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Recent studies on enzyme application in animal feeding

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SUMMARY - The addition of enzymes to poultry diets became established commercially during the 1980s. The inclusion of non-starch-polysaccharide-degrading enzymes (NSPDE) in monogastric diets has allowed a higher use of cereals different from maize -mainly barley and wheat, which have significant levels of antinutritive factors such as β-glucans and pentosans-. However, an accurate utilization of these enzymes requires the knowledge of the main activities present and their amounts in the preparations and in finished feeds, their stability, and the effects that these enzymes produce on productive and physiological parameters. Several trials have been conducted in our Department using some commercial enzymes in barley based diets for broilers chicks and laying hens. At the beginning, the studies were focused on the effect of non starch-polysaccharide-degrading-enzymes (NSPDE), specially β-glucanases, on performance of broiler chicks fed high levels of barley (growth, feed consumption, feed efficiency, water intake, etc.). Later, the studies were directed to study the use of enzymes in laying hens, where the effect of enzymes were less clear and more controversial. Finally, experiments were planned to elucidate the effect of high-viscosity diets and enzyme addition on some physiological parameters, such as digesta viscosity, rate of feed passage, endogenous digestive enzymes, survival of β-glucanases in the digestive tract and ileal nutrient digestibility. An overview of the more recent work conducted in the Department is presented. Additionally, analytical procedures to evaluate the activities present in the commercial enzymatic products, in feeds and their stability to technological processes and storage conditions have been developed. There are different methods to analyse the carbohydrase activities (β-glucanase, cellulase, xylanase, etc.) present in enzymatic products, such as viscommetry, radial gel diffusion, use of coloured azo-substrates or measurement with dinitrosalicilic acid (DNS) of reducing sugars released by the action of enzyme on pure substrates. The units of expression of activity of an enzymatic product can be very different, depending on the method used and the conditions of measurement, because pH, temperature and the substrate affect strongly the kinetics of the reaction, making the comparison between commercial products difficult. The determination of NSPDE activity present in finished feeds becomes more difficult due to some additional problems, such as the low level of enzyme incorporation in feeds and the complexity of the diets. The Azo-substrate method performed with a standard addition curve is the technique that shows most accurate results. The activity and stability of the enzymatic products used in feeds can be affected by different parameters: the origin of microorganism, the type of coating used in the commercial preparations, the temperature and conditions of pelleting of feed, the action of endogenous digestive enzymes, and the time and conditions of storage. The incidence of these parameters on the stability of NSPD enzymes are discussed.

Key words: Enzyme supplementation, barley, broilers, laying hens, analytical procedures.

RESUME *- "Etudes récentes sur l'application des enzymes dans l'alimentation animale". L'addition d'enzymes aux régimes avicoles a été établie commercialement pendant les années 1980. L'incorporation d'enzymes dégradant les polysaccharides non amylacés (NSPDE) dans les régimes pour monogastriques a permis une utilisation plus élevée de céréales autres que le maïs - principalement l'orge et le blé, qui ont des niveaux significatifs de facteurs antinutritionnels tels que les ß-glucanes et les pentosanes. Cependant, pour une utilisation exacte de ces enzymes il est nécessaire de connaître les principales activités présentes et leurs quantités dans les préparations et les produits finis, leur stabilité, et les effets qu'ont ces enzymes sur les paramètres productifs et physiologiques. Plusieurs essais ont été menés dans notre Département en utilisant des enzymes commerciales dans des régimes à base d'orge pour des poulets de chair et poules pondeuses. Au début, les études étaient axées sur l'effet des enzymes dégradant les polysaccharides non amylacés (NSPDE), spécialement les ß-glucanases, sur les performances des poulets de chair recevant de hauts niveaux d'orge (croissance, consommation d'aliment, efficacité alimentaire, ingestion d'eau, etc.). Plus tard, les études se sont orientées vers l'utilisation des enzymes chez les poules pondeuses, où l'effet des enzymes était moins clair et plus controversé. Finalement, des expériences ont été menées pour éclaircir l'effet de régimes à haute viscosité ainsi que l'addition d'enzymes sur certains paramètres physiologiques, tels que la viscosité des digesta, vitesse de transit des aliments, enzymes digestives endogènes, survie des ß-glucanases dans le tractus digestif et digestibilité iléale des nutriments. Les travaux les plus récents du Département sont passés en revue. En outre, des procédures analytiques ont été mises au*

point pour évaluer les activités présentes dans les produits enzymatiques commerciaux, dans les aliments ainsi que leur stabilité au cours des processus technologiques et selon les conditions de stockage. Il existe différentes méthodes pour analyser les activités des carbohydrases (ß-glucanase, cellulase, xylanase, etc.) présentes dans les produits enzymatiques, telles que la viscométrie, la diffusion radiale en gel, l'utilisation d'azo-substrats colorés ou les mesures avec de l'acide dinotrosalicylique (DNS) des sucres réduits libérés par l'action de l'enzyme sur les substrats purs. Les unités exprimant l'activité d'un produit enzymatique peuvent être très différentes, selon la méthode utilisée et les conditions de mesure, car le pH, la température et le substrat affectent fortement la cinétique de la réaction, en rendant ainsi difficile la comparaison entre produits commerciaux. La détermination de l'activité NSPDE présente dans les produits finis devient plus difficile en raison de quelques problèmes additionnels, tels que le faible niveau d'incorporation de l'enzyme dans les aliments et la complexité des régimes. La méthode de l'azo-substrat effectuée avec une courbe d'addition standard est la technique qui donne les résultats les plus exacts. L'activité et la stabilité des produits enzymatiques utilisés dans les aliments peut être affectée par divers paramètres : l'origine du microorganisme, le type de revêtement utilisé dans les préparations commerciales, la température et les conditions de granulation de l'aliment, l'action des enzymes digestives endogènes, le temps et les conditions de stockage. L'incidence de ces paramètres sur la stabilité des enzymes NSPD est analysée.

Mots-clés : *Supplémentation enzymatique, orge, poulets de chair, poules pondeuses, procédure analytique.*

Analytical methods to evaluate enzymes used in animal nutrition

The inclusion of non-starch-polysaccharide-degrading enzymes (NSPDE) in monogastric diets has allowed a higher use of cereals different from maize -mainly barley and wheat, which have significant levels of antinutritive factors as β-glucans and pentosans-. However, an accurate utilization of these enzymes requires the knowledge of the main activities present and their amounts in the products and in finished feeds. An evaluation of the effects of technological processes such as pelleting and storage conditions on the stability of these enzymes is also necessary.

Suitable methods which facilitate the detection and accurate quantitation of the NSP activities present in commercial products have been reported. Several methods have been used for the determination of β-glucanase activity:

(i) Viscommetric methods, in which the enzymatic activity is evaluated by the reduction of viscosity of a pure β-glucan solution over time (Wood and Weisz, 1987; Buckee *et al.,* 1988).

(ii) Radial Gel Diffusion. In these methods, β-glucan is incorporated into agar gel, and the enzyme diffuses from a solution placed in well cuts in the gel. Enzyme activity may then be assessed visually by the breakdown of a colored β-glucan-dye complex, resulting in decolorated halos surrounding the well. The bleached area is proportional to enzyme concentration (Edney *et al.,* 1986; Wood and Weisz, 1987).

(iii) Dye-labelling methods. The complex formed by precipitation of β-glucans from barley with Congo Red or Remazolbrilliant Blue may be used as an insoluble substrate to assay β-glucanase activity. These substrates remain essentially insoluble in the absence of enzyme, but in the presence of enzyme, they release soluble dyed products linearly over time. The rate of release of dye is proportional to enzyme activity (McCleary and Shameer, 1987; Wood and Jorgensen, 1988).

(iv) Measurement of the rate of increase of reducing sugar equivalents. The enzyme activity is evaluated by measuring the reducing sugars liberated in defined conditions of pH and temperature, with some reagents such as DNS.

Although there are several methods to analyze NSPDE activities, the comparison of commercial products according the activities described in them is very difficult for several reasons:

(i) The activity is defined according to the method of analysis used. The viscommetric methods, those which use the measurement of reducing sugars with DNS or those which use colored substrates show different definitions of unit of activity. These activity units are not equivalent.

(ii) For a specified technique, for example, quantitation of xylanase by measurement with DNS of reducing sugars released from a pure xylan, several substrates have been used: oat spelt xylan,

birchwood xylan and larchwood xylan, which could present different reaction kinetics with the enzyme.

(iii) Sometimes, one method (for example, measurement of reducing sugars with DNS) has been described with different assay conditions: temperatures ranging from 30 to 50ºC, reaction times from 5 to 10 minutes, wavelengths from 490 to 540, which influence in great manner the final activity units measured.

Some additional problems have to be taken into account when the NSPDE activities have to be measured in feeds. Enzymes are normally incorporated into feeds at low levels of up to one kilo of enzyme per tone of feed. The subsequent extraction and preparation for assay may result in the effective dilution of the enzyme concentrate by a factor greater than one in one million (Headon, 1993). Then the method used for its analysis has to be sensitive enough.

Additionally, there are many substances present naturally in feed which interfere in the determination. Reducing sugars produced by the action of carbohydrase enzymes may be conveniently estimated by reaction with DNS. However, all feedstuffs naturally contain very high levels of such reducing sugars. The detection of any additional reducing sugar produced by the enzyme assay is rendered impractical due to the high background values. Nevertheless, these reducing sugars which contribute to high background values are of low molecular weight, and may be removed by gel filtration chromatography using Sephadex G-25 (Headon, 1993). Molecules of high molecular weight such as enzymes are eluted from the column quickly while mono- and oligosaccharides are more retained on the column.

In addition to the presence of enzyme, the feed also contains considerable amounts of enzyme substrate which could interfere during analysis, mainly if a synthetic substrate is used to assay the activity, because significant quantities of the native substrate may be present and may compete with the synthetic substrate for catalytic reaction. Enzyme activity as determined with the synthetic substrate may differ from that detected with the native substrate. Such difficulties can be overcome by including a standard curve in the determination of enzyme activity from different feeds, performed by incorporating different levels of enzyme in a control feed sample without exogenous enzyme.

This procedure allows the study of the effects of some factors on the activity and stability of enzyme preparations, such as origin and type of enzyme, coating used to protect it, composition of the diet, pelleting and storage conditions, and action of endogenous enzymes.

Figure 1 shows the standard curves obtained by including different concentrations of three β glucanases (from three different sources) to one single barley diet. The slopes of the standard curves of each enzyme are not comparable, due to each enzyme shows a specific kinetics in its reaction with the synthetic substrate. For this reason when this method is used to determine NSPDE activities the access to the enzyme preparation included in feeds is necessary. When one enzyme preparation is added to some diets differing in their composition the curves obtained not present such big differences.

The type of coating used to protect the enzyme has great influence in its stability when feed is pelleted. This fact can be observed in the data presented in Fig. 2. Six xylanase preparations differing only in their coating were incorporated to one single wheat diet. The diets were pelleted at two temperatures: 60ºC and 80ºC. The recoveries of the enzymes were very different ranging from 58 to 102% at 60ºC and from 6 to 42% at 80ºC. Enzyme E-2 was the most stable while coatings of E-3 and E-6 were the less effective in the protection of enzyme and these results were independent of pelleting temperature.

During the pelleting process, the highest reduction of enzymatic activity has been observed when the feed is in the conditioning chamber, as can be seen in Fig. 3. In this figure the β-glucanase recoveries obtained when a barley diet was pelleted at 65ºC or at 80ºC and some samples of feed were taken from different points over the pelleting line are represented. Minor reductions of activity have been detected when feed pass through the dye.

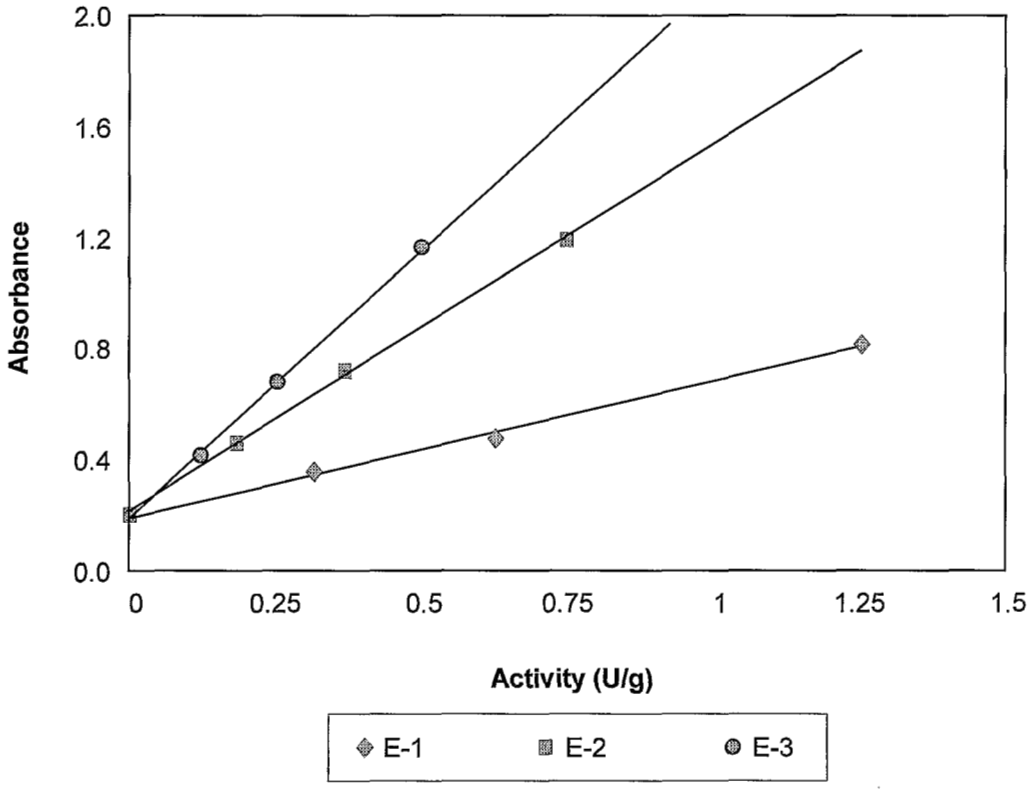

Fig. 1. Effect of enzyme origin on β-glucanase activity measured by Azo-barley glucan substrate method.

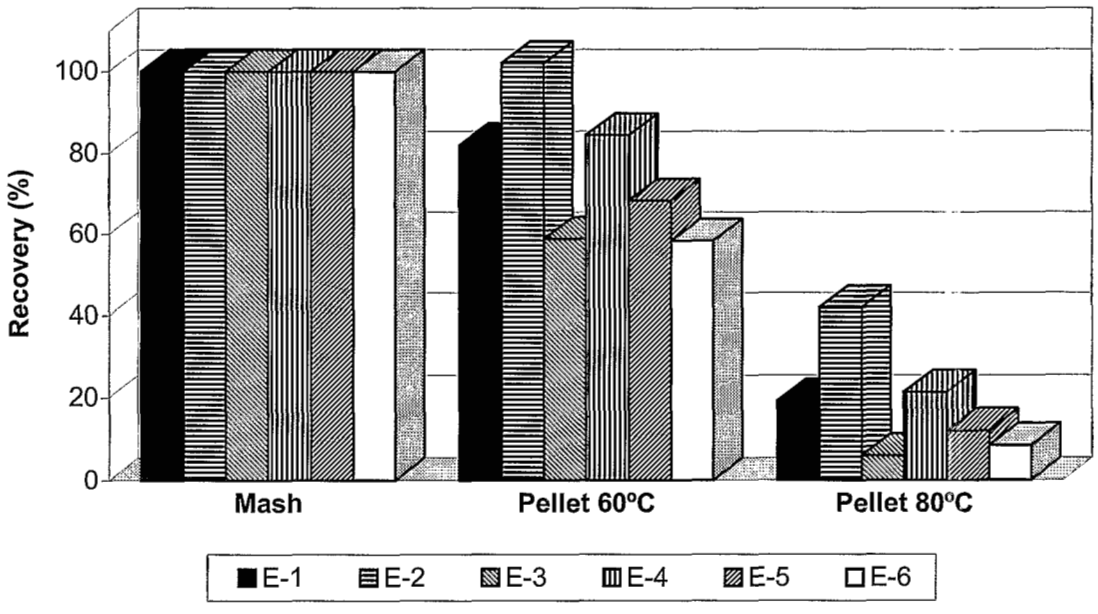

Fig. 2. Effect of type of coating on the stability of xylanase incorporated in pelleted feeds.

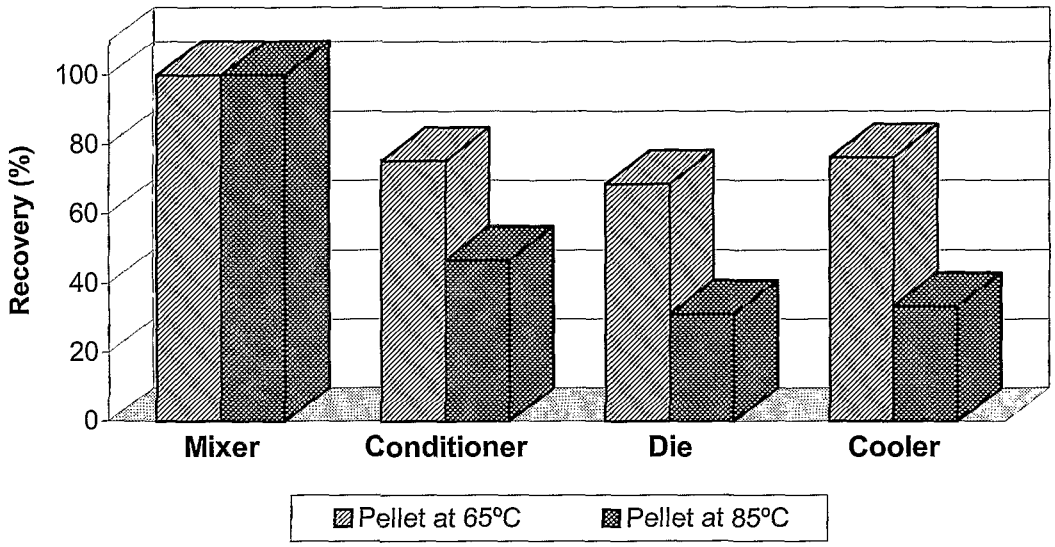

Fig. 3. Termostability of β-glucanase to pelleting process.

In order to study the effect of storage conditions on the activity of enzymes, a β-glucanase preparation was included in broiler and pig diets, which were stored for one month at different temperatures: -18°C, 4°C, 20°C, 30°C and 40°C. The recoveries of enzyme are shown in Fig. 4. A significant reduction of activity is detected when feeds were stored above 30°C.

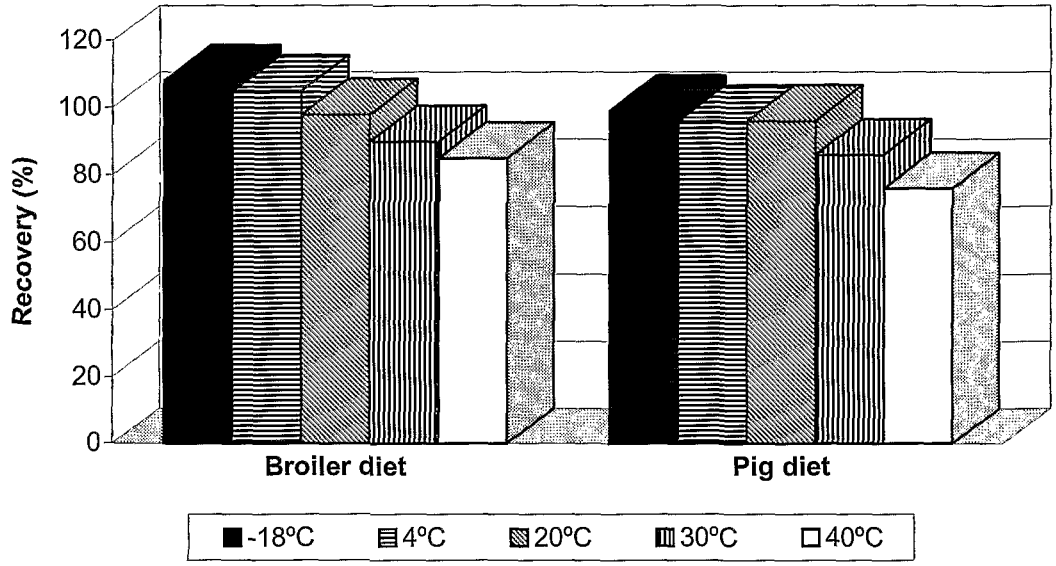

Fig. 4. Effect of storage conditions on the β-glucanase activity present in mash feeds. (Cos, 1995; unpublished data)

When enzymes are fed to poultry, once inside the gastrointestinal tract, the enzymes have to withstand conditions which can cause inactivation, such as an acidic pH of the stomach in the presence of pepsin, and further proteolitic attack from pancreatic proteases. The effects of gastrointestinal conditions on the stability of a β-glucanase preparation were studied *in vitro* (Almirall and Esteve-García, 1995). The results obtained in this experiment are presented in Table 1. The

β-glucanase activity determined after 30 minutes of incubation at 40°C with pepsin at pH 3.2 or with pancreatin at pH 7, was respectively 96% and 27% of the activity measured initially. These results indicate that the β-glucanase enzyme should survive, at least, after passing the proventriculus.

There are different factors that could greatly affect the activity and stability of NSPDE and their effects could be evaluated with analytical techniques based on the use of specific dye-labeled substrates, which included a standard curve obtained by incorporating different concentrations of the enzyme in a control sample of the feed to be analyzed. These techniques are sensitive and selective to allow the detection and quantitation of such enzymes at low levels.

Table 1. *In vitro* initial and residual β-glucanase activity after incubation at 40°C for 30 minutes at pH 3.2 with pepsin, or at pH 7 with pancreatin† (Almirall and Esteve-García, 1994)

Treatment	β-glucanase activity	
	U/ml	Relative activity (%)
Initial at pH 5	4081 + 147[a]	100
Pepsin at pH 3.2	3946 + 182[a]	96
Pancreatin at pH 7	1290 + 65[b]	27

†Means not sharing a letter in a column are significantly different at P<0.05

Effects of non-starch polysaccharide-degrading enzymes (NSPDE) on productive and physiological parameters

Young and adult birds responds differently to the antinutritive factors present in barley

It is generally accepted that adult bird are less affected by the presence of high amount of barley in the diet. For this reason a series of trials were planed to determine whether viscosity caused by mixed linked barley β-glucan depresses ileal nutrient digestibility and digestive enzyme activities and to determine the interaction of intestinal viscosity, digestive enzyme activities and ileal nutrient digestibility of poultry in different ages (Almirall *et al.,* 1995). Moreover, to determine the effect of barley diets on rate of feed passage at different ages and the effect of β-glucanase supplementation (Almirall and Esteve-García, 1994).

One-day broiler chicks and one-year-old cocks were fed diets containing 60% corn, low and high viscosity barley (7.2 and 13.3 cps, respectively) with or without β-glucanase addition, for 3 weeks. Both types of barley increased intestinal viscosity in poultry of both ages, and their values were closely related to the viscosity of the barley used. However, viscosity values were lower in mature chickens than in young birds (Fig. 5). β-glucanase addition reduced digesta viscosity significantly in both type of birds.

Barley viscosity affected in different way the rate of feed passage of broiler chicks and adult cocks. In broiler chicks, the maximum excretion of marquer occurred in the 4th hour, whereas in cocks occurred 1 hour before (Fig. 6A and B). When β-glucanase was added, the age-related difference disappeared and rates become similar. Moreover, in broilers chicks T50 (time of 50% chromium excretion) and T1 (time of 1% chromium excretion) decreased with β-glucanase addition, whereas in cocks increased. This suggest that older birds are capable of transporting viscous materials more easily than younger birds.

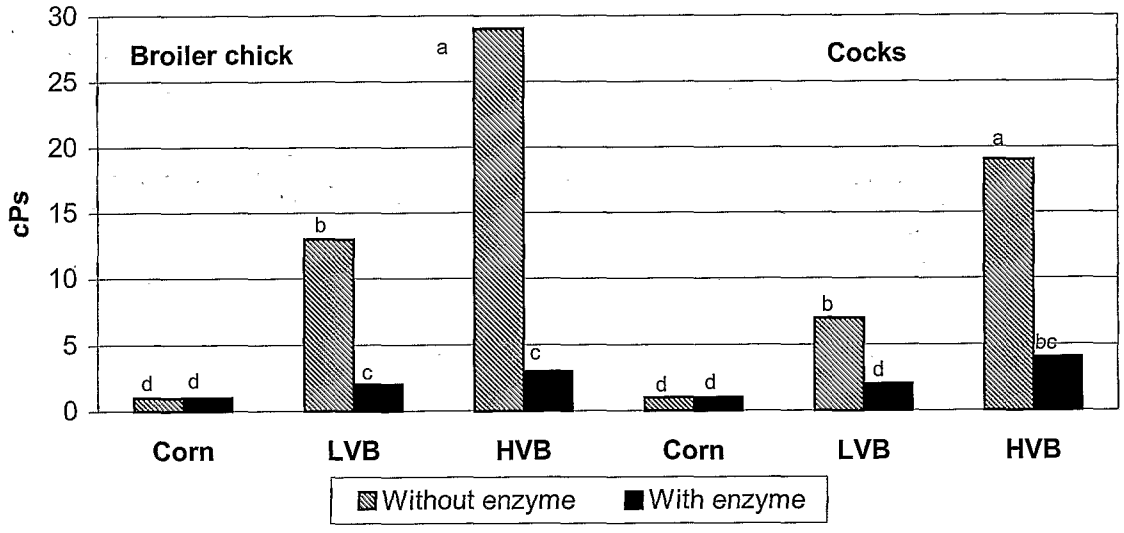

Fig. 5. Digesta viscosity along the digestive tract of broilers fed corn, low-viscosity (LVB) and high-viscosity barley (HVB) diets with or without β-glucanase addition. (Almirall *et al.*, 1995)

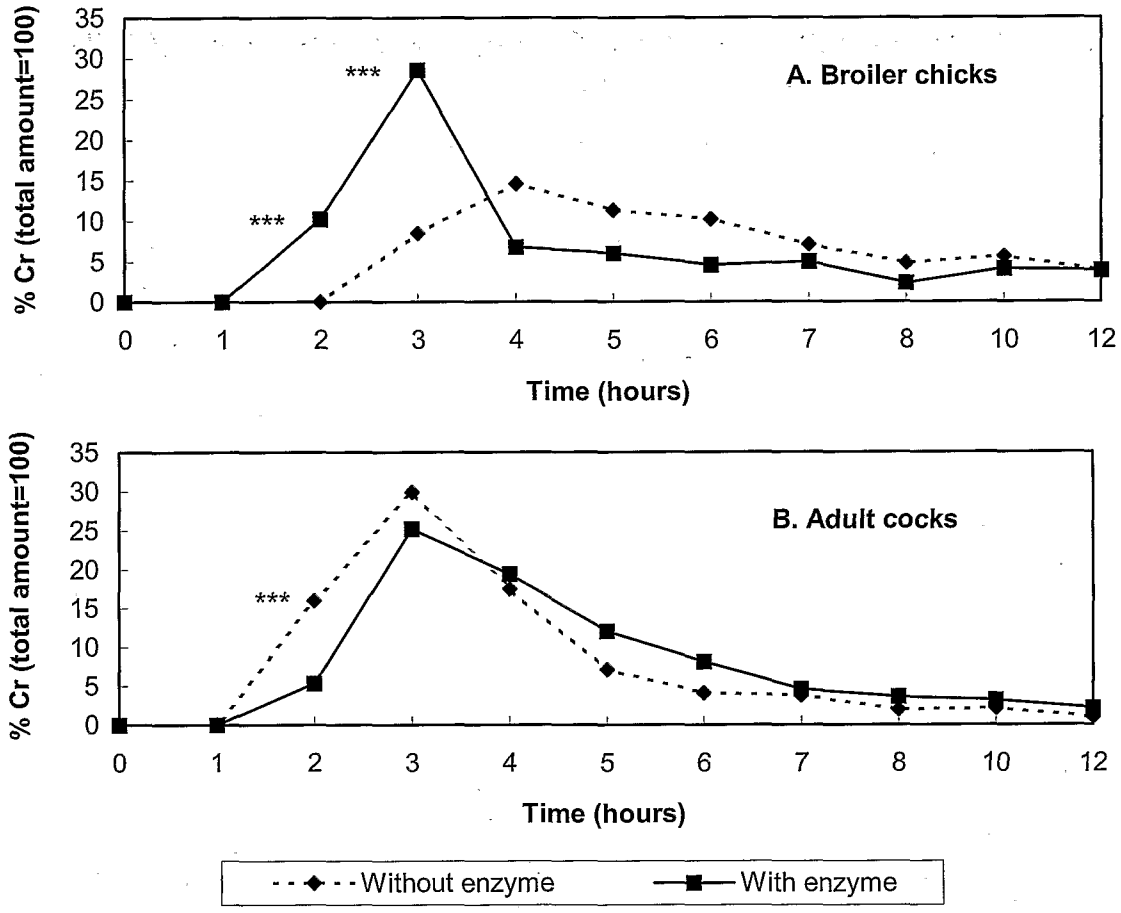

Fig. 6. Non-cumulative marker excretion in broiler chicks (A) and adult cocks (B) fed barley diet and barley with β-glucanase. Asterisks indicate significant difference ($P<0.01$). (Almirall and Esteve-García, 1994).

The substitution of corn by barley in the diet affected endogenous enzymes activities, specially in broiler chicks and in a to lesser extend in adult cocks. Amylase and lipase activities in the digesta of broiler chicks were significantly lower when they were fed high viscosity barley than corn, while β-glucanase increased both activities and that of trypsin as well (Table 2). In cocks, only the lipase activity was reduced significantly with the use of high viscosity barley, while β-glucanse increased it.

Table 2. Digestive enzyme activities in small intestinal contents of broiler chicks and cocks fed corn or barley diets with or without β-glucanase[1] (Almirall *et al.*, 1995)

Diet	Amylase $Ux10^{-4}/g$	Trypsin $Ux10^{-2}/g$	Lipase $Ux10^{-3}/g$
Broiler chick at 21 d			
Corn	22.7^{a}	8.5^{b}	39.5^{a}
HV barley	15.7^{b}	9.8^{ab}	24.9^{c}
HV barley + β-glucanase	24.2^{a}	12.5^{a}	31.6^{b}
Pooled SEM	1.55	1.03	1.59
Cocks			
Corn	24.6	11.5	14.0^{a}
HV barley	27.9	15.1	7.8^{b}
HV barley + β-glucanase	27.8	12.7	10.9^{ab}
Pooled SEM	1.32	1.66	1.46

[1]Means not sharing a letter in a column within type of poultry are significantly different at P<0.05
HV: High viscosity barley

In broiler, protein and lipid ileal digestibilities of the barley diet were lower than of the corn diet, while no significant differences in cocks were observed. Ileal starch digestibility was not affected by barley nor β-glucanase addition in both type of animals. Enzyme addition increased protein and lipid digestibilities in broilers and it did not show any effect in adult birds (Table 3).

Table 3. Ileal nutrient digestibilities in broiler chicks and cocks fed corn or barley diets with or without β-glucanase[1] (Almirall *et al.*, 1995)

Diet	Crude protein	Crude fat	Sartch
	g/100 g dry wt		
Broiler chick at 21 d			
Corn	87.0^{a}	83.8^{a}	95.7
Corn + β-glucanase	86.5^{a}	82.4^{a}	94.8
HV barley	69.4^{c}	76.4^{c}	94.6
HV barley + β-glucanase	80.9^{b}	78.8^{b}	96.5
Cocks			
Corn	82.5	78.5	97.6
Corn + β-glucanase	82.2	79.8	97.2
HV barley	77.9	75.2	96.4
HV barley + β-glucanase	80.2	73.8	97.3

[1]Means not sharing a letter in a column within type of poultry are significantly different at P<0.05
HV: High viscosity barley

Stability of the β-glucanase along the digestive tract of the chickens

In order to obtain more information about the site where β-glucanases act in the digestive tract, broilers chickens were fed a barley diet (60%) with increasing levels (0, 40 and 100 ppm) of an enzyme preparation containing 70.667 β-glucanase units/g. At 21 days birds were killed and gut samples from different sections were taken to measure the β-glucanase activity (values are presented in Fig. 7). A reduction of activity in the crop and proventriculus in comparison to the β-glucanase activity present in the feed was observed, followed by an increase of enzyme present in the duodenum. The β-glucanase activity in the ileum decreased from the proximal to distal part and it almost disappeared at the end of the small intestine. It seems that the principal site of action of the enzyme added is in the proximal part of small intestine. In the ceca, a great increase of β-glucanase activity was observed even without enzyme addition. This probably has to be attributed to the microbes present in the ceca.

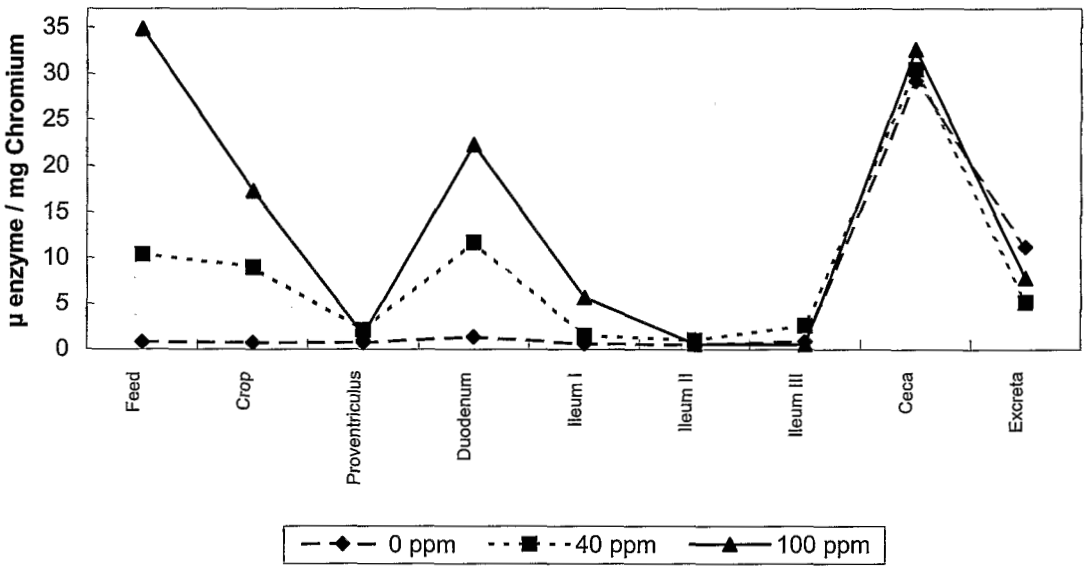

Fig. 7. β-glucanase activity along the digestive tract of broilers fed barley diet with or without β-glucanase (expressed as a µg enzyme/mg Chromium in digesta).

Effect of enzyme supplementation on laying hen performance

Feeding high levels of barley to laying hens may cause problems of wet litter, high water consumption and dirty eggs. These problems could be magnified further enhanced using considerable amounts of sunflower meal in diet, which contains high levels of pectins from cell wall residues. It is widely accepted that older birds are less sensitive to the deleterious effects of β-glucans from barley and that the response to enzyme addition is less important in laying hens than in broilers chickens. In spite of that, results from recent experiments at IRTA have shown that egg weight during the early laying period is improved by enzyme addition to a barley-soybean or barley-sunflower meal based diets (Table 4) (Brufau *et al.,* 1994; Francesch *et al.,* 1995a; Francesch *et al.,* 1995b). In addition, enzyme supplementation increased the percentage of eggs heavier than 60 g in the early stages of lay, when small egg size in young laying hens is an important economic problem.

In an other experiment, the effect of increasing levels (0, 80 and 160 ppm) of an enzyme complex on productive parameters, egg quality and water consumption of laying hens fed with two type of diets was evaluated from 21 to 52 weeks of age. The diets were: a normal energy diet (2750 kcal/kg) containing barley (57%) and soybean meal, and a low energy diet containing barley (42%), soybean meal, peas (18%) and sunflower meal (14%). Enzyme improved significantly feed efficiency and reduced dirty eggs in the overall experiment (Table 5) (Francesch *et al.,* 1995b).

Table 4. Effect of enzyme addition in laying hen diets on egg weight and commercial grading of eggs[†]

Enzyme	Egg weight (g)	Egg grading (% egg >60 g)
Experiment 1[††]	(26-29 wk)	(29 wk)
0 (g)	55.3[a]	17.0[b]
0.5 (g)	55.4[a]	16.8[b]
0.75 (g)	55.8[a]	20.7[ab]
1 (g)	56.9[b]	27.0[a]
Experiment 2[†††]	(21-32 wk)	(28 wk)
0 (ppm)	58.8[a]	50.8
80 (ppm)	59.6[b]	55.3
160 (ppm)	59.6[b]	54.3
Experiment 3[††††]	(31-34 wk)	(21-37 wk)
0 (ppm)	60.2	44.7
100 (ppm)	60.2	47.3
200 (ppm)	61.7	49.9

[†]Means not sharing a letter in a column within trial are significantly different at P<0.05
[††](Francesch *et al.*, 1995a) barley + sunflower diet
[†††](Francesch *et al.*, 1995b) barley + sunflower + peas diet
[††††](Brufau *et al.*, 1994) barley diet

Table 5. Effect of enzyme supplementation on laying hen performance fed a barley-soybean or barley-sunflower-peas diet (from 21 to 52 weeks), (Francesch *et al.*, 1995b)

Diet[†]	Dose enzyme (ppm)	Feed efficiency (g feed/g egg)	Feed intake (g/day)	Egg laying (%)	Egg weight (g)	Dirty eggs (%)
A	0	2.072	114.1	88.3	62.4	4.0
A	80	2.043	111.7	88.5	61.8	3.6
A	160	2.033	113.0	89.0	62.5	3.4
B	0	2.182	117.8	87.5	61.8	7.7
B	80	2.143	117.8	88.2	62.3	6.0
B	160	2.141	119.1	89.3	62.3	6.0
Factorial analysis						
Diet		**	**	NS	NS	**
Enzyme		*	NS	NS	NS	*
Interaction		NS	NS	NS	NS	NS

[†]Diet A: 57% barley; Diet B: 42% barley + 14% sunflower + 18% peas. Values are means of four replicates of 75 laying hens
NS: Non significant; *P<0.05; **P<0.01

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