ninhydrin reaction) from hemoglobin during the incubation time. Total cathepsin D was assayed as indicated for free cathepsin D, but pretreating the homogenate with Triton X-100 0.2% in order to break the lysosomal membrane.

Cathepsin A was assayed by the method of IODICE and WEINSTOCK (12), the steps involved being very similar to those mentioned for the cathepsin D, with the difference that N-CBZ-glutamyl-L-tyrosine, incubated in acetic acid-acetate

buffer (pH 5.0) was used as the substrate. As indicated above, the tyrosine liberation was measured to evaluate free and total cathepsin A activities in samples of liver and muscle. Vitamin E, Triton X-100, hemoglobin and N-CBZ-L-glutamil-L-tyrosine were purchased from Sigma.

Statistical evaluations were carried out by analysis of the variance. For body weight changes, food intake and liver weight, two-way (diet and vitamin E) analysis of the variance was used. For enzymatic activities, three-way (diet, vitamin E and age) analysis of the variance was used. Differences ($p < 0.05$) from the values of the control group were calculated (26).

Table I. *Composition of the experimental diets (%).*

Diet	Control	*Vicia faba*	*Vicia faba* + vitamin E
Raw *Vicia faba*[1]	—	50	50
Vitamin E	—	—	0.25
Maize meal	30	35	35
Fish meal	3	11	11
Barley meal	36	2	2
Heated soybean	29	—	—
$CaCO_3$	1	1	1
$CaHPO_4$	0.50	0.50	0.50
NaCl	0.30	0.30	0.30
Supplement[2]	0.20	0.20	0.20
Total protein (N × 6.25)	20.9	20.2	20.2

[1] *Vicia faba* composition (%): protein, 22.3; ether extract, 2.1; ash, 2.8; crude fiber, 6.7.
[2] The supplement for 1 kg of diet contained: vitamin A, 5250 IU; vitamin D_3, 520 IU; riboflavin, 4 mg; nicotinic acid, 20 mg; Cu, 3 mg; Fe, 35 Mg; Mg, 300 mg; and, Mn, 50 mg.

Results and Discussion

Results of the experiment are summarized in tables II and III. Table II shows that, as compared to control birds, a significant reduction ($p < 0.05$) both in growth rate and liver weight was observed in the legume-fed chickens. This finding agrees with previously reported data from this laboratory (23) and others (16, 17) and shows the marked growth inhibition of chickens fed diets in which the raw field bean is the main source of protein. The same table shows that addition of vitamin E to the legume diet had no effect in restoring either growth or liver weight. Several hypotheses have been postulated in order to explain this antinutritive effect. First, legume protein has long been known to be deficient in sulphur amino acids, principally methionine and cysteine (5, 15, 27). Attemps to improve the nutritional value of legume proteins, especially in farm animals, by adding these amino acids to diets where legume proteins are intended to be the chief source of protein, are well known (2). It is of interest to point out that, expressed per unit of body mass, no differences were found in the amount of food intake among the different experimental groups (table II). Therefore, the growth differences displayed by the three groups of animals cannot be attributed to a higher or lower feed consumption (that, in principle, could be related to the palability or other organoleptic properties of the experimental diets) or to differences in the amount of energy or protein consumed by the experimental animals. This correlates with previously reported data from our laboratory (22, 23). On the other hand, raw field bean contains a number of compounds known to be antinutritive factors: tannins, globulins, trypsin and chymotrypsin inhibitiors, lectins, several glucosides, hemagglutinins, etc. (13). Reports from us (27) and others (17) showed that tannin content and digestive-enzymes inhibitors of the raw field bean might be responsible for the growth inhibition displayed by young animals fed the raw legume. Impairment

of the physiological process of intestinal absorption of sugars and amino acids seems to be one of the main mechanism by which legume-antinutritive compounds alter the rate of growth in rats and chickens (4, 19, 20).

Table III shows that, independently of age, as compared to control birds, those fed the field bean diet exhibited a marked increase ($p < 0.05$) in the activities of cathepsin A and D, both in liver and muscle. This finding correlates with recently published evidence (24) and with the fact that feeding growing animals on diets elaborated with raw legumes brings about an increase of protein catabolism. Previously reported data (6, 22) have shown increases in the activity of a number of hepatic amino-acid-degrading enzymes in rats and chickens fed a raw field bean diet. As mentioned in the introduction, it was published by the present authors (21) that rats fed a raw *Vicia faba* diet exhibited a significant reduction in the sarcoplasmic nitrogenous fraction, together with a marked increase in the non-protein nitrogenous fraction of the skeletal musculature. However, the myofibrillar protein turnover, as measured by the urinary excretion of 3-methylhistidine (23), remained unchanged. In order to explain, at least in part, this catabolic effect caused by raw field bean, the results of this experiment showed that the

Table II. *Food intake, weight gain and liver weight in chickens fed for 60 days 20% protein diets containing either heated soybean (control) or raw field bean* (Vicia faba *L.*), *with or without added vitamin E (250 mg/100 g diet), as main protein sources.*

Entries are mean values (± SEM) for 10 chickens in each group.

Diet	Food intake g/100 g body weight	Body weight gain g/day	Liver weight g/100 g body weight
Control	190±10	28.7±4.2	3.7±0.2
Vicia faba	200±15	16.3±3.7*	2.7±0.1*
Vicia faba + vitamin E	205±16	14.9±3.5*	2.8±0.2*

*$p < 0.05$, as compared to control-diet-fed chickens (two-way ANOVA).

Table III. *Liver and muscle cathepsin A and D activities in chickens fed 20% protein diets containing either heated soybean (control) or raw field bean* (Vicia faba *L.*), *with or without added vitamin E (250 mg/100 g diet), as main protein sources.*

Entries are mean values of enzymatic units ($\times 10^2$)/mg of protein of 10 chickens.

		Liver Cathepsin A		Liver Cathepsin D		Muscle Cathepsin A		Muscle Cathepsin D	
Diet	Age days	Total	Free	Total	Free	Total	Free	Total	Free
Control	1	54±2	27±2	45±4	26±2	14±1	10±1	24±2	18±2
	30	38±1*	18±1*	34±2*	20±2*	12±2*	6±1*	22±1	17±3
	60	53±3	18±1*	53±3*	26±1	11±1*	8±1	19±2*	16±1
Vicia faba	1	54±3	29±2	46±3	28±1	16±1	12±1	27±3	19±2
	30	52±3*[1]	25±1*[1]	57±4*[1]	35±2*[1]	19±2[1]	10±1[1]	30±3*[1]	21±1[1]
	60	61±4*[1]	33±2*[1]	63±3*[1]	38±2*[1]	19±2[1]	14±2[1]	28±2[1]	23±3*[1]
Vicia faba + vit. E	1	56±4	29±3	47±5	28±1	16±3	12±1	27±3	18±1
	30	48±3*[1]	25±1*[1]	52±4*[1]	35±2*[1]	17±2[1]	11±1[1]	26±4[1]	16±2
	60	61±5*[1]	30±3[1]	62±4*[1]	41±4*[1]	15±1[1]	9±1*	24±1[1]	21±2*[1]

* $p < 0.05$, as compared to values for one-day old animals within each dietary group.
[1] $p < 0.05$, as compared to the corresponding age-value for control-diet-fed animals. (Three-way ANOVA).

marked increase in liver and muscle proteolytic activity must be taken into account. Addition of vitamin E has no effect in restoring either liver or muscle proteolytic activity (tables III and IV). The well known antioxidant properties of tocopherols in preventing polyunsaturated fatty acids oxidation by molecular oxygen (8), thereby protecting biological membranes, seems to be completely inefficient, at the doses used in this experiment, when growing chickens are fed a raw field bean diet.

The mechanism by which raw field bean originates such a marked increase in liver and muscle proteolytic activity is not yet fully understood. Some workers have found that nutritional stresses such as starvation (7), and protein malnutrition (1, 18) are normally accompanied by increases in the activities of lysosomal proteolytic enzymes: intracellular proteins begin to break down in order to supply the body with the essential amino acids that are lacking in the diet; in these cases, a weakening or an increase in the fragility of the lysosomal membrane has been observed (18). On the other hand, cathepsin A has been reported to greatly increase (12) in the skeletal muscle from rabbits suffering from nutritional muscular distrophy. In our experiment, apart from the aforementioned sulphur amino acid deficiency, some factors contained in the raw legume might have interacted and subsequently breaking down the lysosomal membrane, releasing into the cytoplasm its proteolytic enzymes. Such a mechanism has been postulated by several investigators (28).

In conclusion, the results of this experiment showed that liver and muscle proteolytic activities are markedly increased in growing chickens fed a raw field bean diet. Addition of vitamin E did not prevent this effect. It is suggested that the antinutritive factors contained in the raw field bean seeds might principally account for these effects.

Resumen

Se mide en pollos macho en crecimiento, alimentados durante 30 ó 60 días con dietas elaboradas a base de harina de soja calentada (HSB, control) o de harina cruda de habas (RFB, *Vicia faba* L.), suplementada o no con vitamina E (250 mg/100 g dieta), la actividad de las catepsinas A y D hepáticas y musculares. Los animales alimentados con la dieta RFB, independientemente de la adición de vitamina E, muestran una reducción significativa del crecimiento y un aumento también significativo de la actividad de ambas catepsinas en hígado y músculo. Se comenta la naturaleza probable de estos efectos.

Palabras clave: Factores antinutritivos en leguminosas, Haba (*Vicia faba* L.), Catepsina A, Catepsina D, Vitamina E, Hígado, Músculo, Crecimiento.

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