Anexo.

UTILIZACIÓN DE ARCILLAS EN LA ALIMENTACIÓN DE LAS AVES: ASPECTOS NUTRICIONALES

Las arcillas son componentes estrructurales de la tierra que incluyen, entre otros la caolinita, las bentonitas, las zeolitas y la sepiolita, cuya clasificación físicoquímica figura en las revisiones de Taylor (1998) y Castaing (1998). Sus usos en la alimentación animal se encuentran cada vez más extendidos debido a propiedades tecnológicas como lubricantes para mejorar el rendimiento de las granuladoras, o aglomerantes para mejorar la dureza del granulo. Junto a estas propiedades, en los últimos años se han ido añadiendo posibles efectos positivos de la incorporación de estas arcillas sobre la digestión y asimilación de los nutrientes por las aves (Evans y Ferrell, 1993; Melcion, 1995; Olver, 1997).

1. PROPIEDADES DE LA SEPIOLITA COMO AGLOMERANTE

Según Alvarez (1984), la sepiolita es una arcilla ($SI_{12}Mg_8O_{30}(OH)_4(H_2O)_4 8H_2O$) de estructura tabular, caracterizada por una estructura porosa (Wolter y col., 1990) y una superficie específica de 633 m^2/g, con una afinidad a la absorción de líquidos (1 a 1.5 su peso) y de intercambio iónico (10 a 20 meq/g). La capacidad de adsorber líquidos permite a la sepiolita ser incorporada sobre todo en dietas elevadas en grasas destinadas a alimentar animales de engorde (Lopèz y Alvarez, 1991; Castaing, 1994; Angulo y col., 1995, 1996).

1.1. Aspectos tecnológicos

El uso de aglomerantes en alimentación animal tiene por objetivo aumentar la cohesión de los gránulos (dureza), característica que dependen de: i/ el nivel de inclusión de cada uno de los ingredientes (Wood, 1987; Angulo y col., 1996) sobre todo materia grasa y fibra, ii/ el tamaño

del pellet a conseguir (Reese y col., 1986; Angulo y col., 1996), y iii/ el nivel de inclusión de aglomerante (Bobohm, 1992; Gill, 1993).

Según Angulo y col. (1995), la aglomeración con 2% de sepiolita reduce la variabilidad de la dureza de los gránulos en un 19.6% en formulas cuyos contenidos de grasa añadida varia de 5 a 40g/kg de pienso.

1.2. Aspectos zootecnicos

Debido a los altos niveles de energía necesarios para alcanzar los requerimientos de los broilers, los fabricantes de alimentos recurren a grasas añadidas que requieren la incorporación tecnológica de aglomerantes en la mayoría de los casos. Desde la década de los 70, diversos silicatos han sido objeto de pruebas en la tecnología de fabricación de piensos. De las arcillas estudiadas figuran las zeolitas naturales y sintéticas (Willis y col., 1982; Roland y col., 1985; Houssain y col., 1994; Cornejo y col., 1995; Yalcin y col., 1995), la bentonita (Miles y col.,1986), y más recientemente la sepiolita (Tortuero, 1983; Alvarez y Peréz, 1982; Wang y col., 1989; Castaing, 1989; Tortuero y col., 1992; Fernandez y col., 1994; Schutte y Langhout, 1998; Parisini y col., 1999; Ouhida y col., 2000a,b). En general, junto a la mejora de manejo del alimento granulado, algunos trabajos de investigación presentan mejoras en los resultados productivos de los animales, en algunos casos debidos a mayor ingestión del alimento (Scheilder, 1992; Capdevila, 1993) y en otros a mejoras en la eficiencia de conversión de la dieta (Resultados presentados en la Tabla I.22).

1.2.1. Parámetros productivos y calidad de la canal de los broilers

En broilers, la dilución de la dieta con 1 ó 2% de sepiolita ha dado lugar a resultados de crecimiento e índices de conversión atractivos. Según Tortuero y col. (1992), la inclusión de 1.5% de sepiolita en la dieta no provocó modificación significativa ni de los crecimientos ni de los índices de conversión de dietas base de maíz-soja lo que significa, dada la carencia de valor nutritivo de la arcilla “per se”, una ganancia no despreciable de eficiencia en la utilización de la materia organica ingerida. En trabajos más recientes, con aves (Fernandez y

col., 1994) y cerdos (Parisini y col., 1999) se sugiere la existencia del mismo efecto con la incorporación de un nivel más elevado (2%). En pavos criados hasta 16 semanas, Castaing (1995) observó que la suplementación de la dieta con 2% de sepiolita se asoció a pesos finales más elevados e índices de conversión más reducidos.

Junto a posibles mejoras en la eficiencia digestiva y metabolica de utilización de la MO de la dieta, otros autores destacan otros efectos como puede ser una reducción de la humedad de las heces (Schutte y Langhout, 1998), una mayor absorción de los minerales (Tortuero y col., 1993; Houssain y col., 1994; Yelcin y col., 1995) y un perfil de grasa/muslo más apropiado (Cornejo y col., 1995). Esta última hipótesis no ha sido confirmada con sepiolita añadida en dietas de pollos. Sin embargo fué observada en un estudio realizado por Cornejo y col. (1995), donde observaron una mejora significativa de la proporción de grasa del pollo con un 2% zeolita en la dieta. En pavos y según Castaing (1995), la dilución de la ración con un 2% de sepiolita mejora el peso final de la canal (+2.6%), con menos proporción de grasa en la carne.

Tabla 1.22. *Efecto de la dilución de la dieta con arcillas sobre los resultados productivos de broilers (ganancia de peso vivo GMD e índices de conversión IC)*

Arcilla	*Aplicación g/ kg pienso*	*Dieta*	*Edad (semana)*	*Porcentaje de GPV*	*Porcentaje de IC*	*Referencia*
Zeolita	10	*Maíz*	0-3	-0.8	-2.8	Willis y col. (1982)
			0-7	1.6	-1.1	
Sepiolita	15	*Maíz*	0-6	0.8	7.3	Tortuero y col. (1992)
Sepiolita	20	*Trigo*	0-2	2	0	Schutte y col. (1995, no publicados)
			0-3	1.7	0	
Sepiolita	20	*Trigo*	0-2	-2.1	-0.6	Schutte y Langhout (1998)
			0-5	0.6	-0.2	
Sepiolita	20	*Trigo*	0-3	0.8	2.6	Magnin (1996, no publicados)
			0-6	0.7	0.1	Ouhida y col. (2000a)
Sepiolita	20	*Maiz*	0-3	0.1	-0.6	Ouhida y col. (2000b)
		Trigo/cebada	0-3	0.7	1.3	
Sepiolita	20	*Trigo/cebada*	0-3	0.6	-2.7	

1.2.2. Parámetros de puesta y calidad del huevo en gallinas ponedoras

En gallinas ponedoras, algunos trabajos muestran que la dilución de la dieta con arcillas (Sepiolita: Tortuero y col., 1993; Roland y col., 1989) no afecta los parámetros de producción. Este resultado refleja que la inclusión de silicato de aluminio mejora la utilización de la materia organica de la dieta. Por su parte, Olver (1997) observó que la inclusión de 5% de clinoptelita mejora en diferentes estirpes de ponedoras de forma significativa la tasa de puesta ($P<0.05$) y tiende a mejorar la humedad de heces.

En cuanto a la calidad de composición de los huevos, Tortuero y col. (1993) no observaron modificación alguna con 1.5% de sepiolita en la dieta, salvo una mejora en el deposito de calcio en la cascarilla.

1.2.3. Digestibilidad de los nutrientes y viscosidad de la digesta

Los resultados de digestibilidad son tambien variables y a veces contradictorios. Gonzalez y col. (1996) citaron que la incorporación de un 5% de zeolita en dietas destinadas a broilers aumentó en 6 u.p. la digestibilidad aparente de la materia seca (82 vs 88%) y en 6 u.p. la retención de nitrógeno (77 vs 81%). Sin embargo, Monetti y col. (1996) observaron un descenso en la digestibilidad de la materia seca (83.9 vs 82.7%) en cerdos alimentados con una dieta diluida con 2% de zeolita que fue sin embargo parcialmente compensada por una tendencia a la mejora de retención de nitrógeno (N. retenido/ingerido = 0.77 vs 0.82 $g/kg^{0.75}$). Una mejora significativa en la retención de nitrogeno (+5.3%) y de energia (+6.1) ha sido descrita recientemente por Parisini y col. (1999) en cerdos de 12 a 35 kg de peso vivo alimentados con una dieta comercial diluida con 2% de sepiolita.

2. MECANISMOS DIGESTIVOS ATRIBUIDOS AL USO DE LAS ARCILLAS

Debido a la elevada capacidad de retener agua, algunos autores suponen que la inclusión de arcillas en el pienso puede provocar las siguientes modificaciones, i/ puede reducir la viscosidad de la digesta intestinal en animales monogástricos alimentados con dietas de altos niveles en PNAs (Schutte y Langhout, 1998), ii/ alargar el tiempo de tránsito en el tracto digestivo (Mayer, 1980; Tortuero y col., 1992; Evans y Ferrell, 1993;Van Der Klis y Van Woorst, 1993), iii/ limitar el desarrollo de la flora microbiana en el tracto digestivo (Schutte y Langhout, 1998; Ouhida y col., 2000b), y proteger la mucosa gástrica e intestinal, previniendo diarreas (Castaing, 1998).

La primera hipótesis ha sido poca descrita, y entre ellos Schutte y Langhout (1998) encontrarón una tendencia a la reducción de la viscosidad ileal y un incremento en la relación consumo de agua: alimento (Tabla I.23) tras diluir una dieta base de trigo (35%) con 2% de sepiolita.

Tabla I.23. *Efectos de la inclusión de 2% de sepiolita en la dieta de broilers sobre la viscosidad ileal y la relación de consumo de agua:consumo de alimento*

Tratamiento	*Viscosidad ileal (cPs)*	*Relación consumo agua /alimento (g/g)*
Control	3.13	2.08
Control + 2% sepiolita	2.64	2.03
P<	0.07	0.07

cPs : centipoises
Fuente: Schutte y Langhout (1998)

En cuanto a las modificaciones del tránsito intestinal de la digesta, la mayoría de los trabajos realizados muestran una prolongación del mismo. La suplementación con un 1.5% de sepiolita en substitución del maíz en una dieta de broilers, conllevo la prolongación de 2 a 3h del tiempo medio de retención de la digesta (Tortuero y col., 1992). Los mismos resultados han sido observados por Wiseman (resultados no publicados) con 2% de sepiolita en una dieta base de trigo (60%) y por Evans y Ferrell (1993) con 3% de zeolita en una dieta de ponedoras.

Por otro lado, algunos estudios in vitro (Cabezas y col., 1991) sugieren que las arcillas pueden adsorber los enzimas pancreaticos formando complejos activos a un rango muy amplio de pH.

En este estudio se ha observado que, aun siendo menos activos, los complejos resultantes (enzima-sepiolita) pueden mejorar la hidrólisis de los componentes de la dieta, debido a su mayor independencia del pH intestinal.

Desde el punto de vista del sistema imunitario es importante considerar la interacción de los silicatos con la flora y toxinas presentes en el tracto gastro-intestinal. Se especula que el poder adsorbente de los silicatos puede permitir modificar el equilibrio de la flora intestinal (Schutte y Langhut, 1998) y tal vez secuestrar toxinas presentes en el pienso y digesta, sobre todo de las micotoxinas (Philips y col., 1988; Ramos y col., 1996). En ensayos prácticos (Schutte y Langhout, 1998), la inclusión de 2% de sepiolita en una dieta base de trigo (50%) provocó una reducción en la concentración en ileon de ácidos grasos volátiles, utilizados como indicadores de la proliferación microbiana en los últimos tramos del tracto digestivo. Yen y Pond (1990) sugieren que la dilución con arcillas puede tener los mismos efectos que suplementación de la dieta con antibióticos.

En el mismo sentido, se ha propuesto que las arcillas incluidas en la dieta del porcino pueden reducir la producción de amoniaco (NH_3) en la digesta, y por tanto la irritación del epitelio intestinal (Poulsen y Oksbjerg, 1995). Así, Bernal y Lopez-Real (1993) cuantificando la capacidad de retención de amoniaco, sugieren que 100 g de clinoptelita son capaces de retener 135meq (NH_4^+) un equivalente de 1.89 g de nitrógeno. Del mismo modo, Bueno y col. (1984) estimaron que 1g de sepiolita retiene 50 mg de NH_3. Como resultado inmediato, Poulsen y Oksbjerg (1995) sugieren que la inclusión de arcillas en los piensos ingeridos por el ganado porcino aumenta la excreción de nitrógeno en las heces y la reduce a nivel urinario. Tanto Shurson y col. (1984) como Cahn y col. (1984) mostrarón en el ganado porcino una reducción del 7% en la emisión de nitrógeno en la orina como efecto de la dilución de la dieta con 2% sepiolita.

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OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

El presente trabajo experimental tiene por objetivo general estudiar estrategias de tipo enzimático para mejorar la utilización de la dieta por parte de los pollos en crecimiento. En especial atenderemos a los grandes grupos de ingredientes i/ los cereales y ii/ las leguminosas. En este marco general planteamos la realización de diferentes experimentos "*in vivo*" e "*in vitro*" con objetivos particulares que a continuación se describen.

En un primer objetivo se estudiará la efectividad de los enzimas y la sepiolita como aditivos mejorantes de dietas con altos niveles de cereales. Junto a la determinación del valor añadido de ambos aditivos, se prestará un especial interés al estudió de los mecanismos de actuación individuales o de ambos suplementados de forma simultanea. En particular, se estudiará el efecto de la viscosidad de la digesta intestinal y la cinética de transito digestivo sobre la digestibilidad de los nutrientes.

Contrastadas las dimensiones de las mejoras que pueden alcanzarse mediante la suplementación con enzimas, nos planteamos en un segundo bloque de objetivos abordar el estudio del alcance que dichas mejoras podrían tener en el caso las leguminosas. En este objetivo planteamos el desarrollo de las siguientes etapas; i/ caracterizar con detalle los principales carbohidratos que forman parte de la composición de las leguminosas en general y la torta de soja en particular, ii/ valorar la accesibilidad "*in vitro"* de diferentes enzimas carbohidrasas y proteasa sobre la fracción de carbohidratos y proteína de las leguminosas y iii/ valorar "*in vivo*" los complejos más efectivos "*in vitro"*.

Con detalle, la consecución de estos objetivos se planteó en tres etapas experimentales:

- En la primera de ellas se caracterizarán los polisacáridos de la torta de soja (no-tostada vs tostada), siguiendo técnicas de fraccionamiento y analíticas rutinarias. En concreto se determinará su composición en monosacáridos.

- En una segunda etapa, se realizará una amplia batería de incubaciones *in vitro* con enzimas individuales o combinaciones de las mismas. Se estudiará la actividad enzimática sobre las estructuras originales, pero también sobre diferentes fracciones aisladas de la torta de soja. Mediante esta estrategia pretendemos valorar la efectividad intrínseca de los enzimas, así

como las limitaciones establecidas por la accesibilidad estructural del substrato. La actividad especifica de un determinado complejo enzimático se evaluará mediante la determinación del porcentaje de liberación de monosacáridos y aminoácidos (en relación al total presente en cada fracción).

Una vez seleccionados los complejos enzimáticos efectivos "*in vitro"*, abordaremos la realización de un experimento "*in vivo"* con el que valorar las posibilidades de mejora del valor nutritivo de la torta de soja en broilers en crecimiento. En particular, se estudiarán los efectos sobre los resultados productivos de los animales y la digestibilidad de los nutrientes. Así mismo se valorará una posible reducción de la proliferación microbiana en los tramos del intestino delgado.

CAPITULO 2

Enzymes (β-glucanase and arabinoxylanase) and/or sepiolite supplementation and the nutritive value of maize-barley-wheat based diets for broiler chickens

Abstract

1. Two experiments were conducted to study the effects of crude enzyme preparations (β-glucanase and arabinoxylanase) and/ or 20g/kg Sepiolite (Exal®) on the performance and nutrient digestibility of broiler chickens fed maize-barley-wheat based diets.

2. In experiment one, enzymes improved daily bodyweight gain (14%, $p<0.001$) and feed:gain ratios (8%, $P<0.001$). Sepiolite improved bodyweight gain with the diets not supplemented simultaneously with enzymes (5.6% on 21 day-old chickens, $P<0.05$), but reduced it for the enzymes supplemented diets. Changes in the productive performances with both additives were associated with changes in the dietary organic matter and fat digestibility, and the N balance.

3. In experiment two; enzyme supplementation reduced viscosity in jejunum and ileum digesta, and the mean retention time of digesta in gut. Sepiolite inclusion reduced significantly the jejunum digesta viscosity and modified the retention times of digesta in the gut depending upon the presence of enzymes. There was a decreased retention time without enzymes but an increase with enzyme supplementation.

4. Although different mechanisms are presumed for enzymes and sepiolite, both seem to counteract the negative effects of soluble NSP in the diet by modifying jejunum viscosity and improving organic matter digestibility.

INTRODUCTION

The use of barley and wheat in commercial broiler diets has been traditionally limited, due to their low and variable energy value (Campbell et al., 1989) and their effects on the prevalence of sticky droppings, especially with barley (Esteve-Garcia et al., 1997). These problems have been extensively attributed to the viscosity increasing effects of the water-soluble non-starch polysaccharides (sNSP), mainly (1-3), (1-4) β-glucans in barley grain and arabinoxylans in wheat grain. However, a widespread incorporation of both cereals in poultry diets has been recently facilitated by the availability of feed enzymes (β-glucan and arabinoxylan degrading enzymes). Their use has been shown to increase the AME value of barley and wheat based diets, specially when using batches with a low feeding value (Fuente et al., 1998), and it improves litter quality through the reduction of the water holding capacity of soluble non-starch polysaccharides (sNSP; Esteve-Garcia et al., 1997).

Alternatively, other feed additives, such as antibiotics and clays, have been proposed in order to override the detrimental effects associated to the sNSP. Antibiotic supplementation increases the nutritive value of diets containing sNSP (Vukic Vranjes and Wenk, 1995), which implicates the likely contribution of microorganisms in the small intestine and a reduction in nutrient digestibility. However, clays have effects on the physical and kinetic parameters of digesta (Tortuero et al., 1992) which may explain their effects on the productive performance of broiler chickens and laying hens (Tortuero, 1982; Miles et al., 1986). The objectives of this study were to evaluate the growth performance (body weights, feed:gain conversion rates and nutrient digestibilities; Exp 1) and digestive parameters (viscosity and digesta retention times, Exp 2) associated with the incorporation of enzymes (β-glucanase and arabinoxylanase) and clays (sepiolite) to maize-barley-wheat based diets.

MATERIALS AND METHODS

Both experiments were performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review of this

institution.

Experiment 1

One-day-old male broiler chickens were fed a proprietary starter diet for a 5 days pre-experimental period and on day 6 (initial BW=102.0g ± 2.80) were randomly allocated to 48 cages (4 birds per cage, 50 x 60cm^2). The body weight of each group was recorded and the group was randomly assigned to one of four dietary treatments. Treatments consisted of a barley, wheat and maize based diet (formulated according to NRC, 1994; Table 1) either unsupplemented (Control) or supplemented with 20g/kg sepiolite (EXAL® UE-562, TOLSA, S.A. Madrid), 0.44 g/kg of β-glucanase and arabinoxylanase 50:50 (1mg/g of wheat+barley; Capsozyme C and T, ®EC-3216 and ®EC-3218 respectively, ITPSA, S.A. Barcelona), or both simultaneously. The experiment consisted of 12 replicates per treatment, each cage being the experimental unit. Food and water were supplied *ad libitum* and 23h light: 1h dark light programme was given. Group body weight and feed intake were recorded on day 21 and 42. On day 21-22 and 41-42, feed intake was recorded and total excreta were collected quantitatively once daily over a 48h period. Both feed and excreta samples were dried in an forced-air oven and samples were stored until analysis of OM, CP, and fat.

Experiment 2

One-day-old male broiler chickens were fed a proprietary starter diet for a 5-day pre-experimental period, allocated on day 6 (initial BW=102.0g ± 2.80) to 12 cages (four animals per cage) and randomly assigned to four dietary treatments. Treatments consisted of a maize-barley-wheat based diet (Table 1), either unsupplemented (Control) or supplemented with 20 g/kg sepiolite (EXAL®, Tolsa), 0.60 g/kg of enzymes (dose: 1mg/g of wheat plus barley; Capsozyme C and T, ITPSA, Barcelona) and both enzymes and sepiolite together. A different source of supplementary fat (sunflower oil vs tallow in Experiment 1) was included in this experiment in order to reduce the likely detrimental effects of saturated fatty acids on nutrient digestibility in the young broiler. The following experimental procedures were followed: sticky droppings were scored on day 12 and food intakes and body weights were recorded from day 6 to 22. Non-cumulative faecal excretion of TiO_2 was determined on day 20 and, on day 20-21, a balance trial was performed by measuring feed consumption and collecting excreta for a 40h period. On day 22, birds

were slaughtered by cervical dislocation and digesta removed for direct measurements of viscosity and digesta retention time.

Digesta kinetic measurements

Indirect measurements of digesta retention time (non-cumulative faecal excretion curves of Ti) were obtained as follows: On day 19, experimental diets containing 0.5% TiO_2 were administered for a 24h period, after which a marker-free diet was included and the non-cumulative faecal excretion curve (Ti contents) obtained following an hourly (0-8h) sampling of faeces. Curves were fitted by least square mean deviation to the equation derived from Van der Klis and Van Voorst (1993)

$$[TiO2] = a\left(1 - \frac{1}{1 + e^{-b\,(t-m)}}\right)$$

where "a" is the marker concentration when infusion ceased (mg Ti/ g digesta); "b", the dilution rate (h^{-1}); and "m" , the delay time (h). Time required for excretion of 5 and 50% of the total marker contained in the gastrointestinal tract was obtained from the following cumulative excretion equation , assuming a continuous (DM/h) faecal excretion.

$$\int_0^8 [TiO2]\,dt = a * \frac{[\ln(1 + e^{(b*m)}) - \ln(1 + e^{b*(m-8)})]}{b}$$

and mean retention time (MRT, min) was estimated as

$$MRT = \frac{1}{b} + m$$

Direct measurement of digesta kinetics on Day 22 was obtained as follows: On Day 20, diets containing 0.5%, TiO_2 were readministered for a 40h period until the animals were slaughtered. The gastrointestinal tract was immediately removed, weighed and segmented into gizzard, duodenum, jejunum, ileum and caeca. Each compartment was then weighed, total digesta collected and weighed, and contents of every two animals of similar weight

pooled (6 samples per treatment), frozen and stored until analysis of Ti and water relative viscosity. Mean retention time in the small intestine and the whole digestive tract (gizzard + small intestine + caeca; (RTa, n=6) was estimated from Ti contents in each compartment (Qa) and the hourly intake of Ti on day 20-22 (Ia; mg/h) in each treatment using the following equation

$$RTa = \frac{Qa}{Ia}$$

Ia was measured in steady state conditions with the amount of marker flowing daily (input/output) through each part of the digestive tract.

Analysis

Chemical analysis of the diet and excreta were undertaken following the methods of the Association of Official Analytical Chemists (1984) for DM, ash, CP and crude fat. Titanium was determined colorimetrically after the acid extraction of marker from the ash following the method proposed by Short et al. (1996). Water relative viscosity of jejunum and ileum digesta was determined by flow rates measurements with a CANNON-FENX viscometer according to the procedure of Choct and Annison (1992).

Statistical Analysis

Values were examined by a factorial (enzyme x sepiolite) analysis of variance. Treatments sums of squares were partitioned into the factors of presence of enzymes (enzymes +, enzymes -), sepiolite supplementation (0 vs 20 g/kg) and their interactions. When significant differences were found, the least square mean difference test was used to compare treatment means (Steel and Torrie, 1980). The percent of birds with no-, moderate-or heavily adhered

faeces were compared between treatments using a Chi-square test (with Yates correction=0.1500, DF=1)

Table 1. *Ingredient composition (g/kg) and determined analysis of basal diets for Experiment 1 and 2*

Experiments	**Experiment 1**	**Experiment 2**
Composition		
Maize grain	204	-
Barley grain	220	300
Wheat grain	220	308
Soya bean meal (460g/kg)	280	197
Full fat soybean meal	-	121
Sunflower seed oil	-	35
Tallow	35	-
Dl-methionine	2.0	2.0
L-lysine	1.0	1.8
Sodium chloride	3.6	3.6
Dicalcium phosphate	19.4	18.0
Calcium carbonate	9.2	8.9
Vitamin and trace element premix[1]	4.8	4.5
Analysis		
Crude protein (g/kg)	205.4	207.1
Crude fat (g/kg)	62.7	90.6
Ash	72.8	68.4

[1]***The active ingredients contained in the vitamin-mineral premix (/kg): All-trans retinol, 667mg; Cholecalciferol, 310mg; DL-α-tocopherol, 15g; menadione, 750; cyanocobalmin, 3mg; riboflavin, 1.25g; calcium pantothenate, 4.25g; niacin, 12.5g; choline chloride 62.5g; folic acid 0.37g; pantothenic acid, 4.3g; biotin 12.5mg. Co, 400mg; Se, 90mg; Fe, 8.75g; Cu, 5.0g; Mn, 2.4g; Zn, 2.4g; and Ethoxyquin, 15.1mg.***

RESULTS

Bird Performance

There were no significant differences in feed intakes due to any dietary treatment in Experiment 1 (Table 2). Both, enzymes and sepiolite caused differences in the average body weight (BW) of animals on day 21 and 42, but there was an interaction between these two treatment factors ($P<0.01$). While enzyme supplementation gave a 14% increase ($P<0.001$) in

BW with the diets without sepiolite, there were no significant difference in body weight due to enzyme supplementation of the sepiolite containing diets. Feed: gain ratios were significantly decreased by enzyme addition (P<0.01), but were not significantly altered by addition of sepiolite.

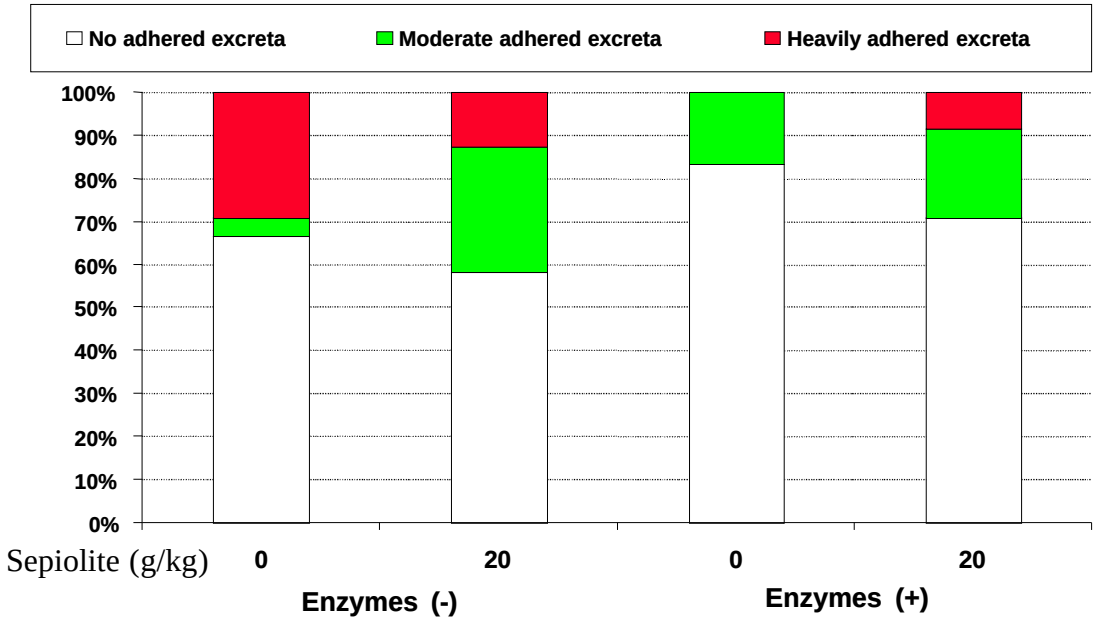
Table 2. *The effects of enzyme (β-glucanase and arabinoxylanase) and/or sepiolite supplementation on feed intake (g/bird) from day 6 to 21 and from day 22 to 42, bodyweight (g/bird) on day 21 and 42, and feed conversion rates (Feed:gain ratios) of broilers (Experiment 1).*

Enzymes level	Enzymes (-)		Enzymes (+)		RSD	Enz.	Sep.	Enz*Sep
Sepiolite level (g/kg)	0	20	0	20	*df* = 44			
Feed intake								
6 to 21 day	976	1000	1025	1009	64.3	NS	NS	NS
22 to 42 day	2981	2952	3115	2953	122.9	NS	NS	NS
6 to 42 day	3957	3952	4140	3963	154.2	NS	NS	NS
Bodyweight								
Day 21	696^{c}	735^{b}	793^{a}	751^{b}	46.1	***	NS	**
Day 42	2235^{c}	2325^{bc}	2541^{a}	2417^{b}	129.7	***	NS	**
Feed:gain								
6 to 21 day	1.66	1.58	1.49	1.56	0.102	***	NS	NS
22 to 42 day	1.91	1.86	1.78	1.77	0.116	**	NS	NS
6 to 42 day	1.84	1.78	1.70	1.71	0.082	**	NS	NS

RSD= Residual Standard Deviation ; *df*: Error degrees of freedom
* p<0.05 ; ** p<0.01 ; *** p<0.001 ; NS= Not Significantly different
a-c Means within rows with no common superscripts (a-c) are significantly different (P<0.05)

In Experiment 2 (Table 4), broiler performance between day 6 and 21 tended to show similar results to those observed in Experiment 1. Body weight (702 vs 667g, P<0.05) and feed: gain ratios (1.64 vs 1.53, P<0.01) were improved significantly by enzyme supplementation. No significant differences were observed that were associated with sepiolite incorporation. Enzyme supplementation of diets without sepiolite gave a significant increase (P<0.01) in the proportion of birds without adhered excreta (sticky droppings; Figure 1). On the other hand, sepiolite supplementation tended to increase (P>0.05) the number of animals with a detectable degree of adhered excreta. However, it is noticeable that the percentage of birds with heavily adhered excreta tended to decrease (P=0.09) in diets without enzymes, and increase with diets simultaneously supplemented with enzymes (P=0.06).

Figure 1. *Effect of enzymes and/or sepiolite supplementation on the incidence of sticky droppings at day 12 (No adhered, Moderate and Heavily adhered excreta; Experiment 2)*



Organic matter and nutrient digestibilities

Chemical analyses of excreta were undertaken for OM, fat and CP, but metabolic N was not quantified. From these results, apparent OM digestibility and N balance were calculated. Both, enzyme and sepiolite addition increased nutrient digestibilities. Enzymes increased ($P<0.01$) the digestibility of OM and fat, and the N balance on day 21 and 42. Sepiolite affected the nutrient digestibility of OM and fat, and the N balance depending on the simultaneous incorporation of enzymes (interaction enzymes x sepiolite, $P<0.05$-0.10). There were greater increases in digestibility in diets not supplemented with enzymes.

Table 3. *The effects of enzyme (β-glucanase and arabinoxylanase) and sepiolite incorporation on the apparent whole tract digestibility (g/g) of organic matter (OM) and fat (EE), and N balance (NB) determined on broilers on day 21 and 42. (Experiment 1)*

Enzymes level		Enzymes (-)		Enzymes (+)		RSD	Enz	Sep	Enz*Sep
Sepiolite level (g/kg)		0	20	0	20	*df*=44			
21-22 days	OM	0.736	0.775	0.794	0.812	0.0158	***	**	NS
	NB	0.723 [c]	0.785[b]	0.848[a]	0.836[a]	0.0212	***	*	**
	EE	0.560 [b]	0.687[a]	0.689[a]	0.713[a]	0.0446	**	**	*
41-42 days	OM	0.787	0.805	0.814	0.813	0.0108	**	NS	0.10
	NB	0.803	0.827	0.838	0.841	0.0124	**	*	0.10
	EE	0.671[b]	0.739[a]	0.756[a]	0.746[a]	0.0244	**	*	*

RSD= Residual Standard Deviation ; *df*: Error degrees of freedom
*p<0.05 ; ** p<0.01 ; *** p<0.001 ; NS= Not Significantly different
a-c Means within rows with no common superscripts (a-c) are significantly different (P<0.05)

As in Experiment 1, enzyme addition (Experiment 2, Table 4) increased the OM and fat digestibility and N balance.

Table 4. *The effects of enzymes and sepiolite on feed intake (g/bird) between day 6-21, bodyweight (g/bird) on day 22, food conversion rates (Feed:gain ratios), digestibility of organic matter (OM), fat (EE) (g/g), nitrogen retention (NB) of diets (Experiment 2), and relative viscosity of the jejunal and ileal digesta.*

Enzymes level		Enzymes (-)		Enzymes (+)		RSD	Enz	Sep	Enz*Sep
Sepiolite level (g/kg)		0	20	0	20				
						df= 8			
Feed intake		934	927	923	971	33.6	***NS***	NS	NS
Bodyweight		667	672	702	714	26.7	*	NS	NS
Feed:gain ratios		1.64	1.62	1.53	1.58	0.037	**	NS	NS
						df= 8			
Digestibility	OM	0.727	0.736	0.763	0.764	0.0109	**	NS	NS
	EE	0.556	0.609	0.616	0.615	0.0249	*	NS	0.09
N. balance	NB	0.749	0.773	0.808	0.806	0.0118	***	NS	0.08
Relative viscosity						*df* =20			
Jejunum		1.089[a]	1.063[b]	1.062[b]	1.063[b]	0.0171	*	*	0.07
Ileum		1.110	1.111	1.067	1.073	0.0304	**	NS	NS

RSD= Residual Standard Deviation ; *df*: Error degrees of freedom
*p<0.05 ; ** p<0.01 ; *** p<0.001 ; NS= Not significantly different
a-b Means within rows with no common superscripts (a-b) are significantly different (P<0.05)

Viscosity and Kinetic parameters

Supplementation with enzymes significantly reduced (P<0.05; Table 4) the viscosity of both jejunum and ileum digesta. Sepiolite supplementation only reduced (P<0.05) the jejunum viscosity of non-enzyme diets.

The titanium content of the excreta in steady state conditions (time 0) increased significantly with enzyme supplementation (0.397 vs 0.350, P<0.01). No significant differences were observed with sepiolite incorporation. Mean digesta retention times showed a significant interaction between enzymes and sepiolite. Enzyme supplementation decreased T50 (12.6min, P<0.05) and MRT (30min, P>0.05) of the diets without sepiolite, but increased these variables for diets with sepiolite. Thus, sepiolite decreased T50 (3min) and MRT (13 min) in diets not supplemented with enzymes, and increased both variables (P<0.05) in those diets supplemented also with enzymes (T50, 16min; and MRT, 38 min). Differences between treatments also tended (P>0.05) to be evident in the direct measurement of digesta retention time in the whole digestive tract. These differences were statistically significant in the small intestine where sepiolite increased (P<0.001) the time of retention by 8 min when no enzymes were present and by 20 min when enzymes were present.

Table 5. *The effect of enzymes and sepiolite on the retention time of digesta estimated from the non-cumulative excretion curves (T5 and T50 time –min; MRT, mean retention time –min) or directly in steady state conditions (Retention time –min- in the small intestine and whole G.I. tract)*

Enzymes level	Enzymes (-)		Enzymes (+)		RSD	Enz	Sep	Enz*Sep
Sepiolite level (g/kg)	0	20	0	20				
					df =8			
T5[1]	11.6	11.4	10.4	11.8	0.90	NS	NS	NS
T50[1]	118.4 [b]	114.7[c]	105.8[d]	121.7[a]	1.06	*	*	*
MRT	274.7 [ab]	261.8[ab]	245.3[b]	282.7[a]	17.20	NS	NS	*
					df=20			
G. I. Tract	177.6	160.9	158.5	178.3	25.36	NS	NS	NS
Small Intestine	104.8	113.3	101.5	121.3	12.55	NS	***	NS

RSD= Residual Standard Deviation ; *df*: Error degrees of freedom
*p<0.05 ; ** p<0.01 ; *** p<0.001 ; NS= Not significantly different
a-d Means within rows with no common superscripts (a-d) are significantly different (P<0.05)
[1]T5 and T50 determined as the time required for excretion of 5% and 50% of marker contained in gut

DISCUSSION

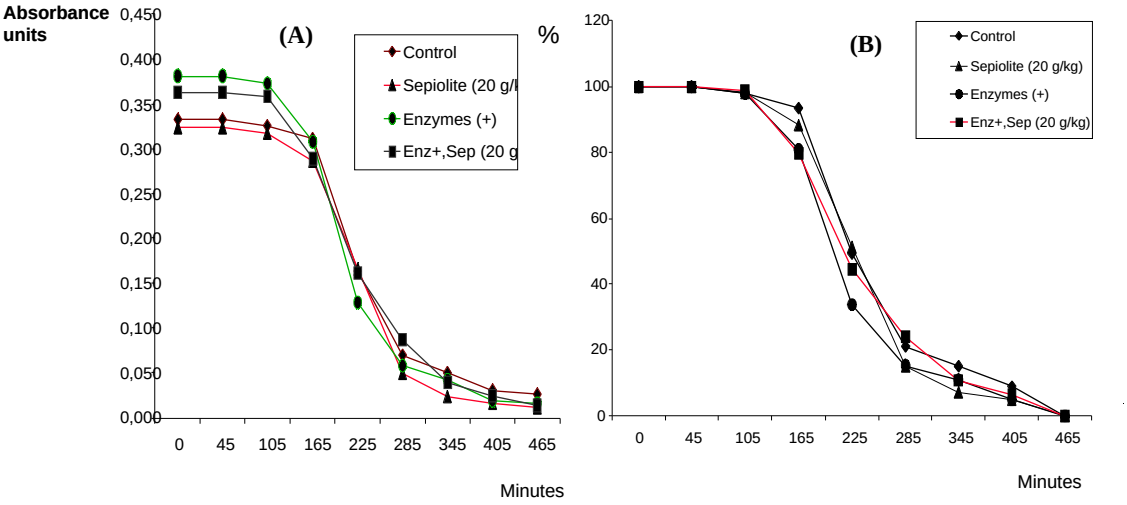
Enzyme supplementation

Soluble NSP in barley and wheat are known to adversely affect nutrient utilization and give rise to highly viscous conditions in the small intestine (Choct et al., 1996). These effects can be overcome by including exogenous enzyme preparations to the diets (Almirall et al., 1995; Van der Klis et al., 1995). In the present experiment the supplementation of a maize-wheat-barley based diet with β-glucanases and arabinoxylanases improved body weight and feed conversion ratios, probably through increasing the nutrient digestibilities of the animals (Almirall et al., 1995; Marquardt et al., 1996). Although differences were consistent throughout the whole growing period, these were more pronounced with young animals (21 days old). Similar results have been reported previously by Salih et al., (1991); Almirall et al., (1995); Philip et al., (1995), and Danicke et al., (1997) who suggested that there is an age-dependent ability of broilers to reduce the adverse effects of sNSP. Almirall et al., (1995) found higher digesta viscosity in broiler chickens than in adult cockerels when barley-based diets were fed. Non-endogenous enzymes may be involved in this change; likewise the microbial enzyme activity in the small intestine may increase with age and this could cause an increased partial hydrolysis of NSP and important reductions on digesta viscosity.

Different mechanisms have been proposed to explain the effects observed with enzymes included in cereal-based diets. The simplest explanation is that β-glucans and arabinoxylans from the endosperm wall of cereal cells act to restrict access of the endogenous enzymes to nutrients found in the endosperm. However, the increases observed on fat digestibility, a non-endosperm stored nutrient, give doubts about this theory. The most widely accepted mechanism is the clear reduction of viscosity in the jejunal and ileal digesta (Choct et al., 1996). Our results confirm this relationship as both jejunum and ileum digesta viscosity were significantly reduced by enzyme supplementation. A partial hydrolysis of β-glucan and arabinoxylan structures could reduce considerably their water holding capacity and associated viscosity, increasing the diffusion of enzymes and nutrients, and improving absorption through a reduction of the unstirred water layer lining the intestinal mucosa (Johnson and Gee, 1981).

Reductions on the digesta viscosity have also been related to significant decreases on the time of digesta retention in the gastrointestinal tract (Almirall and Esteve-Garcia, 1994) and increases on the voluntary intake (Albustany, 1996). The present results agree with other findings that enzyme incorporation to maize-barley-wheat based diets reduced digesta retention time in the whole tract. However, the direct measurements did not confirm that these changes occurred exclusively in the small intestine retention time. No consistent differences were obtained in feed consumption, which suggests that most of the differences in bird performance were related to the improvement of the digestibility of the diets.

Figure 2. *Non cumulative curves of faecal excretion of Ti on animals fed the control treatment or supplemented with enzymes (β-glucanase and arabinoxylanase) and/or sepiolite (expressed as mean absolute values (A), or refered to 100% of concentration at time : 0 (B))*



Sepiolite supplementation

Sepiolite is a hydrated magnesium silicate clay ($Si_{12}Mg_8O_{30}$ $(OH)_4(H_2O)_4$ $8H_2O$) which presents a high specific surface (Wolter et al., 1990) and a moderate absorption and NH_3 retention capacity. These properties were basic for its first technological application as a lubricant of ground diets and a pelleting agent during feed processing procedures. Through these technological applications, sepiolite was found to improve feed consumption and feed:gain ratios (Angulo et al., 1996) in monogastric animals. However,

recent reports suggest a direct effect of sepiolite on the nutrient digestibility and bird performances (Tortuero, 1982). Our results support this hypothesis. Although theoretically a 20g/kg dilution with a non-digestible additive should cause an increase on the feed:gain ratios, sepiolite tended (P>0.05) to decrease this variable, especially with diets not supplemented with enzymes. These results can be explained by the significant increases on the OM and fat digestibilities, and N balance, counteracting the simultaneous effect of sepiolite dilution. However, sepiolite dilution of a highly digestible diet (for example with enzymes) may negate this effect or it is possible that both additives are effective on similar organic matter fractions.

Different mechanisms have been proposed to explain the effect of sepiolite in broiler diets. Sepiolite is thought to promote significant increases on the digesta retention time in the gut of pigs and poultry (Tortuero et al., 1992). Thus, an increased transit time of digesta would allow the endogenous enzyme activity to be more effective in the digestion of fat, protein and carbohydrates. Both, direct and indirect measurements showed that sepiolite reduced the rate of passage of digesta, especially through the small intestine that is the main site of digestion and absorption. However, smaller differences were observed with the non-enzymatic treatments than with the diets simultaneously supplemented with enzymes, which suggests that sepiolite may have reduced the rate of passage of non-viscous- (as those studied by Tortuero et al., 1992) more than of viscous digesta. The present experiments did not establish a quantitative relationship between the time of retention of digesta in the gastrointestinal tract and digestibility of OM. Sepiolite supplementation of barley-wheat based diets has been related to significant decreases on ileal viscosity of 30 day old broilers (Schutte and Langhout, 1998). Sepiolite changes on the physical-chemical properties of digesta may allow a reduction of the antinutritive effects of high viscosity. In the present experiment, sepiolite promoted significant decreases in the viscosity of jejunum digesta, reaching similar values to those observed with the enzyme supplementation. No differences were observed with the ileal digesta. From jejunum to ileum, there is a net absorption of DM and water, which progressively decreases the moisture content of digesta. Although these decreases were relatively low (82.2 vs 77.7% respectively in jejunum and ileal digesta in the control diet), clear differences were observed between a fluid jejunal digesta and a more "pasty" ileum digesta. It may be speculated that a sepiolite interaction with digesta could be more pronounced as higher levels of moisture and solutes

are present in digesta. Although ileum digesta is frequently used to measure digesta viscosity (Choct et al., 1996), the jejunum is considered the small intestine segment where most digestion and absorption processes occur.

It can be concluded that supplementation with enzyme mixtures improved broiler performance (growth) on maize-wheat-barley based diets. Sepiolite dilution at a 20g/kg level tended also to increase weight gain, and improve feed: gain ratios of diets not supplemented simultaneously with enzymes. Both, enzyme and sepiolite decreased intestinal viscosity, which was related to the increases in nutrient digestibility (especially fat and N balance) and the reduced percentage of animals with adhered excreta on the perineal region.

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CAPITULO 3

Soybean (***Glycine max***.) cell wall composition and availability to feed enzymes

ABSTRACT

Defatted untoasted soybean cotyledons and hulls were fractionated as water solutes (WSc and WSh) and water unextractable (WUc and WUh). Further fractionation of WUc through deproteinization yielded the isolation of a water unextractable solid (WUS) fraction that was mainly composed of galactose (28.1% monosaccharides^{-1}), glucose (27.8%), arabinose (13.3%) and uronic acids (17.6%), which accounted for 76% of the water insoluble polysaccharides (%WUc-monosaccharides^{-1}) in soybean cotyledons. The cell wall (WUS) was sequentially fractionated with chelating agents (chelating agent soluble solids, ChSS) and a gradient of agents (dilute alkali, DASS; 1 M alkali, 1M ASS; and 4M alkali, 4 M ASS), which gave a final cellulosic residue. The ChSS and DASS extracts were characterised as pectin-rich fractions, whereas 1 M ASS and 4 M ASS were hemicellulose- and cellulose-rich fractions.

Incubation *in vitro* of the WUc fraction with pectinase, cellulase and xylanase resulted in the release of low amounts (not more than 5 percent bound basis^{-1}) of monosaccharides, mostly uronic acids, xylose and arabinose. Protein extraction hardly increased this release after enzymatic incubation (< 7%). However, progressive fractionation of the cell wall matrix markedly increased the release of monosaccharides from pectin- (ChSS and DASS), and hemicellulose-rich fractions (1 and 4M ASS). Significant degradation of cellulose (until 20%) was achieved only after complete protein, pectin and hemicellulose extraction.

Keywords: Soybean meal; cell wall components; polysaccharides; enzymes

INTRODUCTION

Feed enzymes are widely used as additives, with an established role in animal nutrition. Although several cloned enzymes are available, they are only applied to improve cereal-based diets for broilers, and, to a lesser extent, piglets. In fact, feed enzymes are used when profitable uses have been evidenced. For example, the availability of β-glucan- and arabinoxylan- degrading enzymes has fostered the incorporation of barley and wheat in poultry diets. They increase the metabolizable energy (ME) value of these diets, especially in batches with low nutritional value (Bedford and Morgan, 1996; Esteve-Garcia et al., 1997; Fuente et al., 1998).

In general, feed enzymes have proved beneficial when effective enzymatic activities target a defined problem (e.g. glucanase and xylanase partial hydrolysis of water-soluble glucans and xylans to reduce digesta viscosity; Ouhida et al., 2000). On the other hand, most efforts to improve the utilisation of insoluble and otherwise unavailable plant cell wall components with feed enzymes have proved ineffective. The plant cell wall consists of a series of polysaccharides often associated with or replaced by proteins and phenolic compounds, like the phenolic polymer lignin in some cells (Theander and Westerlund, 1993). The main polysaccharides of the plant cell wall are cellulose, arabinoxylans, mixed linked β-(1-3; 1-4)-D-glucans (β-glucans), xyloglucans, xylans, rhamnogalacturonans and arabinogalactans (Morita, 1965a,b; Aspinall et al., 1967a; Labavitch et al., 1976).

Most of these carbohydrates are only partially digested or poorly utilised by the digestive enzymes. However, a disruption of these structures through processing (e.g. extrusion; Marsman et al., 1995) or degrading enzymes (Marsman et al., 1997a) can improve their nutrient availability. At present, the data available are contradictory. Zanella et al., (1999) reported that a mixture of enzymes improved the nutritional value of corn-SBM diets for broilers. However, multi-enzyme preparations designed for soybean non-starch polysaccharides have failed to improve the growth performance of broilers fed diets containing SBM as the main protein source (Irish and Balnave, 1993; Marsman et al., 1995; Marsman et al., 1997a; Douglas and Parsons, 2000). It is not clear

whether the proper enzymes were missing or a complex structure remained insoluble and inaccesible.

The aim of this study was to evaluate the efficiency of cell wall degrading enzymes (pectinase, xylanase and cellulase) in hydrolysing soybean meal carbohydrates. Disruption of the cell wall polysaccharide network by sequential extraction may increase the degradability of extracts and residuals by enzymes, thus reflecting the influence of the structural network on the low cell wall degradability. We studied soybean meal, which is a major protein source for livestock and human feeding (World production ~101 millons tonnes, FAOSTAT, Agriculture 2000). We used a sequential extraction of cell walls proposed by Redgwell and Selvendran (1986) in milder conditions, which prevent the chemical degradation of carbohydrates (Aspinall et al., 1967a, b).

MATERIAL AND METHODS

Plant material. Solvent extracted soybean meal (53.9 %CP) was obtained from a local market and was physically separated on hulls and cotyledons by gradient sieving.

Sequential fractionation of untoasted SBM. **Defatted solvent extracted cotyledons (1000 g) and hulls (100 g) were ground to pass through a 0.59-mm sieve. The meals were respectively suspended in 3.75 l and 375 ml of distilled water containing 50 mg chloramphenicol/l for 2h at room temperature and centrifuged at 11000 x *g* for 30 min. The recovered Water Unextractable (WU) fractions, from cotyledons (WUc) and hulls (WUh), were resuspended and the procedure was repeated four times. The combined supernatants were freeze-dried (soluble water from hulls, WSh, and from cotyledons, WSc).**

The residue was suspended in 3 l of solution containing 1.5% sodium dodecylsulphate and 10 mM 1-4-dithiothreitol for 3h at room temperature to extract the protein from the WUc fraction. After centrifugation (11000 x *g*; 30 min), this extraction was repeated three times. The final pellet was washed twice in distilled water.

Subsequently, starch was removed as follows: the pellet was suspended in 1 l of distilled water (pH 5.0) at 85ºC for 1h and centrifuged at 11000x *g* for 30min. The residue was suspended in 1 l of buffer solution (pH 6.5) containing 10 mM maleic acid, 10 mM NaCl, 1 mM $CaCl_2$, and 50 mg chloramphenicol. Porcine pancreatic alpha-amylase (2 mg; EC 3.2.1.1 heat stable) was added and the mixture was incubated at 30ºC for 19h. After centrifugation

(11000x *g*; 30 min), the residue was washed in 1 l of hot water (65ºC) and centrifuged again. Alpha-amylase digestion and hot water washing were repeated. The combined supernatants were discarded and the remaining unextractable residue was freeze-dried (WUS).

Soybean WUS was sequentially extracted following Huisman et al., (1998), with 0.05M 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) and 0.05 M NH_4-oxalate in 0.05 M NaAc-buffer, pH 5.2 (8 times 600 ml) at 70ºC for 1 h (chelating agent soluble solids, ChSS); washed in distilled water (twice 600 ml) and these extracts were added to the ChSS fraction; extracted with 0.05 M NaOH (3 times 600 ml) at 2ºC for 1h (dilute alkali soluble solids, DASS); extracted with 1 M KOH + 20 mM $NaBH_4$ (5 times 600 ml) at room temperature for 2 h (1 M alkali soluble solids, 1M ASS); and 4 M KOH + 20 mM $NaBH_4$ (3 times 600 ml) at room temperature for 2 h (4M alkali soluble solids, 4M ASS). After each extraction, the solubilised fractions were separated from the residue (Pellet1 after ChSS-, Pellet2 after DASS-, Pellet3 after 1M ASS-, and last residual "RES" after 4M ASS-extraction) by centrifugation at 19000x *g* for 30 min. All dialysable fractions and pellets were concentrated, dialysed and freeze-dried.

Enzymatic degradation of soybean polysaccharides. **A small amount of substrate was weighed (30 mg) in 2mL-Kimax tubes with a screw cap, containing 0.05 M sodium acetate buffer (pH 5.0) and 0.05 mg/mL chloramphenicol, and incubated with specific enzymes at 40ºC for 12h in a horizontal shaking waterbath. The substrates obtained from the sequential fractionation (WUc, WUS, ChSS, DASS, 1 M ASS, 4 M ASS) and the intermediary pellets (Pellets 1, 2, 3 and RES) were used for incubations. The enzymes tested were from Quest International Company (Ireland): Biocellulase A**

Conc® (8750 cellulase units/g), Biopectinase NKP 120P® (endopolygalacturonase 830 units/g; containing also cellulase, 1400 units/g; xylanase, 13270 units/g; and β-glucanase 9650 units/g) and Glucanase-xylanase® (β-glucanase, 300000 units/g; and xylanase, 200000 units/g). Enzyme addition was standardised to 1 %DM^{-1}. At the end of each incubation, enzymes were inactivated by boiling (100ºC for 10 min), centrifuged (4000 x *g*; 10 min), and the supernatant was collected and frozen for subsequent free monosaccharide analysis.

Analytical methods. **Previous to the analysis of soybean fractions, samples were lyophilised to constant weight. Protein content was assessed by the Kjeldahl method, using selenium as a catalyst, with a conversion factor of 6.25. Fat was determined by the Soxhlet method.**

Solvent extracted SBM, WSh, WSc, WUh, WUc, WUS, ChSS, DASS, 1M ASS, 4M ASS and RES were analysed for their neutral sugar composition following Theander (1991) using inositol as internal standard. After treatment with 72% w/w H_2SO_4 (1 h, 30ºC) followed by incubation in autoclave (1h, 125ºC) and immediate filtration (Poro 2), monosaccharides were reduced to alditols with KBH_4 and converted to their alditol acetates using 1-methylimidazole and acetic anhydride. Alditol acetates were separated on a GLC column (25m x 0.25mm x 0.25μm; Hewlett Packard 19091 C-131) using D-glucose, D-galactose, D-xylose, L-arabinose, D-mannose, fucose, and rhamnose as standards. The acidic cell wall polysaccharides released during enzyme incubations and total uronic acid in the cell wall polysaccharides were quantified as uronic acids, following Theander, (1991).

RESULTS AND DISCUSSION

Yield and composition. **Table 1 shows the composition of soybean meal hulls and cotyledons, as obtained after extraction with distilled water. Soybean hull solubility was 16.7%DM^{-1}, yielding a substrate (WSh) containing 26.0% crude protein and 37.2% carbohydrates. Soluble carbohydrates were mainly composed of mannose (36%), glucose (27%), galactose (20%) and uronic acids (8%), whereas the water insoluble**

fraction (WUh) contained low amounts of mannose (4.5%) and galactose (3.0%). Soluble polysaccharides in hulls consisted of galactomannans (Aspinall and Whyte, 1964) or beta-mannans, a mannose backbone linked to galactose units in a 2:3 ratio. Meanwhile, the higher glucose content in WUh carbohydrates (66%) may point to a large amount of cellulose (mainly β-1,4 glucosidic linkages). Soybean hulls contain two additional polysaccharides, pectins (linked α-1,4 D-galactopyranosyl uronic acids, most of which are extractable with water) and two hemicelluloses; unbranched xylans "hemicellulose A" and arabinoglucurunoxylan "hemicellulose B" (Aspinall, 1988).

Cotyledon water solubility was 56%DM^{-1}, yielding a substrate (WSc), containing 57.2% crude protein (CP) and 17.9% carbohydrates. Thus, cotyledon CP was mainly composed of water soluble proteins (59.4%), as reported by Huisman et al., (1998) for dehulled and defatted untoasted soybean meal, in which most of the material was water-soluble (59 %DM^{-1} and 67 %CP^{-1}). The dehulled soybean meal contains 24.9% carbohydrates (DM^{-1}), determined as the sum of neutral sugars and uronic acids. A relatively large fraction (40%) of carbohydrates was also recovered in the WSc fraction. Water-soluble carbohydrates were mainly composed of mannose (5.1%), galactose (39.6%) and glucose (49.4%), which reveals a large amount of extracted α-galactosides (Leske et al., 1993) and β-mannan polysaccharides, which probably result from incomplete removal of hulls from the cotyledons. In fact, the sucrose and oligosaccharide content in the soybean meal was 72 and 53g/kg DM, respectively, and stachyose (±40g/kg DM) and raffinose (±14g/ kg DM) were the major alpha-galactosides. Although WSc extracts contained high amounts of sucrose (88 g/kg DM) and oligosaccharides (68g raffinose + stachyose /kg DM), water extraction did not remove them all. In a study on soybean meal alpha-galactosides, Irish et al., (1995) removed with water only 18% of oligosaccharides extracted by ethanol and water sequential extraction.

Protein extraction with sodium dodecyl sulphate (SDS) allowed 96.7% removal of WUc proteins and 24% of carbohydrates. However, the monosaccharide composition of the deproteinized residues (WUS) was similar to that observed in WUc, which suggests non-

selective carbohydrate extraction of SDS. Thus, carbohydrates in the WUS fraction were considered representative of the total cell wall carbohydrates. Sugar composition (WUS) mainly consisted of galactose (28.1%), glucose (27.8%), arabinose (13.3%) and uronic acids (17.6%), which reflects the galactan chains and the three structurally major domains in the cell wall (cellulose-xyloglucan framework and pectic polysaccharides; Carpita and Gibeaut, 1993). Most of the uronic acids contained in the native dehulled SBM (90%) and WUc (96%) were recovered in the WUS fraction, in agreement with Huisman et al., (1998). Thus, pectins from soybean meal are less soluble than those from other plants like onions (Redgwell and Selvandran, 1986), apples (Schols et al., 1995) and olives (Huisman et al., 1996).

Table 1**: Yield and composition of soybean meal and fractions (Percent Weight). Sugar composition expressed as percent mol.**

	Soybean hulls		Dehulled SBM			
Fractions[a]	*WSh*	*WUh*	*SBM*	*WSc*	*WUc*	*WUS*
Yield (%)	**16.7**	**83.3**	**100**	**56.0**	**44.0**	**13.9**
Crude protein	**26.0**	**10.7**	**53.9**	**57.2**	**49.6**	**5.2**
Crude fat	**0.3**	**0.4**	**0.7**	**0.4**	**1.0**	**1.5**
Ash	**12.8**	**2.6**	**6.9**	**10.5**	**2.4**	**2.9**
Total monosaccharides	**37.2**	**63.5**	**24.9**	**17.9**	**37.5**	**90.3**
Sugar composition						
Rhamnose	**2.8**	**3.3**	**2.6**	**2.2**	**5.5**	**3.6**
Fucose	**0.2**	**0.5**	**1.3**	**0**	**2.1**	**2.1**
Arabinose	**3.7**	**7.3**	**10.4**	**1.8**	**13.3**	**13.3**
Xylose	**1.3**	**12.6**	**4.3**	**0**	**5.9**	**5.9**
Mannose	**36.2**	**4.5**	**3.5**	**5.1**	**2.2**	**1.5**
Galactose	**20.4**	**3.0**	**31.9**	**39.6**	**29.6**	**28.1**
Glucose	**26.9**	**66.3**	**37.7**	**49.4**	**28.2**	**27.8**
Uronic Acids	**8.4**	**2.6**	**8.2**	**1.8**	**13.2**	**17.6**

[a]SBM= dehulled soybean meal; WSc= water soluble from cotyledons; WSh= water soluble from hulls; WUc= water unextractable from cotyledons; WUh= water unextractable from hulls; WUS= water unextractable solids.

Table 2 presents the yield and composition of substrates extracted from the fractionation of WUS. Sequential extractions solubilized 33.7% ChSS, 8% DASS, 13.2% 1M ASS and 18.5% 4 M ASS, accounting for an 84 %DM^{-1} recovery.

The fractions extracted with chelating agents (ChSS) and diluted acids (DASS) had the same composition: 32-33% galactose, 12-18% glucose, 14-16% arabinose, 4-5% xylose and 22-26% uronic acids. CDTA and NH_4-oxalate solubilise pectic polysaccharides by abstracting Ca^{2+} from the cell walls and disrupting ionic cross-links. The galactose:arabinose (2.1:1 – 2.3:1) and uronic acids:rhamnose (5.5:1 – 5.0:1) ratios indicate the presence of arabinogalactan (Aspinall,1988) and rhamnogalacturonan polysaccharides (Lau et al., 1985).

Table 2: **Yield and composition of fractions extracted from WUS (Percent Weight, sugar composition expressed as Percent mol)**

WUS fractions[a]	ChSS	DASS	1M ASS	4 MASS	RES	***Recovery % of WUS***
Yield (%)	**33.7**	**8.0**	**13.2**	**18.5**	**10.7**	***84.1***
Protein content (%)	**15.7**	**0**	**0**	**0**	**0**	
Total monosaccharides	**54.0**	**68.3**	**71.1**	**83.1**	**71.8**	
Rhamnose	**4.1**	**5.2**	**4.5**	**4.9**	**2.2**	***98.9***
Fucose	**2.1**	**2.6**	**2.1**	**2.6**	**0.3**	***84.0***
Arabinose	**14.2**	**16.7**	**13.4**	**17.1**	**4.2**	***85.7***
Xylose	**4.3**	**5.0**	**15.6**	**7.1**	**2.9**	***93.2***
Mannose	**1.2**	**0.3**	**1.8**	**0.3**	**2.2**	***64.9***
Galactose	**33.0**	**31.9**	**27.1**	**39.1**	**4.8**	***88.2***
Glucose	**18.3**	**12.4**	**25.6**	**15.7**	**76.3**	***77.8***
Uronic Acids	**22.7**	**25.8**	**10.0**	**13.1**	**7.3**	***80.8***

[a]ChSS= chelating agent soluble solids; DASS= dilute alkali soluble solids; 1M ASS= 1 M alkali soluble solids; 4M ASS= 4 M alkali soluble solids; RES= residual; WUS= water unextractable solids.

Extractions with stronger alkali (1 and 4M KOH) further solubilize the amount of pectins and hemicelluloses. The 1M ASS and 4M ASS fractions contained, respectively, mainly galactose (27 and 39%), glucose (16 and 26%), arabinose (13 and 17%), xylose (7 and 16%) and uronic acids (10 and 13%), indicative of remaining pectic polysaccharides, probably ester cross-linked within the wall matrix. However, the uronic acids:rhamnose ratio (2.2:1-2.6:1) was sligthly lower than those of ChSS and DASS. It may reflect the presence of higher amounts of rhamnogalacturonan containing many arabinosyl and galactosyl-side chains, often referred to as "hairy region" (Aman and Westerlund, 1996).

The final residue (RES) contained 76% glucose, probably in the cellulose framework, and low amounts of uronic acids (7.3%) and other neutral monosaccharides. The remaining rhamnose (2.2%) and uronic acids in the last residue suggest the presence of rhamnogalacturonan polymers tightly bound to the cellulose network.

Enzymatic degradation of cell wall polysaccharides. **Table 3 shows the amount of neutral sugars and uronic acids released (Percent Weight) over a blank from the insoluble soybean meal (WUc) and cell wall polysaccharide (WUS) fractions, after incubation with three enzyme preparations containing an identified activity of pectinase, cellulase and xylanase. Enzyme incubations of WUc and WUS fractions released an amount of monosaccharides lower than 7%. However, a slight increase was observed after the SDS protein extraction. As to uronic acids, their release was duplicated with pectinase (11.4 vs 20.4%) and xylanase (6.3 vs 11.4) incubations, but not cellulase. This reflects the relatively higher accessibility of pectins and arabinan chains. On the other hand, the low values observed for glucose and galactose from WUS indicates that the cell wall network was not hydrolysed.**

Table 3. **Amount of solubilized neutral sugar (NS) and uronic acids (UA) (Percent bound basis) after 12h incubation of insoluble SBM fractions with pectinase, cellulase and xylanase.**

Soybean fractions[a]	WUc	WUS

Enzymes	*Pectinase*	*Cellulase*	*Xylanase*	*Pectinase*	*Cellulase*	*Xylanase*
∑ ***NS + UA***	**3.8**	**5.4**	**5.0**	**6.9**	**6.8**	**6.3**
Rhamnose	**6.8**	**nd**	**nd**	**nd**	**nd**	**1.3**
Fucose	**nd[b]**	**nd**	**nd**	**nd**	**nd**	**nd**
Arabinose	**3.7**	**2.6**	**18.6**	**5.8**	**2.6**	**19.6**
Xylose	**1.5**	**1.5**	**6.3**	**4.0**	**2.3**	**9.6**
Mannose	**nd**	**9.1**	**6.5**	**3.5**	**9.3**	**nd**
Galactose	**1.3**	**1.0**	**0.8**	**4.4**	**1.1**	**nd**
Glucose	**5.1**	**7.2**	**4.0**	**7.0**	**9.6**	**5.8**
Uronic Acids	**11.4**	**23.7**	**6.3**	**20.4**	**23.5**	**11.4**

[a]WUc= water unextractable from cotyledons; WUS= water unextractable solids

[b]nd: not detected

Marsman et al. (1997b) have also studied the *in vitro* accessibility of the water unextractable (WU) fraction from untreated, toasted and extruded soybean meals for various enzyme activities. Proteolytic and cell wall degrading enzymes promoted the release of approximately 15% of monosaccharides, but nearly 50% of neutral sugars or polysaccharide fragments. After incubation with polysaccharide degrading enzymes, 85% of the released polysaccharide fragments were composed of galactose, arabinose and uronic acids, while limited amounts of glucose, mannose and xylose were found. After an initial reaction over accessible material with the intermediate formation of soluble polymeric material (Düstechöft et al., 1993), the enzymatic attack on insoluble substrates may occur by the direct and slow release of mono- or dimeric products.

Tables 4 and 5 present the free monosaccharides (Percent bound basis^{-1}) released by the incubation of WUS, solubilized fractions (ChSS, DASS, 1 and 4M ASS) and their residual pellets (pellet 1, 2, 3 and RES) with pectinase and cellulase, respectively. To evaluate the influence of structural disruption on enzyme efficiency, pectinase and cellulase incubations were focused on the fractions mostly containing pectins or cellulose: the fractions WUS, ChSS, Pellet 1, DASS, Pellet 2, 1 M ASS for pectinase incubations (Table 3) and WUS, 1M ASS, Pellet 3, 4M ASS and RES for cellulase incubations (Table 4).

Table 4. **Amount of solubilized neutral sugar (NS) and uronic acids (UA) (Percent bound basis) after 12h-incubation of WUS, ChSS, DASS, 1M ASS, Pellet 1, and Pellet2 with pectinase.**

WUS-SBM fractions [a]	WUS	ChSS	Pellet 1	DASS	Pellet 2	1MASS
Yield (% of WUS)	**100**	**33.7**	**76.3[b]**	**8.0**	**58.3[c]**	**13.2**
$\sum$ *NS + UA*	**6.9**	**15.7**	**10.8**	**21.9**	**8.7**	**19.0**
Rhamnose	**nd[d]**	**nd**	**6.2**	**nd**	**nd**	**5.3**
Fucose	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Arabinose	**5.8**	**16.4**	**19.1**	**21.5**	**13.6**	**10.4**
Xylose	**4.0**	**nd**	**3.4**	**nd**	**3.6**	**6.6**
Mannose	**3.5**	**4.4**	**nd**	**nd**	**nd**	**10.6**
Galactose	**4.4**	**7.4**	**4.9**	**5.5**	**4.8**	**4.9**
Glucose	**7.0**	**18.3**	**5.0**	**12.6**	**4.3**	**13.6**
Uronic Acids	**20.4**	**35.7**	**42.1**	**48.3**	**36.6**	**37.1**

[a]ChSS= chelating agent soluble solids; DASS= dilute alkali soluble solids; 1M ASS= 1 M alkali soluble solids; WUS= water unextractable solids.

[b]Determined as WUS-Chss

[c]Determined as WUS-(Chss+DASS)

[d]nd: not detected

As mentioned above, pectinase incubation of WUS degraded the cell walls to a minor extent, since only some small neutral degradation products (3-7%) and uronic acid residues (approximately 20%) were released. Disruption of the cell wall network by sequential extraction increased the degradation of pectin-rich fractions (ChSS, DASS and 1 M ASS). The release of uronic acids increased (20 vs 35-48%), as did that of arabinose (5.8 vs. 16-21%), glucose (7 vs 12-18%) and, to lesser extent, galactose (4.4 vs 7.4%). This suggests that pectinase contained side activities of endoarabinase.

Pectin extraction increased the hydrolysis of the residual network (WUS vs Pellet 1 and Pellet 2), especially of uronic acids (20 vs 42 and 36%) and arabinose (5.8 vs 19.1 and 13.6%, respectively). Pectic polysaccharide extraction (ChSS) enhanced the accessibility of the pectin and the arabinan chains remaining in the wall matrix. On the other hand,

glucose was poorly released, which reflects that cellulose structures were still too complex or dense to be penetrated by the applied enzymes.

To evaluate the influence of cellulose complexity on the enzyme hydrolysis, we studied the degradation with cellulase of WUS and substrates obtained after further fractionation with a gradient of alkaly agents (hemicellulose / cellulose) of pellet 2 (Table 5). The fractions extracted (Table 2; 1M ASS and 4M ASS), mostly containing galactan, glucan and arabinan chains, were more degraded by cellulase than WUS (9.2% glucose from WUS vs 18.1 or 21.8% from 1M ASS and 4M ASS, respectively). Cellulase degradation of cell wall polysaccharide residues (pellet 3 and RES) remained very low (10%), even after protein, pectin and partial hemicellulose extraction (1M ASS). Only after 4M ASS extraction of xyloglucan components, cellulose degradation increased (10.7% vs 19.4% of glucose from Pellet 3 and RES, respectively).

Table 5. **Amount of solubilized neutral sugar (NS) and uronic acids (UA) (Percent bound basis) after 12h-incubation of WUS, 1M ASS, 4M ASS, Pellet 3 and RES with cellulase.**

WUS-SBM fractions[a]	WUS	1 MASS	Pellet 3	4 M ASS	RES
Yield (% of WUS)	**100**	**13.2**	**45.1[b]**	**18.5**	**10.7**
∑ NS + UA	**6.8**	**15.4**	**5.7**	**10.6**	**19.7**
Rhamnose	**nd[c]**	**2.5**	**nd**	**nd**	**5.8**
Fucose	**nd**	**nd**	**nd**	**nd**	**nd**
Arabinose	**2.6**	**4.4**	**2.6**	**6.0**	**7.8**
Xylose	**2.3**	**5.8**	**2.4**	**3.9**	**10.3**
Mannose	**9.3**	**26.4**	**nd**	**nd**	**34.0**
Galactose	**1.1**	**1.2**	**0.5**	**2.8**	**3.8**
Glucose	**9.6**	**18.1**	**10.7**	**21.8**	**19.4**
Uronic Acids	**23.5**	**21.9**	**10.8**	**30.1**	**7.7**

[a]1M ASS= 1 M alkali soluble solids; 4M ASS= 4 M alkali soluble solids; RES= residual; WUS= water unextractable solids.

[b]Determined as WUS-(ChSS+DASS+1M ASS)

[c]nd: not detected

Our results show that enzymes hardly degraded the carbohydrates contained in intact soybean cell wall, as reported elsewhere (Marsman et al., 1997b; Huisman et al., 1999; Malathi and Devegowda, 2001). Although the crude enzymes used in the present experiment still contained non-identified side activities, neither pure cloned enzymes (Huisman et al., 1999) nor commercial mixtures (Marsman et al., 1997b) effectively hydrolyse cell wall carbohydrates to their component monosaccharides. However, the analysis of free monomers probably accounted for an incomplete index of cell wall degradation and chain size reduction. Huisman et al. (1999) demonstrated that endoenzymes like endogalactanase, significantly reduce the volume of polymers, whereas exoenzymes release monomeric sugar residues from the polysaccharide without modifying hydrodynamic volume of heterogeneous polysaccharides. Incubation *in vitro* of WU-SBM with several commercial enzyme preparations (Marsman et al., 1997b) resulted in the solubilisation of a large amount (up to 67%) of neutral sugars (fragments of neutral cell wall polysaccharides) but only 12-22% of monomers.

We used free monosaccharides as an index of carbohydrate hydrolysis to obtain information about the ability of the enzyme to reach hydrolysis sites. Our results indicate that sequential fractionation significantly increased enzyme degradation. However, the fractions clearly differed; pectins and arabinan chains were the most accessible carbohydrates, while xylans and cellulose were practically inaccessible. We failed to obtain clear results on galactose accessibility, but Huisman et al. (1999) revealed that combinations of endo- and exo-galactanase, exo-arabinase and arabinofuranosidase that promoted the release of high amounts of arabinose and galactose residues and a large number of oligosaccharides.

From a nutritional point of view, the activity of these enzymes "*in vivo*" or simulating the animal conditions "*in vitro*" (Malathi and Devegowda, 2001) should be evaluated. In

particular, the effective hydrolysis of carbohydrates *in vivo* depends on environmental digestive conditions and times of retention in the foregut. In monogastric animals in general, especially poultry, the times of retention are considerably short for an effective NSP hydrolysis (Almirall and Esteve-Garcia, 1994; Ouhida et al., 2000). However, whether a potential release of cell wall monosaccharides within the small intestine causes an energetical advantage is unclear. Marsman et al. (1997a) confirmed significant changes in the ileum digestibility of NSP in poultry but failed to observe significant increases in the animal performances. A complete hydrolysis of NSP to monosaccharides may expose monosaccharides to absorption in the gastrointestinal (GI)- tract. However, selective absorption of monosaccharides occurs in the GI-tract of a variety of animals (Wilson and Vincent, 1955). Galactose and glucose are efficiently absorbed, but mannose, arabinose and xylose are absorbed at low rates (at only 20% of glucose). Longstaff et al. (1988) estimated significant decreases in the ileal digestibility and the metabolizable energy of diets supplemented with xylose, arabinose, galacturonic and glucuronic acids.

Nevertheless, nutritional effects other than the release of entrapped nutrients should be evaluated. In particular, oligosaccharides rather than monosaccharides are generated when polysaccharide-degrading enzymes, especially endo-enzymes, are used as feed additives. These released neutral sugars may alter the microflora population of the digestive tract. In fact, a variety of oligosaccharides are beneficial in the short-term, since they selectively favour the growth of single bacterial strains or groups within the commensal flora of livestock (Orban et al., 1997). On the other hand, when solubilizing a considerable amount of both protein and cell wall components from legume seeds, it is more effective to add a mixture of protease and cell wall degrading enzymes (Marsman et al., 1997b). Despite the low enzymatic activities described on the cell wall intact structures, further research should focus on the interactions between protein and carbohydrate digestion, and on the generation of soluble oligomers *in vivo* and *in vitro.*

ABREVIATIONS USED

ChSS, chelating agent soluble solids; CP, crude protein; DASS, dilute alkali soluble solids; 1M ASS, 1 M alkali soluble solids; 4M ASS, 4 M alkali soluble solids; RES, residual; SBM, soybean meal; WSc, water soluble from cotyledons; WSh, water soluble from hulls; WUc, water unextractable from cotyledons; WUh, water unextractable from hulls; WUS, water unextractable solids.

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CAPITULO 4

Feed enzyme combinations with an identified cell wall polysaccharidase and proteolytic activity may enhance soybean meal carbohydrate and protein hydrolysis *in vitro*

Abstract

1. Raw defatted soybean meal was toasted at 85ºC for 25 min or at 125ºC for 45 min to yield mildly processed in the first case and overprocessed substrates in the second, which were compared with non-toasted substrates. Gradual toasting at 85º and 125ºC decreased the water solubilization of dry matter (38.8 and 32.4 *vs* 56%), the protein dispersibility index (38.5 and 24.6 *vs* 60.3%) and carbohydrate solubility (28 and 22 *vs* 40%), pointing to conformational changes in both the protein and carbohydrate compact folded structure.

2. Both water solutes (WS) and water unextractable (WU) substrates were tested for their accessibility *in vitro* toward various carbohydrases (with identified activities of pectinase, cellulase, xylanase and mannanase) combined or not with a protease complex.

3. Carbohydrase incubations of the WS effectively decreased raffinose and stachyose oligosaccharides, especially when pectinase and cellulase were combined with protease. Carbohydrase incubations of the water insoluble extracts (WU) released low amounts of monosaccharides (<10%) and amino acids (<10%). However, carbohydrase interacted with protease enzymes, yielding less than 21% monosaccharide and 13% amino acid release.

4. Increases in protein digestion were explained by the exo- and endo-protease activities in the carbohydrase and protease enzyme complexes rather than by protease-carbohydrase interactions. However, the increase in released monosaccharides suggests an improved cell wall carbohydrate hydrolysis derived from protein digestion.

Keywords: Soybean meal; Carbohydrase; Protease; *In vitro*.

1. Introduction

Soybean meal (SBM) is a by-product of industrial oil extraction. It has a high protein content and a relatively well-balanced amino acid pattern. Thus it is a valuable protein source for protein isolates and livestock feeding, but only after a certain amount of heat is applied to inactivate heat labile antinutritional factors, like protease inhibitors and lectins (review Liner, 1994). Moreover, heating increases the nutritional value of the compact folded SBM proteins by breaking non-covalent interactions (protein denaturation) and increasing the accessibility of proteins to enzymatic breakdown (Bhattacharya and Hanna, 1988).

However, the nutritional value of SBM is also limited by the presence of a high amount of oligosaccharides (40-60g/ Kg dry matter; Saini, 1988) and non-starch polysaccharides (210g NSP/kg dry matter; Bach Knudsen, 1997), whose composition and structure are more complex than those from cereals. In particular, α-galactosides are not digested in the small intestine of monogastric animals but degraded by the microflora (Leske and Coon, 1999a,b) to short-chain fatty acids (SCFA). Their fermentation contributes to the energy absorbed by the animal, but it may produce flatulent gases, like carbon dioxide, hydrogen and methane (Rackis, 1975), and reduce the AME of farm animals (Coon et al., 1990). It is particularly hard to hydrolyse cell wall polysaccharides because of their complex structure, especially cellulose, arabinoxylans, β-glucans, xyloglucans, xylans, rhamnogalactorunans and arabinogalactans (Aspinall et al., 1964; Aspinall et al., 1967a,b), which may have antinutritional effects on broiler chicks (Annison and Choct, 1991; Smits and Annison, 1996).

An alternative approach for further increase on the nutritive value of vegetable protein sources is the use of feed enzymes. Protease and cell wall degrading enzymes break these polymeric chains into smaller units *in vitro* (Marsman et al., 1997a; Huisman et al., 1999; Malathi and Devegowada, 2001), especially when feed enzyme mixtures are used.

This study is the first step to evaluate a range of enzyme preparations with specific cell wall degrading and protease activities for their ability to solubilize the proteins and

carbohydrates contained in the SBM *in vitro*. We first screened their potential activities and interactions in optimal conditions before further experiments *in vivo* or *in vitro* by simulating the conditions in the gut (Malathi and Devegowada, 2001). We focused on the ability of feed enzymes to increase protein hydrolysis, as justified by the high cost of vegetable proteins in animal nutrition. The differences between gradient toasting intensity and the accessibility *in vitro* of soluble and insoluble material toward a protease and carbohydrase were also examined.

2. Materials and methods

2.1. Preparation of soybean material

Three substrates of soybean meal (SBM) were prepared following the toasting procedure: no toasting (NT); mild toasting at 85ºC for 25 min (T-85); and intensive toasting at 125ºC for 45 min (T-125). The three substrates were extracted with water to yield a water-soluble extract (WS) and a water unextractable fraction (WU). Briefly, 200 g of substrates was suspended in 800 ml of distilled water containing 50 mg chloramphenicol/l for 2 h at room temperature and centrifuged at 11,000 x *g* for 30 min. The residues were resuspended and the procedure was repeated four times. The combined supernatants and the residues were freeze-dried and stored until analysis.

2.2. Enzyme incubations

Several enzyme preparations were screened for their ability to digest carbohydrates and proteins contained in the WS and WU extracted from SBM. Biopectinase NKP 120P® (830 endopolygalacturonase units/g, containing also 1400 cellulase units/g, 13270 xylanase units/g, and 9650 β-glucanase units/g; produced from selected strains of *Aspergillus niger*), Biocellulase A Conc® (8750 cellulase units/g; produced from selected strain of *Aspergillus niger*), Bioxylanase 10P® (10000 xylanase units/g, produced from *Trichoderma reesei*), Mannase® (1400000 mannanase units/g) and Bioprotease N120P® (120000 protease units/g, produced from a selected strain of *Bacillus subtilis*) were added at a ratio of 10 mg per g of substrate (20 mg/ml) in 0.05 M sodium acetate buffer (pH 5.0). WS and WU were incubated under continuous shaking at 40ºC for 12 h.

Thereafter, the enzymes were inactivated by boiling (100ºC for 10 min), centrifuged at 4000 x *g* for 5 min and the supernatants were frozen at -20ºC until further analysis.

2.3. Analytical methods

Previous to the analysis of soybean fractions, samples were lyophilised to constant weight. Protein content was assessed by the Kjeldahl method, using selenium as a catalyst and 6.25 as conversion factor. Fat was determined by the Soxhlet method.

The protein dispersibility index (PDI) was determined following the AOCS (1979) method. Briefly, 20 g of material was extracted for 10 min in 300 ml water at ±8500 rpm using a waring blender and centrifuged at 2700 x *g* for 5 min, and the protein contents in dried pellet and soluble extracts were determined by the Kjeldahl method. The nitrogen solubility index (NSI) was determined following Dale et al., (1987). Briefly, 500 mg of material was extracted for 20 min in 25 ml of 0.042 M potassium hydroxide at 500 rpm using a magnetic stirrer and centrifuged at 2700 x *g* for 5 min, and the protein contents in dried pellet and soluble extracts were again determined following Kjeldahl. PDI and NSI were calculated as a ratio between the nitrogen extracted in the supernatants and the total nitrogen content of the material.

Free amino acids in liquid supernatants after enzyme incubation were analysed by reverse-phase chromatography (Vendrell and Avilés, 1986), which was performed on 1090 HPLC (Hewlett Packard). Elution was monitored using a diode array detector (DAD UV-Vis).

Carbohydrates from WS and WU and free monosaccharides were analysed for their neutral monomer profile following Theander (1991), with inositol as internal standard. After treatment with 72% w/w H_2SO_4 (1 h, 30ºC), followed by incubation in autoclave (1 h, 125ºC) and immediate filtration (Poro 2), the monosaccharides were reduced with KBH_4 and converted to their alditol acetates using 1-methylimidazole and acetic anhydride. The alditol acetates were separated on a GLC column (25m x 0.25mm x 0.25μ; Hewlett Packard 19091 C-131) using D-glucose, D-galactose, D-xylose, L-arabinose, D-mannose, Fucose, and Rhamnose as standards (Sigma, Sant Louis USA).

Uronic acid release during enzyme incubations and the total content in the cell wall polysaccharides were determined as galacturonic acid following Theander (1991).

Sucrose and α-galactosides (raffinose, stachyose) in soybean samples were determined as described by Gdala et al. (1997). Duplicate 100-mg samples were weighed into 15 ml-test tubes and mixed with 3 ml of water on the magnetic stirrer at room temperature for 20 min. Samples were then kept in an ultrasonic bath for 10 min and after mixing with 7 ml of absolute ethanol, the test tubes were vortexed and centrifuged at 1000 x *g* for 10 min. The liquid supernatants were decanted in glass tubes placed in heating block (60ºC for 10 min) and then evaporated at 40ºC in a rotating evaporator. The residue and supernatants from enzyme incubations were filtered and analysed following HPLC procedures. Briefly, the chromatograph (1090 Hewlett Packard) was equipped with an Alltech NH_2 polymeric column (250 x 4.6 mm., 5μm) and an Alltech NH_2 polymeric guard column (10 x 4.6 mm., 5μm) maintained at 30ºC. Elution was performed with a mixed C_2H_3N/H_2O (70/30) at 1ml/min and monitored using a refractive index detector (HP 1047 A).

Duplicate incubations *in vitro* showed low variability (difference between duplicates lower than 5% for amino acids and 6% for monosaccharides). We thus present the mean values without performing statistical comparison between treatments.

3. Results and discussion

3.1: Soybean meal composition

Table 1 shows the yield and composition of WS and WU obtained from NT, T-85 and T-125 SBM samples. The WS yield varied with toasting (56.0% for NT *vs* 38.6% and 32.4% for T-85 and T-125 SBM, respectively). Thermal processing also decreased the protein dispersibility index (60.3, 38.5 and 24.6% for NT, T-85 and T-125-SBMs, respectively) and Nitrogen Solubility Index (84.3, 72.5 and 63.0%). Similar results have been reported elsewhere for toasting (Visser and Tolman, 1993) and extruding (Hendriks et al., 1994), suggesting that physical processing conditions protein structure "denaturation". Values of NSI lower than 70% or higher than 85% indicate over- or

under-processing, respectively (Araba and Dale, 1990a,b). Our results (63% for T-125) confirm that toasting at 125ºC for 45 min over-processed SBM.

The carbohydrate content of SBM measured as neutral and acidic monosaccharides was about 250 g/kg DM, being glucose (~94 g) and galactose (~80 g) the major sugars. Carbohydrate solubility was around 40% for NT, 28% for T-85 and 22% for T-125 SBMs. Carbohydrate and protein solubility data suggest that thermal processing promoted binding between proteins and carbohydrates in the soybean meal structural matrix. The conformational changes of proteins (Van den Hout, 1997) and carbohydrates (Kikuchi et al., 1971) after soybean meal heat treatment are widely described for mild toasting and overprocessing, which may affect the ability of feed enzymes to digest proteins and cell wall carbohydrates (Marsman et al., 1997a).

Table 1**: Yield (%) and composition (percent weight) of the native defatted soybean, and the water solutes (WS) and water unextractable (WU) fraction extracted from No toasted (NT), Toasted 85ºC (T-85) and Toasted 125ºC(T-125) soybean meals.**

	SBM	NT		T-85		T-125	
		WS	WU	WS	WU	WS	WU
Yield	**100**	**56.0**	**44.0**	**38.6**	**61.4**	**32.4**	**67.6**
Protein content	**53.9**	**57.2**	**49.6**	**60.9**	**41.4**	**59.9**	**45.8**
Crude fat	**0.7**	**0.4**	**1.0**	**0.3**	**0.7**	**0.4**	**0.7**
Ash	**6.9**	**10.5**	**2.4**	**9.1**	**3.3**	**9.2**	**3.2**
Carbohydrates	**24.5**	**17.9**	**37.5**	**17.9**	**35.4**	**17.3**	**36.1**
Oligosaccharides							
Sucrose	**7.25**	**8.85**	**nd**	**7.84**	**nd**	**8.89**	**nd**
Raffinose	**1.40**	**1.78**	**nd**	**1.66**	**nd**	**1.74**	**nd**
Stachyose	**3.95**	**5.02**	**nd**	**3.94**	**nd**	**6.64**	**nd**
Monosaccharide profile							
Rhamnose	**2.6**	**1.4**	**3.8**	**0.4**	**3.8**	**0.4**	**3.7**
Fucose	**1.3**	**0.0**	**2.2**	**0.0**	**2.3**	**0.0**	**2.1**
Arabinose	**10.4**	**2.7**	**17.0**	**1.4**	**16.8**	**2.5**	**16.1**
Xylose	**4.3**	**0.9**	**7.3**	**0.9**	**7.2**	**0.9**	**7.2**
Mannose	**3.5**	**13.0**	**2.2**	**11.5**	**2.0**	**12.7**	**2.2**
Galactose	**31.9**	**32.1**	**29.5**	**33.5**	**29.4**	**30.7**	**27.9**
Glucose	**37.7**	**48.4**	**28.4**	**50.9**	**28.8**	**51.8**	**29.1**
Uronic acids	**8.2**	**1.6**	**9.7**	**1.3**	**10.9**	**1.2**	**11.8**

nd: not determined

Water solute carbohydrates were mainly composed (>93%DM^{-1}) of mannose, galactose and glucose, probably contained in sucrose, α-galactosides and β-mannan polysaccharides. Indeed, the sucrose and α-galactoside contents were 72 and 53g/kg DM; the α-galactosides were composed of stachyose (±39g/kg) and raffinose (±14g/kg). It is remarkable that WS extracts did not account for the total sucrose and oligosaccharide (raffinose + stachyose) SBM content, especially in toasted SBM samples, which showed lower recoveries (40 and 51 vs 71%, in T-85 and T-125 vs NT-SBM, respectively). Similar results have been described by Irish et al. (1995) on removing α-galactosides by sequential ethanol and water extraction of toasted SBM. Water solubilization allowed only 18% extraction of that obtained with ethanol. Differences in solubilization with thermal processing point to interaction between oligosaccharides, proteins and cell wall polysaccharides in the WU fraction during the heating procedure.

3.2. Effect of several enzymes on the hydrolysis of carbohydrates from water-soluble (WS) fractions

Tables 2 and 3 show the efficiency with which various cell wall carbohydrases, as single enzymes or combined with protease, respectively, reduce free α-galactosides in the supernatants (% $content^{-1}$) and release free monosaccharides. Unexpectedly, the sucrose and α-galactoside contents in the supernatants of buffer incubations were lower than after Bioprotease incubation. Thermal processing also increased the amount of free sucrose and oligosaccharides, especially for mild treatment (T-85 SBM). Di- and oligosaccharides in WS were present as free or bound molecules and protease disrupted the bound complexes. These toasting-induced changes confirm the structural and chemical modifications caused by thermal processing, which probably involve insolubilization of the protein-carbohydrate complex and the fact that they are not recovered in the WS fraction.

Table 2: *Amount of free (mg/g content) sucrose (D2) and oligosaccharides (D3: raffinose and D4: stachyose) in the supernatants obtained after incubation of WS fractions from NT, T-85 and T-125 SBM with cell wall degrading enzymes with or without protease (Prot.) combination.*

	Bioprotease		Biopectinase		Biocellulase		Bioxylanase		Mannase	
	D2	*D3+D4*	*D2*	*D3+D4*	*D2*	*D3+D4*	*D2*	*D3+D4*	*D2*	*D3+D4*
NT-SBM										
Bioprot (-)[a]	**216**	**351**	**21**	**156**	**68**	**355**	**328**	**634**	**69**	**676**
Bioprot (+)	**521**	**835**	**20**	**143**	**73**	**388**	**408**	**669**	**42**	**599**
T-85 SBM										
Bioprot (-)	**318**	**605**	**28**	**337**	**72**	**420**	**380**	**730**	**92**	**819**
Bioprot (+)	**605**	**1018**	**24**	**417**	**82**	**462**	**611**	**629**	**66**	**1018**
T-125 SBM										
Bioprot (-)	**454**	**492**	**52**	**187**	**46**	**249**	**579**	**561**	**103**	**413**
Bioprot (+)	**614**	**508**	**47**	**147**	**82**	**251**	**673**	**524**	**84**	**422**

[a] Bioprotease x Bioprot(-)=Blank

Single cell wall carbohydrase incubation also modified the free oligosaccharide content in the WS supernatants. In particular, Biopectinase, Biocellulase and Mannase significantly decreased sucrose, indicating a side sucrase activity that was not observed in Bioxylanase. On the other hand, the free α-galactoside concentration (*vs* control) was lower for Biopectinase and Biocellulase and higher for Bioxylanase and Mannase incubations. Biopectinase and Biocellulase allowed the hydrolysis of α-galactosides, whereas Bioxylanase and Mannase may have released soluble bound oligosaccharides, probably through a side protease activity. Combined incubation of cell wall carbohydrase enzymes with Bioprotease may provide further evidence of the α-galactoside hydrolysis. Both Biopectinase and Biocellulase with Bioprotease greatly decreased raffinose and stachyose. No consistent decreases were observed with Bioxylanase or Mannase. Disappearance of α-galactosides may be promoted by cleavage of the fructose and glucose moieties (invertase activity; Slominski, 1994) or the distal α-(1, 6)-galactose units (α-galactosidase activity; Irish et al., 1995), with intermediary conversion from stachyose to raffinose. Biopectinase with Bioprotease incubation promoted similar decreases in free raffinose and stachyose (more than 60% content^{-1}), paralleled by a high glucose release (84% content^{-1}; Table 3), which suggests a pronounced invertase activity. On the other hand, Biocellulase with Bioprotease reduced stachyose (74% content^{-1}) more than raffinose (42% content^{-1}), together with higher releases of galactose (38% content^{-1}; Table 3), suggesting a side α-galactosidase activity.

Table 3. ***Amount of free major neutral monosaccharides (% content^{-1}) solubilized[a] from the mildly toasted SBM (T-85)-WS fraction after cell wall degrading enzyme incubation with or without Bioprotease (Prot.) combination.***

	Bioprotease			Biopectinase			Biocellulase			Bioxylanase			Mannanase		
	Man[a]	*Gal*	*Glu*	*Man*	*Gal*	*Glu*	*Man*	*Gal*	*Glu*	*Man*	*Gal*	*Glu*	*Man*	*Gal*	*Glu*
Prot (-)[b]	**30.8**	**14.9**	**28.4**	**95.6**	**22.7**	**82.3**	**76.1**	**31.2**	**69.7**	**37.0**	**17.8**	**37.2**	**67.4**	**17.9**	**68.2**
Prot (+)	**36.3**	**21.6**	**34.5**	**99.8**	**26.8**	**84.2**	**90.7**	**38.5**	**81.8**	**36.0**	**20.5**	**35.1**	**70.9**	**21.6**	**71.0**

[a] Man: mannose; Gal: galactose; Glu: glucose.

[b] Bioprotease x Prot (-)= blank

Among monosaccharides, glucose and mannose were more released than galactose, being Biopectinase more efficient than Biocellulase, Mannase and Bioxylanase. Then, mannanase activity was also evidenced in the enzyme complex identified as Biopectinase and Biocellulase.

3.3: Effect of several enzymes on the solubilization of monosaccharides and amino acids from the water-insoluble (WU) fractions

Table 4 presents the amounts of neutral and acidic sugars, and free amino acids released from WU by various single cell wall carbohydrase and protease enzymes. On average, monosaccharide release was lower than 20%, whereas total amino acid release was lower than 13% of the values in the WU fraction. The results obtained by Malathi and Devegowda (2001) *in vitro* were even lower, and no more than 1% of monosaccharides bound in the NSP fraction were released after incubation of soybean meal with enzyme combinations containing cellulase, pectinase or xylanase. Our values and those obtained by Malathi and Devegowda (2001) were also significantly lower than the 60% and 50% reported by Marsman et al. (1997a) for neutral sugars and soluble proteins, respectively. However, these differences may be due to the analytical procedures used rather than to the efficacy of feed enzymes. Indeed, Marsman et al. (1997a) measured the total fragments of neutral cell wall polysaccharides released during enzyme incubations by the automated orcinol method (Tollier and Robin, 1979), which measures monomers and oligomers. Further characterization by high performance size exclusion chromatography (HPSEC) showed that the amount of

released monomers ranged from 2 to 22%, as obtained in the present incubations. The higher soluble nitrogen content observed by Marsman et al. (1997a) compared with the results obtained in the present study after free amino acid analysis may also be due to differences in the analytical procedures. Feed enzymes break polymeric chains into smaller molecules, but in practice, added enzymes rarely cause total hydrolysis of the target substrate. As result, large amounts of peptides and oligosaccharides are released.

Thermal processing did not alter the amount of neutral monosaccharides released from WU by the enzyme incubations, but the amount of amino acids released from toasted SBM slightly increased. This may be due to differences in the composition of insolubilized substrates associated with thermal processing or to conformational changes, like disruption of the non-covalent interactions. On the other hand, Marsman et al. (1997a) reported slight increases in the release of neutral sugars and amino acids with toasting and extrusion. This increase was more marked with extruded than with toasted SBM, suggesting that at intense shear forces and excess mechanical energy, covalent disulfide bounds between proteins are easily broken (Arêas, 1992) and proteins tend to aggregate after cooling without the renaturation phenomenon described for toasting (Jaenicke, 1965).

As expected, Bioprotease showed very low carbohydrate hydrolysis, whereas measurable monosaccharides were released by the cell wall degrading enzymes, especially for Biopectinase, Biocellulase and Mannase (approximately 10%) and less so for Bioxylanase (4-5%). However, significant interaction was observed when carbohydrase was incubated with Bioprotease, as reflected by the increase in the amount of monosaccharides released by Biopectinase (19-21%), Biocellulase (12-16%) and Mannase (17-21%). Bioprotease may increase the accessibility of the cell wall matrix to the carbohydrate-degrading enzymes.

Table 4. ***Amount of total monosaccharides (neutral and acidic monosaccharides; NS+UA) and free amino acids "AA" (% content^{-1}) solubilized from SBM (NT: no toasted; T-85: toasted 85ºC; T-125: toasted at 125ºC)-WU fractions and casein after cell wall degrading enzyme incubation with or without Bioprotease (Prot) combination.***

	Bioprotease		Biopectinase		Biocellulase		Bioxylanase		Mannase	
	AA	NS+UA	AA	NS+UA	AA	NS+UA	AA	NS+UA	AA	NS+UA
NT-SBM										
Prot (-)[a]	1.0	0.8	5.5	9.2	7.1	11.7	1.0	5.0	2.8	12.5
Prot (+)	2.5	2.5	9.2	21.9	8.5	16.0	3.9	4.2	7.9	20.6
T-85-SBM										
Prot (-)	1.6	0.5	6.0	7.0	9.7	8.9	1.8	4.0	4.3	8.3
Prot (+)	2.7	2.7	10.4	19.8	11.7	12.0	6.4	4.7	9.6	17.8
T-125-SBM										
Prot (-)	1.5	1.1	5.9	9.3	8.6	10.8	1.6	4.1	3.6	8.6
Prot (+)	3.2	3.2	12.3	21.0	11.1	14.0	7.4	4.3	7.5	17.8
Casein										
Prot (-)	0.0	-	8.3	-	16.3	-	0.0	-	3.6	-
Prot (+)	2.3	-	18.7	-	28.8	-	4.6	-	9.2	-

[a]Bioprotease (+)*Prot (-): blank

On the other hand, Bioprotease released an unexpectedly low amount of amino acids (lower than 3.5%), even lower than carbohydrase incubations, like Biopectinase (5.5-6.0%) or Biocellulase (7.0-10.0%). Again, simultaneous incubation with carbohydrase and protease mixtures significantly increased the amino acid release in an additive (Biocellulase) or synergic (Biopectinase, Bioxylanase and Mannase) manner. Similar results were obtained by Marsman et al. (1997a), who concluded that to solubilize considerable amounts of both protein and cell wall components, a mixture of a protease and a cell wall degrading enzyme at lower concentrations is more efficient than separate addition of the preparations at higher doses. Protease and carbohydrase may interact and thus allow the enzymes to penetrate and reach the complex cell wall proteins and carbohydrates sites for hydrolysis (Marsman et al., 1997a).

To test this hypothesis, we measured the efficiency of feed enzymes to digest casein, as a model of a single purified protein source (Table 4). Similar results regarding the

interaction between protease and carbohydrase activities were observed, which may reflect an interaction between proteases rather than a protease-carbohydrase interaction. Endo-protease activity in the protease enzymes and exo-protease activity in the carbohydrase enzymes increased the amount of amino acids released from both casein and SBM. On the other hand, the significant interaction observed upon monosaccharide release from SBM may also point to physical benefits for the cell wall carbohydrate hydrolysis derived from protein hydrolysis.

Table 5 shows the release of monomers and amino acids, as obtained after a more descriptive analysis of carbohydrate and protein digestion. Monosaccharides are presented according to the composition of WU-SBM: mainly glucose (28%), galactose (30%), arabinose (17%) and uronic acids (10%); while released nutritional essential amino acids, such as lysine, methionine and threonine were chosen as protein hydrolysis index. As expected, different incubations led to differences in the main monosaccharides released: glucose and uronic acids for Biopectinase, glucose for Biocellulase and Bioxylanase, and arabinose for Mannase. However, we observed closer changes among carbohydrase enzymes, which were associated with the simultaneous protease incubation, as reflected by the significant increases in galactose release with Biopectinase, Biocellulase and Mannase. Further increases in uronic acids for pectinase and arabinose for mannanase were also observed. It appears that enzymes act on cell wall material at several stages, attacking first the potentially accessible material, as shown in the present incubations, and then pectic compounds or highly substituted galactomannans (Düsterhöft et al., 1993). Biopectinase, but also Biocellulase and Mannase, efficiently cleaved the alpha- and beta-galactan chains progressively exposed by protein digestion. On the other hand, cellulosic structures were hardly accessible in the hidden cell wall matrix (Marsman et al., 1997a; Huisman et al., 1999). As to nutritional essential amino acid release, we would like to highlight the significant differences between amino acids: for instance, lysine and methionine were released to a higher extent than threonine. Exo-proteases of Biopectinase and Biocellulase also promoted a higher release of lysine than of methionine, but the latter was widely released upon simultaneous incubation with the endo-proteases of Bioprotease. Proteases have specific affinities for hydrolyzing the binding of certain amino acids (e.g. Lys and Arg for the tripsine enzyme). Clear differences were also observed between

SBM and casein, as reflected by the higher accessibility of casein *vs*. the insoluble and carbohydrate protected SBM-water unextractable proteins.

*Table 5**: Individual neutral sugars, uronic acids and free amino-acids (% content^{-1}) solubilized from SBM (T-85: toasted 85ºC)-WU fraction and casein after cell wall degrading enzyme incubation with or without Bioprotease combination.***

	Bioprotease		**Biopectinase**		**Biocellulase**		**Bioxylanase**		**Mannase**	
Bioprotease	**(-)**	**(+)**	**(-)**	**(+)**	**(-)**	**(+)**	**(-)**	**(+)**	**(-)**	**(+)**
T-85-SBM										
Monosaccharides										
Arabinose	**nd**	**nd**	**5.5**	**9.3**	**4.4**	**6.1**	**0.4**	**0.5**	**20.0**	**37.3**
Galactose	**0.3**	**2.5**	**3.3**	**35.1**	**3.0**	**17.6**	**0.9**	**2.7**	**1.8**	**17.1**
Glucose	**0.5**	**3.8**	**9.8**	**11.2**	**15.0**	**15.7**	**8.8**	**8.8**	**8.3**	**10.4**
Uronic acids	**1.6**	**2.8**	**9.2**	**17.7**	**5.3**	**10.1**	**1.3**	**2.7**	**2.5**	**3.9**
Amino acids										
Lysine	**1.8**	**10.4**	**8.6**	**14.5**	**15.7**	**18.1**	**1.9**	**10.3**	**5.2**	**12.6**
Methionine	**1.9**	**12.3**	**6.5**	**14.8**	**11.3**	**18.1**	**1.9**	**12.0**	**6.2**	**15.3**
Threonine	**0.6**	**5.6**	**3.4**	**4.6**	**4.0**	**4.8**	**1.5**	**2.9**	**2.8**	**4.9**
Casein										
Lysine	**nd**	**nd**	**23.7**	**42.9**	**49.3**	**68.7**	**nd**	**3.8**	**2.2**	**2.5**
Methionine	**nd**	**nd**	**9.0**	**40.2**	**18.7**	**85.1**	**nd**	**nd**	**nd**	**3.7**
Threonine	**nd**	**nd**	**5.6**	**13.0**	**7.0**	**16.6**	**nd**	**1.4**	**2.4**	**5.4**

nd: not detected

The present results confirm that commercial enzymes consist of enzyme complexes rather than of purified enzymes with a single specific activity. In particular, we evidenced high exo-protease activities in several carbohydrase enzymes with identified pectinase and cellulase activities. However, further combinations of enzymes, especially between identified protease or carbohydrase enzymatic activities may also prove more efficient than single enzymatic incubations for protein digestion and hydrolysis of cell wall carbohydrates.

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CAPITULO 5

Effect of β-mannanase in soybean meal diets on broiler performances and ileal and whole-tract digestibility.

ABSTRACT

1. An experimental trial with broiler chicks was conducted to investigate the effects of a mannanase enzyme on the performance and nutrient digestion of maize-soybean diets, with an especial emphasis on changes in chyme characteristics along the digestive tract. Mannanase was added at three levels, (0, 350 and 875ppm) combined or not with a protease enzyme (0 and 350ppm).

2. Neither bodyweight gain nor feed intake were affected by feed enzymes, but food:gain ratios trend to be decreased (1.55 vs 1.59; P=0.07) by protease supplementation.

3. Not consistent effects were observed on ileal and faecal organic matter (OM) and crude protein (CP) digestibility.

4. Mannanase reduced ileal digesta microbial fermentation measured as purine bases concentration in ileum, but not effects were detected at caecal level.

1. INTRODUCTION

Soybean meal (SBM) is known to have a high content of protein and a well-balanced amino-acid pattern, which makes it the single most important source in livestock feeding (World trade of soybean ≈101 millions tonnes, reaching 60% of concentrate legume seeds; FAOSTAT, agriculture, 2000). However the nutritional value of SBM is not potentially exploited, and out of antinutritional factors (Liener, 1994) and thermal over- or under-processing (Araba and Dale, 1990a,b), the digestibility of the dry matter and carbohydrates by poultry is considered low (NRC, 1994). In fact, the presence of some carbohydrates, such as oligosaccharides (±6 $\%DM^{-1}$; Bach Knudsen, 1997), is associated with decreases of the metabolizable energy (AME) of SBM diets fed to broiler chicks (Leske et al., 1999a,b). Other polysaccharides, such as β-mannans (±2 $\%DM^{-1}$SBM; Chesson, 1987), may also cause antinutritive effects in monogastric animals. Inclusion of 2 to 4% of galactomanan in feed is known to retard growth and decrease feed efficiency in broilers (Couch et al., 1967; Ray et al., 1982; Verma and McNab, 1982; Iji et al., 2000). Among mechanisms involved, β-mannans may alter the intestinal enzyme synthesis (Iji et al., 2000) and interfer with glucose metabolism and insulin secretion rates (Leeds et al., 1980; Sambrook and Rainbird, 1985), which may impair intestinal uptake and utilisation of glucose and amino acids by peripheral tissues.

One possible mean of improving the nutritive value of mannan-rich-ingredients is the use of enzymes. In fact, this would be specially profitable if protein digestibility was improved. However, in our knowledge, at now very few reports has been published (Ward and Fodge, 1996; McNaugton et al., 1998), which investigated the response of broiler chicks fed on cereal-soy diets to mannanase supplementation.

The present experiment was designed to evaluate the effects of an inclusion of mannanase in the diet (corn-SBM) on the growth performances, apparent faecal and ileal nutrient digestibility, and chyme characteristics in broiler chicks. A dose response study was performed either combined or not with protease to focus on protein digestion. The highest mannanase supplementation was fixed at 0.87g/kg, because higher doses (1.74 g/kg) have been previously to show in our lab detrimental effects on feed intake and feed conversion rates.

2. MATERIAL AND METHODS

2.1. Animals and housing

One-day-old broiler chickens of the Ross strain were fed a starter diet and on day 6 (body weight = 85.9 g/bird) were distributed by 6 into 36 pens (50 x 60 cm^2 each one) and offered the experimental treatments. Ambient temperature was gradually decreased from 32ºC at day 1 to 22ºC at the end of the growth period (42 d). An alternative artificial light program (23: 1) was applied.

2.2. Experimental procedures

The experiment comprised 6 treatments in a 3 x 2 factorial design, with 6 replicates per treatment. A cage was considered as a replicate experimental unit. Treatments consisted of maize-SBM based diet (formulated according to NRC, 1994, Table 1) supplemented with three levels (0, 350 and 875 ppm) of Mannase® (1400000 mannanase units/g) and two levels (0 and 350 ppm) of Bioprotease N120P® (120000 protease units/g, produced from a selected strain of *Bacillus subtilis*). Both enzyme complexes were provided by Quest International Company (Ireland). Cr_2O_3 was added (10 mg/Kg) as an indigestible marker.

2.3. Data recording and analysis

Body weight (BW) and feed intake (FI) per replicate was recorded at 6, 21 and 42 day of age. At day 22 and 42, samples of faeces were collected and dried in an air forced oven (105ºC) and stored until analysis of Chromium, OM and CP. After faeces collection, three chicks from each cage were slaughtered by an intracardiac injection of 1 ml sodium pentobarbital and ileum (15 cm starting from the ileocaecal junction) and caeca digesta were collected for chemical analysis. A fraction of caeca digesta was immediately acidified with H_3PO_4 (approximately 2 g fresh weight digesta/ 1ml of 5% H_3PO_4, 50 mM 3 methyl valerate as internal standard) for Short Chain Volatile Fatty Acids (SCVFA) analysis. Ileum and caecal digesta samples were freeze-dried and grounded (0.2mm) for chemical analysis of chromium, OM, CP and purine bases.

Table 1. Ingredient composition (g/kg) and analysis of the basal diet.

Ingredients and analysis	g/kg
Corn grain	593
Soybean meal (44 %CP)	258
Fulfat soybean meal	100
Sunflower oil	10
DL-methionine	2.3
Dicalcium phosphate	17.7
Calcium carbonate	10.7
Premix[1]	7.8
Calculated analysis	
CP	195
AME (kcal/ kg)	3150
Total Lys	11.0
Total Met + Cys	8.5
Calcium	10.0
Available phosphorus	4.5

[1]Premix contained per kilogram of diet: NaCl, 4.8g; Vitamin A, 10000 IU; Vitamin D3, 3000 IU; Vitamin E (α-tocopherol), 15.3 mg; Vitamin K_3, 15μg; Vitamin B_1, 2mg; Vitamin B_2, 4.5mg; Vitamin B_6, 3mg; Vitamin B_{12}, 15μg; Pantothenic acid, 8mg; Choline chloride, 230mg; Folic acid, 0.5mg; Nicotinic acid, 25mg; Biotin, 30μg. Minerals: Co, 0.25mg; Se, 0.2mg; Fe, 40mg; Cu, 8mg; Mn, 150mg; Zn, 80mg; I, 1mg; and Ethoxiquin, 0.9mg.

DM of digesta was determined by drying to constant weight at 105ºC and OM by ashing at 550ºC for 8h. Total nitrogen was analysed with semi-automated Kjeldahl method using Se as catalyst. SCVFA concentrations in caecal digesta were determined by gas liquid chromatography, following the method proposed by Jouany (1982). Purine bases in ileal and caecal digesta were determined by HPLC after acid hydrolysis with 2 ml 2N-perchloric acid at 100ºC for 1h, adding 0.75 μmol of allopurinol and neutralising immediately with 4.5 M KOH (Makkar and Becker, 1999). Chromium concentration was determined following the method of Williams et al. (1962).

2.4. Statistical Analysis:

Analysis of variance of data was performed using the General Linear Models procedure of SAS® (SAS Institute, 1996) with cage mean as experimental unit.

3. RESULTS AND DICUSSION

In Table 2 are summarised the broilers performances from 6 to 21d and over 6 to 42 days old. Enzymes supplementation, either mannanase or protease, did not affect feed intake and BW gain. Protease supplementation trend to decrease feed: gain ratio (1.55 vs 1.59; P=0.07) during the first experimental period. However, despite no interaction effects were observed, differences were not consistent among the three levels of mannanase and were especially marked by the slight increases of FCR with 0.35g/Kg of mannanase.

Table 2. Average values of body weight, feed intake and Food conversion ratio (FCR) of broiler chickens fed on diets SBM diets with or without enzyme supplementation.

Enzyme combinations		6-21 days old			6-42 days old		
Mannase (g/kg)	Protease (g/kg)	*Body weight (g)*	*Feed Iintake (g)*	*FCR, (g/g)*	*Body weight (g)*	*Feed Iintake (g)*	*FCR (g/g)*
0	0	704	968	1.57	2035	3377	1.73
0.35	0	703	1000	1.62	2014	3422	1.77
0.87	0	713	984	1.57	2048	3408	1.74
0	0.35	727	1000	1.56	2098	3451	1.72
0.35	0.35	712	967	1.54	2027	3386	1.74
0.87	0.35	713	979	1.56	2060	3415	1.73
Mean		712	983	1.57	2046	3409	1.74
S.E.		15.4	29.4	0.024	45.5	85.3	0.017
				Probability			
Protease		0.40	0.94	0.07	0.43	0.83	0.18
Mannanase		0.86	0.99	0.76	0.58	0.99	0.16
Protease*mannanase		0.75	0.55	0.18	0.81	0.80	0.75

Ward and Fodge (1996) and McNaughton et al. (1998) referred significant increase of the average daily gain and feed efficiency of animals fed on SBM diets supplemented with β-mannanase. Differences were associated with significant increases in the energy digestibility since broilers fed low energy feeds treated with enzyme showed similar bodyweight and feed: gain ratios than broilers fed higher energy feeds without enzyme (McNaughton et al., 1998). In the present experiment basal diet was slightly low in protein (195 g/kg) and also in energy (3150 Kcal/kg), which could preclude productive responses if protein and energy are not simultaneously increased. However, not significant differences were observed in ileum or whole-tract digestibility associated with protease or mannanase supplementation.

Apparent ileal and whole tract organic matter (OM) digestibility and crude protein (CP) retention are showed in Table 3. Ileum OM digestibility was lower in 21 than 42 days old. Average values were 76.8 vs 79.6% for OM and 82.7 vs 84.6% for CP digestibility in 21 and 42d old broilers, respectively. Age differences were not observed in the whole tract data, reflecting the influence of hindgut fermentation of digesta in young animals. Protease and mannanase supplementation did not modify significantly the OM or CP digestibility. However, mannanase supplementation trend to increase (P=0.06) the apparent whole tract OM digestibility in 21 days old animals, likely determined by differences on carbohydrates or lipid digestion. We do not present results on carbohydrate digestion or metabolizable energy, which could help to know the main fraction affected. Marsman et al. (1997a) observed increases on the maiz-SBM NSP digestion with supplemented carbohydrase (Energex), which were not associated with changes on animal productive performances.

Whether a potential release of cell wall monosaccharides within the small intestine causes energy advantage, is not definitively confirmed. From a theoretically point of view, it is suggested that complete hydrolysis of NSP to free monomers may expose them to absorption in the gastro-intestinal tract. Marsman et al., (1997b) estimated a 6% of increases in AME (Kcal/kg) in broiler chickens fed on conventional corn-SBM (60: 35%) diets, assuming that all released NSP-sugars regarded as glucose monomers. However, it has been described that selective absorption of monosaccharides occurs in the small intestine (Wilson and Vincent, 1955). Compared to galactose and glucose, mannose, arabinose and xylose are absorbed and metabolised at lower rates. These results are confirmed by Longstaff et al. (1988), who reported significant decreases in the ileal digestibility and metabolizable energy of diets

supplemented with additional amounts of xylose, arabinose, galacturonic and glucuronic acids.

If a non-direct effect through carbohydrate hydrolysis is accepted, other mechanisms should be involved in the productive responses observed by Ward and Fodge, (1996) and McNaugton et al., (1998). Mechanisms by which soluble SBM-NSP exerts their anti-nutritive effects are complexes. One explanation is that soluble NSP may increase ileal digesta viscosity and decrease digestibility, like β-glucans and arabinoxylans from cereals (review of Smits and Annison, 1996). This hypothesis have been confirmed by Maisonnier et al. (2001), who reported increases on intestinal viscosity and decreases on nutrient absorption by guar gum supplementation, (1 to 3 g/kg foods), to corn-SBM diets. Not significant differences were also observed in productive performances. Guar gum has been described as a beta-manan chains to which D-galactose units are attached in 2:3 ratio, almost identical the chemical structure of SBM β-manans (Whistler and Saarnio, 1957).

Table 3. Ileal and faecal organic matter (OM) and crude protein (CP) digestibilities (%) of corn-SBM diets with or without enzymes supplementation.

Enzyme combinations		21 days old				42 days old			
		Ileum		*Faeces*		*Ileum*		*Faeces*	
Mannase	Protease	*OM*	*CP*	*OM*	*CP*	*OM*	*CP*	*OM*	*CP*
0	0	76.8	82.0	79.6	79.0	79.7	84.3	80.7	78.8
0.35	0	77.4	82.2	79.0	78.7	79.4	84.0	81.1	80.9
0.87	0	76.1	82.1	80.3	79.5	80.7	85.7	80.2	80.6
0	0.35	75.5	83.2	79.6	80.2	79.6	83.8	79.6	80.6
0.35	0.35	76.2	83.1	80.0	78.9	79.5	84.4	80.2	80.4
0.87	0.35	78.5	83.6	80.8	80.6	78.8	85.2	82.2	80.9
Mean		76.8	82.7	79.8	79.5	79.6	84.6	80.7	80.4
S.E.		1.26	1.05	0.47	0.91	0.59	0.64	0.73	1.02
		Probability							
Protease		0.49	0.18	0.24	0.27	0.19	0.72	0.98	0.53
Mannanase		0.98	0.96	0.06	0.37	0.86	0.29	0.38	0.53
Protease*mannanase		0.79	0.96	0.49	0.88	0.22	0.15	0.07	0.52

From this perspective, other digestive parameters, likely related with slightly changes on viscosity could be modified by manans degrading enzymes. In particular, earlier authors have proposed a narrow relationship between soluble NSP and microflora colonisation of small intestine (Fuller, 1984; Choct et al., 1996; Dänicke et al., 1997a,b; Vahjen et al., 1998). Changes on microflora were also associated with significant decreases of the OM digestibility, especially of saturated fatty acids by deconjugation of bile acids by enterococci (Dänicke et al., 1999).

Effects of mannanase and protease supplementation on the microbial colonisation were studied based on the analysis of SCVFA and purines concentration in ileal and caecal digesta (Table 4). SCVFA concentration in caeca digesta was 118 ːmol/g FM, slightly lower than values observed in caecum digesta of pigs (167 ːmol/g FM; Pérez et., 2001) and higher than in rumen liquids (85 ːmol/g FM; Pérez et al., 1997). However, a large variability (59% of CV) was observed, which preclude any comparison among experimental treatments. On the other hand, purine bases concentration in ileum digesta ranged between 4.8 and 9.5 ːmol/gDM, showing a drastic increment from ileum to caeca (from 6.9 to 82 ːmol/gDM). Assuming a relationship of 188 PB/gOM, determined in microflora isolated from rumen liquor (Pérez et al., 1997), microbial content was 34 mg/gDM (30 to 38) vs 402 mg/gDM (388 to 416), respectively in ileal and caecal digesta.

Significant changes were observed on the PB content in ileum and caeca digesta from 21 to 42 days old animals. As an average, PB concentration in ileum digesta was 5.6 vs 7.6 ːmol/g DM ($p<0.001$) in 21 and 42 days old animals. Age dependent differences could reflect an increase on the microbial colonization of the small intestine, likely associated with changes of the digesta water content and viscosity (García, 2000). On the other hand, PB decreases in caeca digesta were concordant with the significant age-dependent increases on ileum digestibility, which suggest a decrease on the supply of fermentable substrates.

Mannanase supplementation promoted significant decreases on the PB content in ileum digesta, 5.28 vs 6.35, ($p<0.05$) with 21d old broilers and 7.17 vs 8.05, ($p=0.6$) with 42d old broilers, supplemented and not supplemented respectively. Differences could reflect changes on the amount of substrates arriving ileum for fermentation. From a theoretical point of view, microflora inhabiting ileum and caeca obtain their energy from dietary sugars escaping

foregut digestion, which could reveal that mannanase was able to promote the release and absorption of significant amounts of sugars, likely from β-manans.

A trend to increased ileal PB concentration with protease supplementation (5.90 vs 5.37, P=0.11; and 8.03 vs 6.90, p=0.16, respectively at 21 and 42 d old broilers) also was detected concomitant with a trend to reduce PB in caeca of 42 days old broilers (p=0.6).

Table 4: Short Chains Volatile Fatty Acids (SCVFA) concentration in caecal digesta and purine bases contents (μmol/g) in ileum and caecum digesta of broilers fed on SBM diets with or without enzymes.

Enzyme combinations		SCVFA		Purine bases			
		21 days	*42 days*	*Ileum*		*Caeca*	
Mannase	Protease			*21 days*	*42 days*	*21 days*	*42 days*
0	0	107	118	5.8	7.1	80.5	76.3
0.35	0	102	127	5.5	6.9	81.5	75.8
0.87	0	113	132	4.8	6.7	75.1	72.6
0	0.35	128	164	6.9	9.0	74.5	73.4
0.35	0.35	125	101	5.9	9.5	78.3	69.8
0.87	0.35	136	95	4.9	5.6	79.1	69.2
Mean		118	123	5.6	7.6	78.2	72.9
S.E		28.2	19.7	0.42	0.74	3.51	2.81
				Probability			
Protease		0.40	0.41	0.11	0.16	0.87	0.08
Mannanase		0.89	0.27	0.04	0.06	0.27	0.38
Protease X mannanase		0.66	0.12	0.48	0.70	0.73	0.84

Previous trials "in vitro" have shown the activity of protease over water-soluble extracts releasing bound α-galactosides as free α-galactosides (100 vs 32% content^{-1}). Meanwhile, protease+mannanase incubation *in vitro* increased slightly the monosaccharides release (17.8 vs 8.3%) of non soluble fractions, mainly mannose, arabinose and galactose (Ouhida et al.,

Chapter 3); and is also supposed to increase significantly the release of oligosaccharides by disrupting cell wall polysaccharides (Marsman et al., 1997a). Changes on the amounts and accessibility of carbohydrates flowing in digesta appear to promote in ileum and caeca changes on the rate and extent of microbial fermentation. Choct et al., (1996) reported co-ordinated changes on the SCVFA concentration in ileal and caecal digesta after incorporating carbohydrases in sorghum based diet supplemented with 66g of wheat soluble NSP extracts/kg. The authors suggest that enzymes supplementation were associated with increases of SCVFA concentration in caeca, while the opposite appers in the present experiment. However, results in caecal digesta (PB and SCVFA) in the present experiment in response to experimental treatments indicate this is not a proper site to check microbial proliferation as an index of soluble NSP negative effects.

The result of this experiment suggest that, still no increases on productive performances were observed, mannanase supplementation have an influence on the gastro-intestinal microbial flora colonisation. We should continue to study if this change likely modify the risk of disbiosis, specially in practical conditions.

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CAPITULO 6

DISCUSIÓN GENERAL

1. LAS CARBOHIDRASAS MEJORAN EL VALOR NUTRITIVO DE LOS CEREALES VISCOSOS.

En la actualidad, la incorporación de cebada y trigo en los piensos destinados a las aves en crecimiento no presenta las limitaciones tradicionales que presentaba hace pocas décadas. En concreto, la suplementación enzimática (β-glucanasas y arabinoxilanasas) ha permitido incrementar la competitividad de ingredientes como la cebada, trigo, triticale, centeno o sorgo en la alimentación de las aves (Choct y col., 1996; Albustany, 1996; Marquardt y col., 1996; Williams y col., 1997; Dänicke y col., 1999; Lázaro, 1999; García, 2000). La mejora en los resultados productivos se asocia en algunos casos con incrementos en la ingestión del alimento (Campbell y col., 1984; Campbell y col., 1989). Si bien, en la mayoría de los trabajos revisados, las mejoras productivas reflejan incrementos significativos en la utilización de los nutrientes (Brenes y col., 1993; Carré y col., 1992; Vranjes and Wenk, 1995; Albustany, 1996; Marquardt y col., 1996; Dänicke y col., 1999). Resultados similares han sido observados en nuestro primer y segundo ensayo (Capitulo 2) en los que las mejoras de los resultados zootécnicos estuvieron determinadas por las mejoras en la digestión y absorción de los nutrientes. Las mejoras en la digestibilidad de la MO y EE, y la retención de N, permitieron incrementar las concentraciones energéticas de la dieta (EMAn) fundamentalmente durante la primera fase de crecimiento de los animales.

Entre los nutrientes mas afectados en su digestibilidad con la incorporación de enzimas a la ración, se distinguen las grasas (Salih y col., 1991; Frieson y col., 1992; Martinez,1992), sobre todo saturadas de origen animal (Martinez,1992; Choct y Annison, 1992; Choct y col., 1996; Dänicke y col., 1999). Este efecto ha sido observado en los balances de digestibilidad realizados en el presente trabajo, coincidiendo plenamente con la bibliografía. En el primer y segundo ensayo se utilizaron respectivamente sebo (elevada en AG saturados) y aceite de girasol (elevada en AG instaurados) y presentaron respectivamente incrementos de +12.9u.p., ($P<0.01$) y 6u.p., ($P>0.05$) con la suplementación de enzimas.

Diferentes autores han sugerido que modificaciones físico-químicas en los procesos de digestión y absorción de los nutrientes en el tracto digestivo explican las mejoras en

digestibilidad con la suplementación enzimática. Entre estas modificaciones se destaca, en el mismo sentido que nuestros resultados experimentales, una reducción de la viscosidad de la digesta intestinal (Viveros y col., 1994a,b; Barrier-Guillot y col., 1995; Bedford, 1996; García, 2000), que puede facilitar la interacción entre los substratos y los enzimas endógenos, y la difusión de los nutrientes a través del epitelio intestinal (Smits y Annison, 1996; Bedford, 1996). Asociado a la incorporación de enzimas, también se ha descrito una reducción de la proliferación de micro-organismos en tramos posteriores del tracto digestivo (Salih y col., 1991; Choct y col., 1996; Dänicke y col., 1997a,b; Dänicke y col., 1999).

Simultáneamente a las mejoras digestivas, la suplementación enzimática aceleró el tránsito digestivo en dietas base de cebada y trigo, determinado tanto a partir de las curvas de excreción fecal del marcador como a partir del marcador presente en la digesta. Resultados similares han sido citados por Salih y col (1991), Jeroch y Dänicke (1993), Almirall y Estevé-Garcia (1994), Zhengkan, (1996), Dänicke y col., 1999, y por Lazaro, (1999). La aceleración del tránsito digestivo podría ser una consecuencia de la hidrólisis de PNAs que supone una reducción en la viscosidad de la digesta y una mejora en los ritmos de difusión y absorción de los nutrientes (revisión de Smits y Annison, 1996 y Annison y Choct., 1996). Por otra parte, es de destacar que la asociación de mejoras en los coeficientes de digestibilidad con descensos en los tiempos de retención, sugieren que las limitaciones digestivas presentadas por los pollos a primera edad con cereales viscosos no están fundamentalmente condicionadas por tiempos de retención cortos en el tracto digestivo, sino por limitaciones en los ritmos de digestión y absorción.

2. CARBOHIDRASAS PARA MEJORAR EL VALOR NUTRITIVO DE LAS LEGUMINOSAS

Dados los elevados contenidos de las leguminosas en polisacáridos no amiloideos, el principal objetivo de este segundo apartado fue valorar el beneficio potencial de incorporar enzimas dirigidos a su hidrólisis.

1.2.1. in vitro

Para abordar este objetivo planteamos desarrollar una técnica rápida "*in vitro*" de evaluación de los complejos enzimáticos, así como la caracterización de los carbohidratos mediante técnicas de extracción y aislamiento. Aunque nuestro análisis se limitó a la determinación del perfil en monosacáridos en cada una de las fracciones aisladas, la incubación con enzimas proporcionó información suplementaria sobre las características y accesibilidad de estas fracciones.

Los resultados reflejan una gran diferencia de composición entre la cascarilla y los cotiledones (Aspinall y White, 1964; Aspinall y col., 1967a,b; Aspinall, 1988). Aunque con una escasa solubilidad, los carbohidratos de la cascarilla de soja presentan una fracción soluble compuesta por manosa, galactosa y glucosa, que posiblemente se integra en las estructuras de mananos (Aspinall y White, 1964). Por otra parte, la abundancia de glucosa insoluble apunta con mucha probabilidad a una fracción celulósica poco accesible a los enzimas. Como consecuencia, la presencia de una mayor o menor proporción de cascarillas en la torta de soja (44 vs 49% de PB), sin olvidar las variabilidades que pueden derivar de la genética y condiciones climáticas de cultivo (KeShun, 1999), afectan a las características globales de los carbohidratos de la torta de soja.

Los cotiledones presentaron un contenido en α-galactósidos de aproximadamente 5.4%, en su mayoría recuperados en la fracción soluble en agua. Sin embargo, su solubilidad como la de la proteína dependió del grado de tostado, lo que parece reflejar un cierto grado de interacción física entre ambos componentes. El fraccionamiento de los carbohidratos insolubles en agua proporcionó una fracción de pectinas (41.7% de ChSS y DASS), una fracción de hemicelulosas (31.5% de 1 y 4 MASS), y otra de celulosa (10.7% de residual) que fueron utilizadas como substratos de valoración de la actividad de los enzimas pectinasa, xilanasa y celulasa.

El desarrollo de las incubaciones reflejó porcentajes muy reducidos de liberación de monosácaridos, que únicamente se incrementaron ligeramente tras el fraccionamiento de las estructuras. Este hecho sugiere que la actividad enzimática sobre la pared vegetal esta limitada fundamentalmente por las dificultades de contacto entre los enzimas y el sustrato, sea por insolubilidad o por la protección entre estructuras. Resultados similares han sido

presentados por otros autores en relación a la digestión de los carbohidratos (Marsman et al., 1997a; Huisman et al., 1999; Malathi y Devegowada, 2001). Sin embargo, otros autores apuntan la posibilidad de mejorar la digestión de la fracción proteica de la soja (Ghazi y col., 1996, 1997a,b; Zanella y col., 1999) mediante procedimientos enzimáticos. Con objetivo de valorar este potencial nos planteamos estudiar el grado de liberación de aminoácidos por la proteasa en su interacción con diferentes carbohidrasas, y en función del grado de tostado de la soja. El tostado no provocó modificaciones en la hidrólisis de los carbohidratos, resultados que fueron similares a los descritos por Marsman y col. (1997b). Por otro lado, el tostado provocó ligeros aumentos en la liberación de aminoácidos, probablemente debido a la desnaturalización de la proteína (Arêas, 1992). Sin embargo, el dato más relevante fue la interacción entre enzimas de actividad carbohidrasa (pectinasa, cellulasa y mananasa) y la proteasa sobre la hidrólisis de la proteína y PNA de la fracción insoluble. Resultados similares han sido observados por Marsman y col. (1997b), lo que sugiere que la actividad de cada enzima se ve facilitada por la presencia de ambas, posiblemente tras incrementar su acceso a las zonas de hidrólisis, o tras reducir el tamaño de las moléculas.

2.2. in vivo

Recientemente se han descrito efectos anti-nutricionales en las aves por la ingestión de los β-mananos contenidos en la torta de soja (Ward y Fodge, 1996; McNaugton y col., 1998). Nos planteamos reducir estos efectos mediante la suplementación enzimática "*in vivo*" (Capitulo 5). En comparación con la suplementación enzimática en dietas base de cebada-trigo, la suplementación con β-mananasa no provocó efectos significativos sobre los parámetros productivos de los pollos. Tampoco, se observaron mejoras significativas en la digestibilidad de los nutrientes, ni a nivel ileal o fecal. No obstante, se observó una tendencia a mejorar la digestibilidad aparente fecal de la dieta ($p=0.06$) a final de la primera fase de crecimiento.

De los efectos destacados derivados de la ingestión de mananos (gluco- o galacto-mananos, con enlaces tipo β), se ha señalado que un incremento de la viscosidad de la digesta intestinal (Iji y col., 2000; Maisonnier y col., 2001) puede en parte explicar los descensos en la digestibilidad de los piensos basados en maíz y soja. Sin embargo, la suplementación con enzimas de estas raciones no provocó reducciones de la viscosidad intestinal (Marsman et al., 1997a) como con trigo y cebada. Este hecho puede deberse a los menores grados de

viscosidad del maíz y la soja, posiblemente en rangos de viscosidad difícilmente detectables con los métodos analíticos rutinarios. A pesar de ello, se apunta que ligeras modificaciones de viscosidad tiende a provocar elevados efectos digestivos, como reflejan las relaciones exponenciales entre la viscosidad de la digesta y la digestión y absorción de los nutrientes (Annison y Choct., 1996; Maisonnier y col., 2001). Por ello, nos planteamos como objetivo principal de la incorporación de enzimas en los piensos de maíz-soja, el valorar si se optimizaban las condiciones de digestión en el tracto digestivo. El estudio de esta optimización lo realizamos mediante la determinación de parámetros indirectos de la fermentación microbiana. En efecto, anteriormente se ha descrito que descensos en la actividad microbiana en el intestino delgado esta relacionada con incrementos en la digestión de los nutrientes, sobre todo grasas en piensos basados en cereales (Choct y Annison, 1992; Choct et al., 1996; Smits y col., 1998; Dänicke et al., 1997a,b; Dänicke, 1999). En el marco de estas sugerencias, los resultados obtenidos en nuestro experimento mostraron que la inclusión de mananasa provoca unos descensos cuantitativos de la flora microbiana activa a nivel ileal (medida a partir de la concentración en bases puricas, Adenina y Guanina), posiblemente por una actividad sobre los mananos de la soja.

Aunque estamos pendientes del análisis de la digestión de carbohidratos y de la energía de la dieta, descensos en la actividad microbiana a nivel ileal pueden ser de gran importancia en las condiciones de producción práctica. En particular, los enzimas se manifiestan como una alternativa a valorar en las condiciones de legislación actual que requiere la búsqueda de alternativas a los antibióticos.

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CONCLUSIONES

A partir de los resultados obtenidos en el segundo capítulo pueden deducirse las siguientes conclusiones.

Primera:

La incorporación de enzimas (β-glucanasas y arabinoxilanasas) en los piensos formulados con elevados porcentajes de cebada y/o trigo mejora los resultados productivos de los broilers, sobre todo durante la primera fase de crecimiento. Las mejoras están determinadas mayoritariamente por una mejora de la digestión y retención de los nutrientes, sobre todo de la materia grasa, que conlleva una mejora de la concentración en energía metabolizable del pienso.

Segunda:

Simultáneamente a un incremento de los coeficientes de digestibilidad, la hidrólisis de los PNAs presentes en los cereales reduce la viscosidad y acelera el transito de la digesta, lo que demuestra una mayor importancia de las condiciones ambientales en las mejoras de digestibilidad, y menor de los tiempos de retención de la digesta.

A partir de los resultados obtenidos en el tercer y cuarto capítulo pueden deducirse las siguientes conclusiones.

Tercera:

El fraccionamiento de la pared vegetal de la torta de soja (tanto en las cascarillas como en los cotiledones) revela la presencia de un entramado insoluble y altamente resistente a la digestión enzimática, formado por hemicelulosas y celulosas, y un entramado de mayor accesibilidad, compuesto esencialmente por las pectinas en los cotiledones y cadenas de mananos solubles en la cascarilla.

Cuarta:

La incubación del material soluble de la torta de soja con carbohidrasas (sobre todo pectinasa y celulasa) en combinación con proteasa reduce de una forma importante la cantidad de α-galactósidos (rafinosa y estaquiosa) recuperados en dicha fracción.

Quinta:

Tanto la combinación de enzimas carbohidrasas (pectinasa, celulasa y mananasa) con proteasas como el fraccionamiento del entramado de carbohidratos de pared vegetal de los cotiledones incrementa la liberación de monosacáridos, lo que demuestra la presencia de restricciones físicas a la hidrólisis enzimática del material insoluble.

A partir de los resultados obtenidos en el quinto capítulo pueden deducirse las siguientes conclusiones.

Sexta:

En las condiciones experimentales, la suplementación enzimática con mananasa y proteasa en piensos basados en maíz-soja no provocó modificaciones significativas en los resultados productivos ni digestivos de los animales.

Séptima:

La suplementación con mananasa en un pienso constituido por maíz y soja puede reducir la carga microbiana en íleon tanto en animales de 21 como de 42 días de edad, sin modificar la concentración microbiana en el contenido digestivo del ciego.