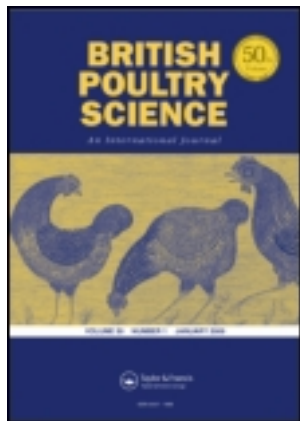


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E. Rueda<sup>a</sup>, M. León<sup>b</sup>, M. Castañeda<sup>a</sup>, A. Mendez<sup>b</sup> & C. Michelangeli<sup>b</sup>

<sup>a</sup> Facultad de Ciencias Veterinarias, Laboratorio de Enzimología y Toxicología, Universidad Central de Venezuela

<sup>b</sup> Facultad de Ciencias Veterinarias, Centro de Bioquímica Nutricional, Universidad Central de Venezuela, Maracay, Estado Aragua, Venezuela

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## Effects of Concanavalin A on intestinal brush border enzyme activity in broiler chickens

E. RUEDA, M. LEÓN<sup>1</sup>, M. CASTAÑEDA, A. MENDEZ<sup>1</sup> AND C. MICHELANGELI<sup>1</sup>

*Facultad de Ciencias Veterinarias, Laboratorio de Enzimología y Toxicología, Universidad Central de Venezuela, , and <sup>1</sup>Facultad de Ciencias Veterinarias, Centro de Bioquímica Nutricional, Universidad Central de Venezuela, Maracay, Estado Aragua, Venezuela*

**Abstract** 1. The effects of Concanavalin A (Con A) on enzymes from the intestinal brush border were studied using membrane vesicles (BBMV) prepared from 3- and 6-week-old broiler chickens.  
2. Maltase, sucrase, phytase, alkaline phosphatase and leucine aminopeptidase activities were assayed in BBMV in the absence (T0) or presence (T1) of Con A, or in the presence of casein (T2). Disaccharidase specific activities were assayed in the presence of Con A that had been pre-incubated with the enzyme (T3) or with the substrate (T4).  
3. Con A significantly affected maltase and sucrase activities in 3-week-old broiler chicken intestinal BBMV. Pre-incubation of the lectin with the maltase or its substrate had no effect on enzyme activity. Pre-incubation of Con A with sucrose reduced enzyme activity.  
4. Con A did not affect phytase, alkaline phosphatase or leucine aminopeptidase activities.  
5. Maltase, alkaline phosphatase and leucine aminopeptidase activities were lower in 6-week-old than in 3-week-old broilers.

### INTRODUCTION

Concanavalin A (Con A) is the lectin present in *Canavalia ensiformis* seeds. Research on the nutritive value of *Canavalia ensiformis* has shown this legume may serve as a primary source of protein and energy for poultry (León *et al.*, 1990) but there is evidence from nutritional studies that it is toxic to the young chick (d'Mello *et al.*, 1989). The adverse effects mostly arise from Con A, a heat-labile lectin that binds specifically to  $\alpha$ -D-mannopyranoside and  $\alpha$ -D-glucopyranoside, to  $\alpha$ -D-mannopyranoside and  $\alpha$ -D-glucopyranoside residues at the non-reducing ends of oligo- or polysaccharides (Goldstein and Poretz, 1986) and, with high specificity, to the trimannosidic core of N-linked glycoproteins [3,6-di-O-( $\alpha$ -D-mannopyranosyl)- $\alpha$ -D-mannopyranoside] (Swaminathan *et al.*, 1998). Significant amounts of ingested

Con A were recovered unaltered from the caecal content of rats 4 h after its oral administration and from faeces (90% recovery) 4 d later (Nakata and Kimura, 1985). This indicates that Con A is quite stable during its passage through the rat gastrointestinal tract. In the intestinal lumen, the Con A can interact with the mucous membrane affecting the processes that take place at this level, such as transport mechanisms and final hydrolysis of dietary components (Gelberg *et al.*, 1992). In rats, Con A reduced the absorption of calcium and sucrase activity (Ayyagari *et al.*, 1993). Nakata and Kimura (1985) reported a decrease in sucrase, alkaline phosphatase and leucine aminopeptidase activities in the presence of Con A in rats. On the other hand, in *Lacnobia oleracea* larvae, Con A increased the trypsin and aminopeptidase activities and did not cause any significant effect of alkaline phosphatase activity (Soler *et al.*, 1998). Studies *in vitro* demonstrated

Correspondence to: E. Rueda, Facultad de Ciencias Veterinarias, Laboratorio de Enzimología y Toxicología, Universidad Central de Venezuela, Maracay Estado Aragua Venezuela. E-mail: emma.rueda@ucv.ve  
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that Con A at 4 mg/ml in the final mixture reaction inhibited the alkaline phosphatase activity by 40% (Ahmad and Gupta, 1992). The present study was undertaken to determine the effect of Con A on the maltase, sucrase, phytase, alkaline phosphatase and leucine aminopeptidase activities in intestinal brush border membrane vesicles isolated from 3- or 6-week-old broiler chickens.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of analytical grade and were purchased from Sigma Chemical (St Louis, MO, USA).

### Concanavalin A isolation and purification

Commercial Con A is very expensive in Venezuela; for this reason, Con A was isolated and purified from *Canavalia ensiformis* seeds through selective precipitation with ammonium sulphate and affinity chromatography according to Ganem and Martín (2000).

### Purity and biological activity of Con A

Purity of Con A was assayed by polyacrylamide gel electrophoresis (SDS-PAGE) conducted under denaturing conditions using 15% polyacrylamide gel. Prior to electrophoresis, the Con A preparation was treated with 2% SDS, 0.6%  $\beta$ -mercaptoethanol, 20% (v/v) glycerol and 40 mM Tris-HCl (pH 6.8) for 5 min at 100°C. The running buffer was 25 mM Tris-HCl (pH 8.3). Protein was stained with 0.25% (w/v) Coomassie blue R-250. Sigma electrophoresis kit standards were used to determine molecular weight. Purity was confirmed by protein immunodetection (Western Blot) using anti Con A/peroxidase after transferring the proteins from the electrophoresis gel to a nitrocellulose membrane applying 24 V for 1.5 h and incubating for 90 min in 500 mM NaCl, 20 mM Tris-HCl, pH 7.5 with 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and 1 mg/ml Con A Sigma C2010. After washing 4 times for 5 min, the membrane was incubated for 60 min on 500 mM NaCl, 20 mM Tris-HCl, pH 7.5 with 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and 50  $\mu\text{g}/\text{ml}$  commercial peroxidase (Sigma P8250). Glycoproteins were revealed using 50 ml buffer,  $\text{H}_2\text{O}_2$  30% v/v and 30 mg 4-chloro-1-naphthol (Towbin *et al.*, 1979). The biological activity was tested by haemagglutination of a suspension of 2% fresh rabbit erythrocytes in 0.9% NaCl after 60 min of incubation at room temperature and compared with the haemagglutination brought about by Con A from Sigma<sup>®</sup> used as a control. In order to

confirm that Con A was active during the enzymatic determinations, haemagglutination was determined in the presence of different enzymatic buffers.

### Animals and diet

A total of 60 1-d-old Ross broiler chicks obtained from Granja La Caridad (Aragua State, Venezuela) were housed in metal cages, receiving water and a standard broiler diet (National Research Council, 1994) on an *ad libitum* basis. At 3 and 6 weeks old, 30 chickens were fasted for 12 h and then killed by cervical dislocation.

### Intestinal mucous homogenate preparation

The duodenum was removed quickly and flushed with ice-cold saline, opened lengthwise and the inner surface scrapped with a glass slide to obtain the mucous layer, which was suspended in 50 mM mannitol and 2 mM HEPES pH 7.1. The suspension was homogenised using a Waring blender at top speed for one minute.

### Brush border membrane vesicles (BBMV); isolation and purification

BBMV were prepared according to Kessler *et al.* (1978) and a modification was made including 0.41 M NaSCN treatment in order to improve the membrane purification (Hopfer *et al.*, 1983). All steps were performed on ice between 0 and 4°C.

### Relative purity of the BBMV fraction

BBMV purity was checked by calculating the enrichment factor as the ratio of specific activity of the vesicles to that of the homogenate.  $\text{Na}^+/\text{K}^+$  ATPase (EC 3.6.3.1), a basolateral marker, and acid phosphatase (EC 3.1.3.2), a lysosomal marker, were systematically assayed according to Proverbio and Del Castillo (1981) and Mircheff and Wright (1976), respectively.

### Enzyme assays

#### *Maltase and sucrase*

Maltase (EC 3.2.1.20) and sucrase (EC 3.2.1.48) activities were assayed according to Dahlqvist (1968) using 0.056 M maltose or sucrose as substrate in 0.1 M sodium maleate pH 6.0. Their specific activities were expressed as nmoles glucose/min/mg protein.

#### *Phytase*

Phytase (EC 3.1.3.8) activity was assayed according to Maenz and Classen (1998) with modifications. Forty microlitres of BBMV were incubated, at

41°C, with 200 µl of 1 mM myoinositol hexakisphosphate and 200 µl of 25 mM MgCl<sub>2</sub> and 50 mM MES pH 6.0. The reaction was stopped with 3.6 ml of a solution with 0.28% ammonium molybdate, 1.1% SDS and 1.1% ascorbic acid. Inorganic phosphate was determined by spectrophotometer at 820 nm. Values were compared to a standard using a curve 0.5 mM KH<sub>2</sub>PO<sub>4</sub>. Phytase activity was expressed in nmoles phosphate/min/mg protein.

#### Alkaline phosphatase

Alkaline phosphate (EC 3.1.3.1) activity was assayed by a modification of the method of Wang and Gilles-Baillien (1992). BBMV (20 µl) were incubated in the presence of 270 µl of 100 mM glycine pH 9.5, 10 mM MgCl<sub>2</sub>, 0.2 mM ZnCl<sub>2</sub> and 20 mM *p*-nitrophenylphosphate as substrate. After 15 min of incubation at 41°C, the reaction was stopped by submerging the tubes in ice-cold water for 5 min, and the contents diluted 1:10 with 0.05 M NaOH. The intensity of the resulting colour was read at 405 nm and compared with the values of a standard curve elaborated from 20 mM *p*-nitrophenol. The enzyme activity was expressed as nmoles *p*-nitrophenol/min/mg protein.

#### Leucine aminopeptidase

Leucine aminopeptidase (EC 3.4.11.2) activity was measured by the method of Kramers and Robinson (1979). The reaction mixture contained 25 µl BBMV, 250 µl of 50 mM imidazole buffer pH 7.5 and 125 µl of 1.6 mM L-leucine-*p*-nitroanilide. After 15 min incubation at 41°C, the reaction was terminated by the addition of 250 µl of 5% (w/v) ZnSO<sub>4</sub>. The absorbance at 405 nm was compared with that of a standard curve using 1.6 mM *p*-nitroaniline solution. The enzyme activity was expressed as nmoles *p*-nitroaniline/min/mg protein.

All enzyme assays were performed in triplicate and in all cases a blank was run in which the samples were added after stopping the reaction.

#### Protein assay

Protein was determined by Bradford (1976) using each buffer as a blank and bovine serum albumin as standard.

#### Treatments

To determine the effect of Con A on maltase and sucrase activities and the possible interaction of the lectin with the enzyme or the corresponding substrate, 5 treatments were assessed: (i) no Con A addition (T0), (ii) Con A addition (T1), (iii) casein addition (T2), (iv) Con A pre-incubated for 30 min

with the enzyme before initiating the reaction (T3) and (v) Con A pre-incubated for 30 min with the substrate before initiating the reaction (T4). All treatments were applied *in vitro* in isolated samples of BBMV from 3- and 6-week-old broiler chicken duodenal mucous. The Con A effects on the phytase, alkaline phosphatase and leucine aminopeptidase activities in BBMV were determined applying three treatments: (i) no Con A addition (T0), (ii) Con A addition (T1) and (iii) casein addition (T2). Con A solutions were prepared in 0.9% (w/v) NaCl and used at a concentration similar to the corresponding substrate (20 mg/ml for disaccharidases, 0.8 mg/ml for phytase, 5.3 mg/ml for alkaline phosphatase and 0.4 mg/ml for leucine aminopeptidase), so the effect of Con A could be determined at the same concentration of the substrate.

Each sample was formed by the BBMV obtained from the pooled duodenum of 5 chickens. The determinations were made in triplicate within each sample.

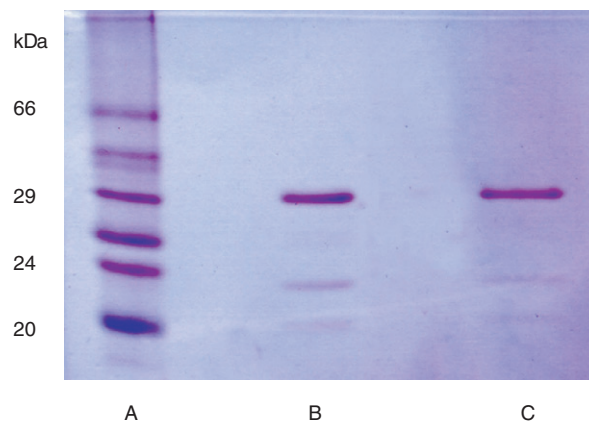
#### Data analysis

Results were expressed as means ± standard deviation and analysed statistically by nonparametric tests (Steel and Torrie, 1988). Comparison between different experimental groups was performed by test of ranks average and were considered statistically different at  $P < 0.05$ .

## RESULTS AND DISCUSSION

#### Con A purity

SDS-PAGE procedures (Figure 1) revealed that under denaturing conditions, Con A isolated and purified ran as one principal band possessing a molecular mass of 29 kDa which corresponded



**Figure 1.** Comparison between purified and commercial Con A using SDS-PAGE. SDS-PAGE was conducted under denaturing conditions and 15% polyacrylamide gel as described under Methods. Lane A, Mw markers; Lane B, Con A Sigma®; Lane C, Con A isolated.

to monomeric Con A. Two small strips of 24 and 26 kDa corresponding to Con A polypeptides were also observed as reported by Hague (1975) and Chrispeels *et al.* (1986) and corroborated by immunodetection test on nitrocellulose membrane (Figure 2). These confirm that our purified Con A was free from other protein fractions and its purity was similar to that of the commercial Con A used as standard.

### Con A biological activity

Con A haemagglutination activity, expressed as a percentage of the total of haemagglutination in different buffers and in physiological saline solution, was similar to that of commercial Con A (Table 1). In the presence of the different buffers, the agglutination capacity of Con A was not affected, guaranteeing that during the enzymatic determinations the lectin maintained its activity. There was no haemagglutination activity of Con A in the presence of 1% casein, possibly because it is a glycoprotein that can bind to the

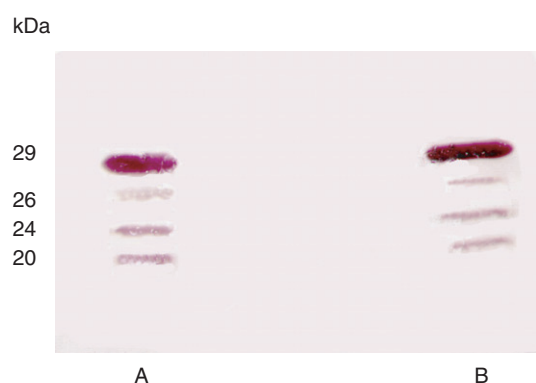
active sites of the lectin and prevent its erythroagglutination action. These results demonstrate that the method used obtained lectin with high purity and of similar biological activity to that reported by Ganem and Martín (2000).

### BBMV purity

Enrichment factors were higher in BBMV from 3-week-old chicks for maltase, sucrase, alkaline phosphatase and leucine aminopeptidase. By contrast, in BBMV from 6-week-old chicks the recovery was greater for phytase and leucine aminopeptidase (Table 2). The values are higher than those reported by Kessler *et al.* (1978) and Torras-Llort *et al.* (1996). The phosphatase alkaline purification factor was similar for both 3 and 6 weeks, with higher values than those reported by Davies and Flett (1978). The enrichment factor of leucine aminopeptidase was similar for both weeks, with values of 11.3 and 11.0 for the third and sixth week, respectively, similar to those reported by Tacnet *et al.* (1990) when obtaining BBMV from pigs' jejunum. The recovery of phytase was significantly higher in week 6 than in week 3. The enrichment factor of Na<sup>+</sup>/K<sup>+</sup> ATPase and acid phosphatase, both indicators of contamination with other membrane fragments, is similar to that reported by several authors (Tacnet *et al.*, 1990; Torras-Llort *et al.*, 1996).

### Effects of Con A on digestive enzyme activities

In BBMV obtained from 3-week-old chickens, significant differences were observed in T1, with a reduction of 82% of maltase activity in comparison with the control (Table 3). In the 6-week BBMV there were no significant differences between treatments; however, a reduction of 23% in maltase activity in comparison with the



**Figure 2.** Western Blot. Protein immunodetection using anti Con A/peroxidase to compare purified and commercial Con A. Western blotting was carried out as described under Methods. Lane A, Con A Sigma<sup>®</sup>; Lane B, Con A isolated.

**Table 1.** Concanavalin A (Con A) haemagglutination activity<sup>1</sup>

Enzymatic buffer	Con A isolated		Con A Sigma <sup>®</sup>	
	Total activity <sup>2</sup>	% of total	Total activity <sup>2</sup>	% of total
Sodium chloride 0.9%	22 ± 0.7	91.7	23 ± 0.4	95.8
0.1 M maleic acid pH 6.0	22 ± 0.7	91.7	23 ± 0.4	95.8
50 mM MES pH 6.0	18 ± 0.7	75.0	22 ± 0.5	91.7
100 mM glycine pH 9.5	22 ± 0.8	91.7	23 ± 0.5	95.8
50 mM imidazole pH 7.5	20 ± 1.1	83.3	12 ± 1.1	50.0
160 mM Tris-HCl pH 7.5	23 ± 0.4	95.8	23 ± 0.5	95.8
0.1 M sodium phosphate pH 7.4	23 ± 0.5	95.8	23 ± 0.5	95.8
1% casein	0	0	0	0

<sup>1</sup>Capacity to produce agglutination in a suspension of 2% rabbit fresh erythrocytes after 60 min of incubation at room temperature.

<sup>2</sup>From a total of 24 cells incubated with dilutions of a solution of Con A prepared in NaCl 0.9% to an initial concentration of 1 mg/ml.

Values are expressed as mean of 5 replicates ± SD.

control was observed in T3. This may be due to changes in the enzyme glycosylation pattern that prevented their binding to Con A. The sucrase activity in 3-week-old BBMV diminished significantly in the presence of Con A (T1) and when the lectin was pre-incubated with the enzyme (T3). Previous studies using *Phaseolus vulgaris* lectins have demonstrated that their specific interaction with membranes depends on the pattern of glycosylation of the mucosa (Begbie and King, 1985; Lafont *et al.*, 1988), which can vary depending on diverse factors such as the age and diet composition (Sharma *et al.*, 1997). These factors may partly explain the results obtained in the present work. When comparing enzymatic activities at different ages a different behaviour between disaccharidases was observed. Thus, the maltase activity was significantly less in 6-week-old BBMV, whereas the sucrase activity did not display significant differences between weeks. These results agree partially with those reported by Iji *et al.* (2001), who found a diminution of disaccharidase specific activity at 21 d old in comparison with the first 7 d of age. The presence of the casein in the incubation mixture did not cause any change in disaccharidase activities. In the BBMV from 3-week-old chickens a statistically significant diminution

( $P < 0.05$ ) of the activity of phytase in the presence of the lectin was observed (Table 4). The specific activity of phytase in the BBMV did not change between the ages studied. Nelson (1976) reported lower phytase activities in chickens and hens after feeding diets with maize in substitution for wheat suggesting that food ingredients can affect phytase activity.

Con A did not affect alkaline phosphatase activity in BBMV at any of the ages studied (Table 4). Alkaline phosphatase is a glycoenzyme and thus might be expected to be inhibited by Con A, as previously observed by Ahmad and Gupta (1992). The results obtained in the present work may have been a consequence of the substrate/lectin ratio. This factor must be taken into account in future studies. Salgado *et al.* (2001) reported a diminution ( $P < 0.05$ ) in alkaline phosphatase specific activity in intestinal mucous homogenate from pigs weaned at 28 d old when Leguminosae seeds were included in their diet, but this effect may have been caused specifically by lectins or other components of the seeds. In *Oleracea lacanobia* larvae fed with semi-purified diets, Con A did not affect alkaline phosphatase activity (Soler *et al.*, 1998). It is possible that the similarity in the effect of Con A on this enzyme in the chickens and the larvae is

**Table 2.** Enrichment factors of integral enzymes of brush border and lysosome and basolateral membrane marker enzymes to assess the purity of the BBMV<sup>1</sup>

Enzyme/age (week)	Enzymatic enrichment factors <sup>2</sup>	
	3-week-old BBMV	6-week-old BBMV
Maltase	18.3 ± 2.8	13.8 ± 1.7
Sucrase	16.7 ± 3.5	11.4 ± 2.5
Phytase	10.0 ± 0.72	20.6 ± 1.1
Alkaline phosphatase	14.1 ± 1.20	14.6 ± 1.6
LeuAMP <sup>3</sup>	11.3 ± 1.68	11.0 ± 1.4
Na <sup>+</sup> /K <sup>+</sup> ATPase	0.6 ± 0.05	0.5 ± 0.03
Acid phosphatase	0.7 ± 0.02	0.5 ± 0.02

<sup>1</sup>BBMV isolated and purified from duodenal homogenate of 3- and 6-week-old broiler chickens.

<sup>2</sup>The enrichment factor was calculated as the relation between the enzyme activity in BBMV and enzyme activity in homogenate in the control groups.

<sup>3</sup>LeuAMP = leucine aminopeptidase.

Values are expressed as mean of 15 repetitions ± SD.

**Table 3.** Effect of Concanavalin A (Con A) on maltase and sucrase activity (nmoles glucose/min/mg protein) in BBMV from 3- and 6-week-old broiler chickens

	T0	T1	T2	T3	T4
<i>Maltase</i>					
3 weeks old	3670 <sup>a</sup> ± 490	670 <sup>d</sup> ± 290	3730 <sup>a</sup> ± 450	3570 <sup>a</sup> ± 460	2980 <sup>ab</sup> ± 450
6 weeks old	1620 <sup>bc</sup> ± 230	1510 <sup>cd</sup> ± 270	1610 <sup>bc</sup> ± 170	1520 <sup>cd</sup> ± 330	1500 <sup>cd</sup> ± 300
<i>Sucrase</i>					
3 weeks old	225 <sup>a</sup> ± 46	56 <sup>d</sup> ± 9	221 <sup>a</sup> ± 54	52 <sup>d</sup> ± 9	137 <sup>ab</sup> ± 14
6 weeks old	109 <sup>abc</sup> ± 14	70 <sup>cd</sup> ± 6	96 <sup>bc</sup> ± 24	74 <sup>cd</sup> ± 6	69 <sup>cd</sup> ± 6

T0 = no Con A addition (Control); T1 = Con A addition; T2 = casein addition; T3 = Con A pre-incubated during 30 min with the enzyme before initiating the reaction; T4 = Con A pre-incubated during 30 min with the substrate before initiating the reaction. The values are expressed as means ± standard deviation ( $n = 18$ ).

Different letters between treatment and between ages in the same enzyme indicate significant differences ( $P < 0.05$ ).

**Table 4.** Effect of Concanavalin A (Con A) on phytase (nmoles phosphate/min/mg protein), alkaline phosphatase (nmoles p-nitrophenol/min/mg protein) and leucine aminopeptidase (nmoles p-nitroaniline/min/mg protein) activities in BBMV from 3- and 6-week-old broiler chickens

	T0	T1	T2
<i>Phytase</i>			
3 weeks old	27 <sup>a</sup> ± 5	20 <sup>bc</sup> ± 4	26 <sup>ab</sup> ± 5
6 weeks old	22 <sup>abc</sup> ± 4	20 <sup>c</sup> ± 4	24 <sup>abc</sup> ± 5
<i>Alkaline phosphatase</i>			
3 weeks old	224 <sup>a</sup> ± 26	226 <sup>a</sup> ± 21	191 <sup>ab</sup> ± 48
6 weeks old	149 <sup>b</sup> ± 21	142 <sup>bc</sup> ± 24	70 <sup>c</sup> ± 9
<i>Leucine aminopeptidase</i>			
3 weeks old	32 <sup>a</sup> ± 7	44 <sup>a</sup> ± 14	38 <sup>a</sup> ± 7
6 weeks old	18 <sup>b</sup> ± 2	18 <sup>b</sup> ± 3	16 <sup>b</sup> ± 3

T0 = no Con A addition (Control); T1 = Con A addition; T2 = casein addition. The values are expressed as mean ± standard deviation ( $n = 15$ ). Different letters between treatments and between ages in the same enzyme indicate significant differences ( $P < 0.05$ ).

a consequence of the frequency in the exposure of the enzyme to foods that contain lectins or of structural characteristics related to the glycoproteins. The alkaline phosphatase specific activity was lower ( $P < 0.05$ ) in the birds of 6-week-old BBMV in comparison with the 3-week-old BBMV. Similar results were reported by Iji *et al.* (2001). In the present work the casein diminished the activity of alkaline phosphatase in BBMV at 6 weeks old. The casein effect may have been due to the high degree of phosphorylation of this glycoprotein acting as an additional substrate in competition with the p-nitrophenylphosphate used as substrate. Con A had no effect on leucine aminopeptidase activity. Additionally, the casein did not affect the activity of this enzyme. In the 6-week-old BBMV leucine aminopeptidase specific activity was significantly lower ( $P < 0.05$ ) in comparison with 3-week-old BBMV. When studying the development of intestinal functions in broiler chickens receiving a commercial diet, Iji *et al.* (2001) reported that the activity of this enzyme diminished with age throughout the first 3 weeks of age.

In conclusion, Con A inhibited the activity of maltase and phytase in 3-week-old chicken duodenal BBMV and that of sucrase in 6-week-old chicken duodenal BBMV but had no effect on alkaline phosphatase and leucine aminopeptidase activities at either age. In general, maltase, alkaline phosphatase and leucine aminopeptidase activities decreased with bird age. These results confirm that Con A, the lectin present in raw jack bean seeds, may interfere with the digestive process in broiler chicks and may help to explain the poorer growth of poultry fed on diets containing raw jack bean.

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