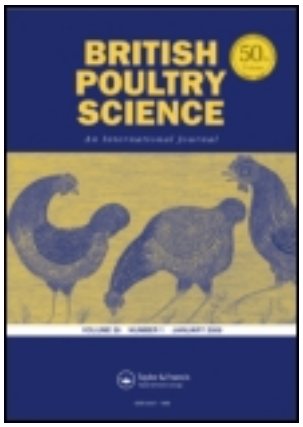

British Poultry Science
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbps20

A family 11 carbohydrate-binding module (CBM) improves the efficacy of a recombinant cellulase used to supplement barley-based diets for broilers at lower dosage rates

T. Ribeiro [a], P.I.P. Ponte [a], C.I.P.D. Guerreiro [a], H.M. Santos [a], L. Falcão [b], J.P.B. Freire [b], L.M.A. Ferreira [a], J.A.M. Prates [a], C.M.G.A. Fontes [a] & M.M. Lordelo [b]

[a] CIISA—Faculdade de Medicina Veterinária, Pólo Universitário do Alto da Ajuda, Avenida da Universidade Técnica, Lisboa, Portugal

[b] Instituto Superior de Agronomia, Tapada da Ajuda, Lisboa, Portugal

Version of record first published: 02 Oct 2008.

To cite this article: T. Ribeiro, P.I.P. Ponte, C.I.P.D. Guerreiro, H.M. Santos, L. Falcão, J.P.B. Freire, L.M.A. Ferreira, J.A.M. Prates, C.M.G.A. Fontes & M.M. Lordelo (2008): A family 11 carbohydrate-binding module (CBM) improves the efficacy of a recombinant cellulase used to supplement barley-based diets for broilers at lower dosage rates, British Poultry Science, 49:5, 600-608

To link to this article: http://dx.doi.org/10.1080/00071660802345749

British Poultry Science Volume 49, Number 5 (September 2008), pp. 600–608

A family 11 carbohydrate-binding module (CBM) improves the efficacy of a recombinant cellulase used to supplement barley-based diets for broilers at lower dosage rates

T. RIBEIRO, P.I.P. PONTE, C.I.P.D. GUERREIRO, H.M. SANTOS, L. FALCÃO[1], J.P.B. FREIRE[1], L.M.A. FERREIRA, J.A.M. PRATES, C.M.G.A. FONTES AND M.M. LORDELO[1]

CIISA–Faculdade de Medicina Veterinária, Pólo Universitário do Alto da Ajuda, Avenida da Universidade Técnica and [1]Instituto Superior de Agronomia, Tapada da Ajuda, Lisboa, Portugal

Abstract 1. Exogenous microbial β-1,3-1,4-glucanases and hemicellulases contribute to improving the nutritive value of cereals rich in soluble non-starch polysaccharides for poultry.
2. In general, plant cell wall hydrolases display a modular structure comprising a catalytic module linked to one or more non-catalytic carbohydrate-binding modules (CBMs). Based on primary structure similarity, CBMs have been classified in 50 different families. CBMs anchor cellulases and hemicellulases into their target substrates, therefore eliciting efficient hydrolysis of recalcitrant polysaccharides.
3. A study was undertaken to investigate the effects of a family 11 β-glucan-binding domain in the function of recombinant derivatives of cellulase *Ct*Lic26A-Cel5E of *Clostridium thermocellum* that were used to supplement a barley-based diet at lower dosage rates.
4. The results showed that birds fed on diets supplemented with the recombinant *Ct*Lic26A-Cel5E modular derivative containing the family 11 CBM or the commercial enzyme mixture Rovabio™ Excel AP tended to display improved performance when compared to birds fed diets not supplemented with exogenous enzymes.
5. It is suggested that at lower than previously reported enzyme dosage (10 U/kg *vs* 30 U/kg of basal diet), the β-glucan-binding domain also elicits the function of the recombinant *Ct*Lic26A-Cel5E derivatives.
6. Finally, the data suggest that exogenous enzymes added to barley-based diets act primarily in the proximal section of the gastrointestinal tract.

INTRODUCTION

In poultry, dietary soluble non-starch polysaccharides (NSP), such as arabinoxylans and β-glucans, are known to increase digesta viscosity leading to a lower efficiency of nutrient digestion and absorption (Smith and Annison, 1996) while contributing to reduce feed passage rates (van der Klis *et al*., 1993). In addition, higher levels of recalcitrant polysaccharides in poultry digesta may cause anaerobic microbial proliferation in the small intestine leading to the production of toxins and deconjugation of the bile salts which are essential for the digestion of fat (Misir and Marquardt, 1978; Langhout, 1998). It is well established that inclusion of microbial plant cell wall hydrolases in wheat, barley and rye-based diets for simple-stomach animals improves the efficiency of feed utilisation, enhances growth and contributes to a better use of low-cost feed ingredients (Chesson, 1993; Bedford, 2000). It is usually agreed that exogenous glycoside hydrolases improve the nutritive value of cereal-based diets rich in NSPs through a variety of mechanisms. Exogenous polysaccharidases efficiently contribute to decrease digesta viscosity that is

Correspondence to: Carlos Fontes, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal. Tel: +351-213652876. Fax: +351-213652889. E-mail: cafontes@fmv.utl.pt

Accepted for publication 8th May 2008.

ISSN 0007–1668(print)/ISSN 1466–1799 (online)/08/050600–9 © 2008 British Poultry Science Ltd
DOI: 10.1080/00071660802345749

associated with the intake of soluble NSPs, and thus have a positive effect on the rate of diffusion of substrates, digestive enzymes and nutrients (White *et al*., 1981; Fengler and Marquardt, 1988; Bedford *et al*., 1991; Bedford and Classen, 1992), and increase feed intake. In addition, cellulases and xylanases may promote the proliferation of beneficial microflora in the final compartments of the monogastric gastrointestinal (GI) tract, by increasing the quantity and/or the quality of the substrates available for fermentation (Bedford and Morgan, 1996; Apajalahti and Bedford, 1999). Finally, plant cell wall hydrolases may mediate their effects by releasing endosperm plant cell wall trapped nutrients that were otherwise unavailable for digestion (Hesselman and Aman, 1986). It is believed that the action of one or a conjunction of the above-mentioned effects may depend on the type of animal, diet and exogenous enzyme used.

Cellulases and hemicellulases (EC 3·2·1) are generally modular enzymes, containing discrete non-catalytic carbohydrate-binding modules (CBMs), which anchor the biocatalysts to the plant cell wall, linked to the enzyme catalytic domains via flexible linker sequences. By mediating a close and prolonged interaction between enzymes and plant carbohydrates, CBMs allow the appended catalytic domains to intimately contact its target substrates, therefore potentiating catalysis (Boraston *et al*., 2004). This proximity and targeting role of CBMs is of extreme importance for cellulase and hemicellulase function, as the complex interactions established between polysaccharides within the plant cell wall restrict their accessibility to enzymatic attack (Guerreiro *et al*., 2006). CBMs are currently grouped into 50 sequence-based families (February 2008; Coutinho and Henrissat, 2003) and have been shown to display a large range of different ligand specificities. Therefore, CBMs that recognise cellulose, β-1,3-1,4-glucans, xylans, mannans, galactans, xyloglucans, arabinans and laminarins have been identified in a variety of cellulases and hemicellulases and the molecular determinates of binding specificity have been elucidated in most cases. However, there is a paucity of information concerning the importance of non-catalytic CBMs in the function of exogenous cellulases used to supplement cereal-based diets for simple-stomach animals. Nevertheless, it has been shown that a family 6 xylan-binding domain is able to improve the efficacy of a microbial recombinant xylanase used to supplement wheat and rye-based diets for poultry (Fontes *et al*., 2004). Animals supplemented with a bi-modular xylanase containing catalytic and xylan-binding domains grew significantly faster than animals fed on diets containing exclusively the xylanase catalytic domain. More recently, we showed that the family 11 β-glucan-binding domain located in the bi-functional cellulase *Ct*Lic26A-Cel5E from *Clostridium thermocellum* is unable to improve the efficacy of the appended catalytic domains (Guerreiro *et al*., 2008). However, it was suggested that the high enzyme dosage rates used in the above-mentioned study may have contributed to attenuate the effect of the non-catalytic β-glucan-binding domain.

The objective of the present work was to determine whether the presence of the CBM module in a truncated *Clostridium thermocellum* cellulase has any influence on its performance in broilers fed on a barley-based diet when the enzyme is used at sub-optimal doses. Together, the data presented here suggest that when microbial enzymes are added at lower concentrations to barley-based diets for broiler chicks the efficacy of the exogenous cellulase is improved by the presence of the family 11 β-glucan-binding domain. The contribution of CBMs to decreasing the incorporation rates of feed enzymes in poultry diets is discussed.

MATERIALS AND METHODS

Enzyme preparation

The molecular architecture of *Ct*Lic26A-Cel5E and its truncated recombinant derivatives used in this study are presented in Figure 1. The enzyme contains an N-terminal β-1,3-1,4-glucanase catalytic domain (GH26), followed by a β-1,4-cellulase second catalytic module (GH5), a family 11 carbohydrate-binding module (CBM11) and a C-terminal dockerin characteristic of other *C. thermocellum* cellulosomal enzymes (Taylor *et al*., 2005). The *Ct*Lic26A-Cel5E truncated derivatives Lic26-Cel5E-CBM11 and Lic26-Cel5E were hyperexpressed in *Escherichia coli* following the protocols described by Taylor *et al*. (2005). The recombinant plasmids, containing the Clostridial genes under the control of T7 promoters in the prokaryotic expression vector pET21a (Novagen, Darmstadt, Germany), were used to transform BL21 *E. coli* cells.

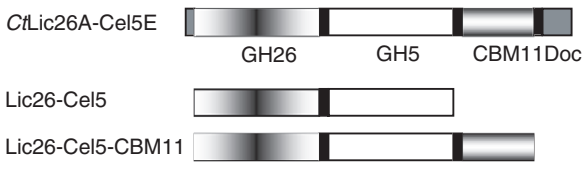

Figure 1. *Domain organisation of* C*tLic26A-Cel5E and its truncated derivatives Lic26-Cel5 and Lic26-Cel5-CBM11 used in this study. The* β*-glucanase (GH26), cellulase (GH5),* β*-glucan-binding domain (CBM11) and the dockerin (Doc) are indicated. The grey and the black boxes represent the linker sequences and the signal peptide, respectively.*

Recombinant *E. coli* strains were grown on Luria Bertani media to mid-exponential phase (A_{600nm} of 0·5) and recombinant gene expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM. Cells were collected after 5 h induction at 37°C and protein extracts prepared by ultrasonication followed by centrifugation. The recombinant proteins were purified by metal-affinity chromatography as described by Fontes *et al*. (2004). Both recombinant proteins, Lic26-Cel5E-CBM11 and Lic26-Cel5E, retain considerable catalytic activity at 40°C and are resistant to proteolytic degradation (Taylor *et al*., 2005).

Animals, diets and management

The barley-based diet used in this study (Table 1) was formulated to contain adequate nutrient levels as defined by NRC (1994). The basal diet was supplemented with no enzyme (C0) or with 10 U/kg of Lic26-Cel5E or Lic26-Cel5E-CBM11. In addition, a 4th treatment corresponded to the supplementation of the basal diet with a calculated 15 U/kg of the commercial enzyme cocktail Rovabio™ Excel AP (Adisseo, Antony, France; treatment termed Rov for simplification), which corresponds to an incorporation ratio of 50 g of enzyme per tonne of feed as recommended by the fabricant. A total of 160 1-d-old chicks (Ross 308) were divided into 40 battery brooders, with a capacity of 4 animals per pen, exposed to constant light for the duration of the trial. Water and a barley-based feed were available *ad libitum* throughout the experiment and were provided via automatic drinking nipples and a hanging feeder, respectively. The brooders were located in an environmentally controlled room, which was daily adjusted to the recommended temperatures, according to standard brooding practice. Birds were individually weighed at the commencement of the experiment and were randomly assigned into one of the 4 treatments, with 10 replicates of 4 birds per treatment. Weekly, feed consumption and individual body weights were recorded. Gain to feed ratios were calculated by dividing the weight gain per pen, per week and at the end of the experiment, including the weight gain of any dead birds, by the total feed consumed during the respective period. Bird mortality was recorded daily. At the end of the experiment, at d 28, one bird per pen was slaughtered by an intravenous injection of an aqueous isotonic solution of 125 mg Tiopental Braun (Braun, Barcelona, Spain). The size of the various GI compartments was measured and digesta samples were collected and stored at −20°C for later analysis. Levels of cellulase and hemicellulase activity in the GI tract were measured as described below.

Table 1. *Ingredient composition and calculated analysis of the cereal-based feed*

Ingredients	g/kg
Barley	550·00
Soybean meal 47%	300·61
Soybean oil	50·73
Maize	50·45
Salt	2·50
Calcium carbonate	8·10
Dicalcium phosphate[1]	10·79
DL-Methionine	1·60
Mineral and vitamin premix[2]	2·00
Estimated nutrient content	
Energy (MJ ME/kg DM)	12
Crude protein	208·0
Ether extract	73·3
Crude cellulose	48·7

[1] Contained 200 g/kg Ca and 180 g/kg P.
[2] Mineral–vitamin premix provided the following per kg of diet: biotin 0·5 mg, calcium pantothenate 10 mg, cholecalciferol 0·05 mg, cyanocobalamin 0·12 mg, folic acid 0·5 mg, menadione 2 mg, nicotinic acid 30 mg, pyridoxine 1·7 mg, retinol 2·7 mg, thiamine 1 mg, α-tocopherol 20 mg, riboflavin 4·2 mg, Co 0·2 mg, Cu 10 mg, Fe 80 mg, I 1 mg, Mn 100 mg, Se 0·3 mg, Zn 80 mg, monensin 0·1 g.

Analytical procedures

Enzyme catalytic activity was determined at 40°C by measuring release of reducing sugars, following the method described by Taylor *et al*. (2005), using barley β-glucan (Megazyme®, Bray, Ireland) as the substrate. One unit of catalytic activity is defined as the amount of enzyme required to release 1 μmole of product per min. The extract containing Rovabio™ Excel AP enzymes was prepared by resuspending 75 mg of the enzyme mixture in 10 ml of 50 mM NaHepes buffer, pH 7·5, which was followed by an overnight incubation at 4°C with gentle agitation and a centrifugation at 13 000 *g* for 5 min. In order to standardise the number of enzyme units used to supplement the basal diet, the catalytic activity of the three exogenous enzymes, including the commercial mixture, were determined in parallel. Previously to detection of β-glucanase activity, digesta samples were centrifuged and the supernatant recovered for analysis. Initially, qualitative analysis of cellulase activity in the digesta samples recovered from the various GI compartments was assessed in agar plates, using barley β-glucan (Megazyme®) at 0·1% (w/v) final concentration, in 10 mM Tris–HCl pH 7·0. Catalytic activity was detected after 16 h incubation at 37°C by the Congo Red assay plate, as described in Ponte *et al*. (2004) and Mourão *et al*. (2006). Zymogram analysis was performed as described by Fontes *et al*. (2004). Briefly, digesta proteins were separated through SDS-PAGE in 10% acrylamide gels containing 0·1% of barley β-glucan (Megazyme®), according

to Laemmli (1970). After electrophoresis, polypeptides were renatured by subjecting the gel to four 30-min washes in 100 mM sodium succinate, pH 6·3, containing 10 mM $CaCl_2$ and 1 mM DTT. The gel was incubated for 36 h at 37°C in the same buffer and proteins were stained in a solution comprising 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 0·4% (w/v) Coomassie Brilliant Blue R. After destaining, β-glucanase activity was detected using a 0·1% (w/v) Congo Red solution, for 15 min and washing with 1 M NaCl until excess dye was removed. Areas of catalytic activity appeared as colourless zones in a dark blue background after a quick wash in a 0·5% (v/v) solution of acetic acid. For measuring the viscosity of small intestine contents, samples were centrifuged for 10 min at 9000 rpm and the viscosity of the supernatant was measured using a Brookfield viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA, USA) whose cup was maintained at 24°C.

Statistical analysis

Statistical analysis of data related to bird performance was conducted by analysis of variance, using the General Linear Models procedure of SAS (SAS Institute, 2004). Means with a significant *F*-ratio were separated by the least significant difference test. The experimental unit was a cage of 4 birds. The number of birds presenting β-glucanase activity in digesta samples collected from various GI compartments was analysed for frequency and significance by chi-square analysis. Unless otherwise stated, differences were considered significant when $P < 0{\cdot}05$, and tendencies to significance were accepted if $0{\cdot}05 < P \leq 0{\cdot}1$.

RESULTS AND DISCUSSION

The importance of *Ct*Lic26A-Cel5E family 11 β-glucan-binding domain in the function of the recombinant cellulase used to supplement a barley-based diet for broiler chicks was evaluated. The basal diet was supplemented with the required enzymes and used to feed broiler chicks *ad libitum* from d 1 to 28. During the experiment, the mortality rate was low (3·75%) and was not related to treatments (anatomo-pathological results not shown).

Bird performance

Values for body weight, weight gain, feed intake and gain : feed ratio are summarised in Table 2. During the entire experimental period, feed intake and gain:feed ratios were not significantly different between birds fed the different diets.

Table 2. *Growth performance of broilers fed on a barley-based diet not supplemented (C0) or supplemented with a commercial cellulase mixture (Rov) or truncated derivatives of* C. thermocellum Ct*Lic26A-Cel5E* β*-glucanase containing (Lic26-Cel5E-CBM11) or not containing (Lic26-Cel5E) a family 11 CBM*

	C0	ROV	Lic26-Cel5E	Lic26-Cel5E-CBM11	SEM	p(*F*)
Body weight (g)						
0 d	45·6	45·4	45·9	45·4	0·187	NS
7 d	156·6	157·6	152·6	160·4	3·256	NS
14 d	382·7	400·0	379·8	405·5	8·853	NS
21 d	693·6	725·4	707·8	737·2	13·367	NS
28 d	1146·5[a]	1205·6[b]	1191·7[ab]	1237·6[b]	24·460	0·075
Weight gain (g)						
0 to 7 d	111·0	112·3	106·8	115·5	3·232	NS
7 to 14 d	226·1	242·4	227·3	245·1	6·611	0·082
14 to 21 d	310·9	325·5	328·0	331·8	7·450	NS
21 to 28 d	452·9	480·3	483·8	500·4	15·419	NS
0 to 28 d	1100·9[a]	1160·3[b]	1145·8[ab]	1192·7[b]	24·515	0·073
Feed intake (g)						
0 to 7 d	139·1	144·1	136·8	137·5	7·267	NS
7 to 14 d	340·4	359·2	341·4	352·8	15·624	NS
14 to 21 d	543·8	572·7	578·3	568·5	17·478	NS
21 to 28 d	909·3	895·6	923·5	925·6	25·480	NS
0 to 28 d	1932·2	1971·7	1980·0	1984·0	58·207	NS
Gain : feed ratio						
0 to 7 d	0·790	0·816	0·781	0·840	0·00214	NS
7 to 14 d	0·665	0·701	0·658	0·695	0·0422	NS
14 to 21 d	0·572	0·571	0·566	0·584	0·0135	NS
21 to 28 d	0·498	0·524	0·520	0·541	0·0143	NS
0 to 28 d	0·569	0·596	0·574	0·601	0·0119	NS

During the 4 weeks of the experiment, weekly body weight and weight gain were not significantly different between the different treatments receiving the exogenous enzymes. However, at the end of the experimental period, at d 28, birds receiving the commercial enzyme mixture and Lic26A-Cel5E-CBM11 tended to perform better than birds on the negative control treatment, both in terms of final body weight and in weight gain. Therefore, even though the enzymes of the commercial mixture included a range of glucanases, cellulases and xylanases with different substrate specificities, which were added at higher levels than the recombinant enzymes, body weight and weight gain were similar between these treatments. Since the recombinant cellulase Lic26A-Cel5E-CBM11 functions as effectively as the commercial enzyme cocktail, it is suggested that accessory non-glucanase activities, such as xylanase or mannanase, become obsolete in improving the nutritive value of barley-based diets such as the one used in the present study. As reported previously by Guerreiro *et al.* (2008), our results highlight the capacity of single recombinant cellulases to improve the nutritive value of barley-based diets for poultry, questioning the need for using enzyme mixtures containing a large array of different enzyme specificities for targeting the anti-nutritive factors present in those diets. This is not completely unexpected, since data previously reported by Philip *et al.* (1995) demonstrated that a recombinant single-domain cellulase, which originates also from the anaerobic bacterium *C. thermocellum*, was as efficient as a complex mixture of cellulases in improving the nutritive value of a barley-based diet for broilers. One of the major actions of feed cellulases is to decrease the degree of polymerisation of soluble β-glucans, through the random cleavage of glycosydic bonds in the polysaccharide backbone. The reduction in carbohydrate chain length contributes to decrease the levels of digesta viscosity (Fengler and Marquardt, 1988; Bedford and Morgan, 1996). In addition, these results indicate that at lower dosage rates (threefold decrease) than the values reported in a previous study (Guerreiro *et al.*, 2008), the β-glucan-binding domain tends to improve the efficacy of the recombinant enzyme containing the CBM11 module ($P < 0{\cdot}1$). It has been previously shown that a modular enzyme containing a family 6 CBM yields better broiler performance (Fontes *et al.*, 2004). In the present study, although birds fed on the modular enzymes with or without the β-glucan-specific CBM did not reveal significant differences in final body weight, there is a slight numerical increase in the weight of birds receiving the diets supplemented with the CBM-containing enzyme.

Dietary fibre can influence the development and the size of digestive organs. It is well known that diets with high levels of soluble NSP induced considerable enlargements of some portions of the GI tract (Brenes *et al.*, 1993; Petersen *et al.*, 1993) and pancreas and stimulated an increase in protein turnover rates (Dänicke *et al.*, 2000). Since enzyme addition decreases digesta viscosity and therefore improves the feed passage rate and nutrient absorption, then, the relative weight of the digestive tract decreases leading to an increase on the carcase yield (Petterson and Aman, 1989; Fuente *et al.*, 1998). Fuente *et al.* (1998) have found an equation relating the empty weight of the digestive tract to digesta viscosity. Therefore, the effects of the different dietary treatments in the relative length or weight of different organs and GI tract compartments of broiler chicken were evaluated and the respective data is presented in Table 3. In the current study, the relative weights of crop, gizzard and liver and the relative length of the duodenum, jejunum, ileum and caecum of birds were not different between birds of the 4 dietary treatments. These data are in contrast with results reported by

Table 3. *Relative weight and length of GI tract and viscosity of digesta samples of broilers fed on a barley-based diet not supplemented (C0) or supplemented with a commercial cellulase mixture (Rov) or truncated derivatives of* C. thermocellum Ct*Lic26A-Cel5E β-glucanase containing (Lic26-Cel5E-CBM11) or not containing (Lic26-Cel5E) a family 11 CBM*

	C0	ROV	Lic26-Cel5E	Lic26-Cel5E-CBM11	SEM	p(*F*)
Relative weight (g/100 g BW)						
Crop	3·674	3·345	3·889	3·827	0·2173	NS
Gizzard	14·014	13·659	12·926	13·477	0·5889	NS
Liver	31·757	31·204	31·842	30·600	1·832	NS
Relative length (cm/kg BW)						
Duodenum	18·622	17·360	19·050	18·240	0·614	NS
Jejunum	54·033	50·020	50·960	51·970	1·392	NS
Ileum	54·800	51·510	54·560	55·110	1·710	NS
Caecum	12·933	11·560	12·150	12·340	0·389	NS
Content viscosity (cpo)						
Duodenum + jejunum	7·450[a]	4·955[b]	6·962[a]	6·149[ab]	0·6503	0·053
Ileum	12·109	9·493	9·077	10·482	1·4476	NS

several other authors who have shown that enzyme addition decreases the digestive tract weight and/or length when expressed as a percentage of live weight (Brenes *et al*., 1993; Peterson *et al*., 1993; Viveros *et al*., 1994). Since the percentages of soluble glucans in barley vary widely, it is possible that the levels of the soluble NSP present in the cereal used in this experiment were too small to have an impact on the size of the bird's GI tract.

Digesta viscosity in the hindgut and foregut of birds fed the different dietary treatments was measured and the results are presented in Table 3. Ileum viscosity was shown to be identical in birds of the different treatments. However, digesta viscosity at the level of the duodenum and jejunum had a tendency to be smaller in birds given the commercial enzyme than in birds receiving Lic26A-Cel5A or the negative control. Although digesta viscosity in birds supplemented with the modular enzymes was not significantly different from the negative control, the viscosity of the duodenum and jejunum contents of birds fed the commercial mixture had a tendency to be similar to the viscosity in birds fed diets containing Lic26A-Cel5E-CBM11. Therefore, the presence of the non-catalytic CBM in the recombinant enzyme may tend to lead to a similar reduction in digesta viscosity when compared with the positive control.

Taken together, our results suggest that single purified recombinant cellulases with a non-catalytic CBM module or enzyme mixtures containing cellulases, can equally improve bird performance. Moreover, the addition of lower doses (10 U/kg *vs* 30 U/kg) of the modular recombinant enzymes to the ones used in a previous study (Guerreiro *et al*., 2008), appear to be equally effective in improving performance of birds fed on barley-based diets when the recombinant enzyme contains a non-catalytic CBM. It is well established that CBMs contribute to enhance the activity of adjacent catalytic modules by increasing enzyme concentration on the substrate surface (Fernandes *et al*., 1999; Gilbert *et al*., 2002). This action is most important in plant cell wall hydrolases that need to be targeted to their specific substrates which are usually less accessible in the complex organisation of the plant cell wall. In fact, Fontes *et al*. (2004) have found that a modular xylanase containing a family 6 CBM yields better animal performance than the enzyme's catalytic module alone. The family 11 CBM of Lic26A-Cel5E binds both β-1,4- and β-1,3-1,4-mixed linked glucans (Carvalho *et al*., 2004). It is also well known that CBMs are particularly important for the hydrolysis of insoluble substrates (Gilbert *et al*., 2002). We have previously shown (Guerreiro *et al*., 2006) that, although the molar activity of the recombinant cellulases *Ct*Lic26-Cel5E-CBM11 and Lic26-Cel5E against barley β-glucan is similar, the presence of the family 11 CBM potentiates the action of the modular cellulase against insoluble cellulose forms, such as Avicel. Since insoluble polysaccharides are less accessible, we envisaged that the major contribution of the family 11 CBM of Lic26A-Cel5E to increase the efficiency of the recombinant enzyme would be related with the targeting of the associated catalytic domains (Lic26A-Cel5E) to the anti-nutritive soluble β-glucans that are abundant in barley-based diets. Our results revealed that when Lic26-Cel5E-CBM11 was supplemented to the diets, the viscosity of the duodenum and jejunum contents was not different from the viscosity of the contents of birds fed the commercial enzyme. In addition, birds fed on diets with Lic26-Cel5E showed no differences in body weight and weight gain in comparison to the negative control, while birds on Lic26-Cel5E-CBM11 achieved higher body weights and weight gains than the negative control but similar to the commercial enzyme treatment. This, again, may indicate that the family 11 CBM positively affects the efficiency of the GH5 and GH26 catalytic domains *in vivo* at the present doses.

Recombinant β-glucanase stability *in vivo*

To evaluate the stability of the exogenous glycoside hydrolases during passage through the GI tract, β-glucanase activity was qualitatively determined in digesta samples collected in the various digestive compartments of 10 animals per treatment. The results, presented in Table 4, demonstrated that while caeca samples collected from birds of the group not receiving exogenous enzymes were positive for cellulase activity, no β-glucan degrading properties were detected in the contents of the other GI compartments. However, β-glucanase activity could be detected along the entire digestive tract of most animals fed on diets supplemented with the plant cell wall hydrolases. Interestingly, β-glucanase activity was not detected in the gizzard of birds fed diets supplemented with the commercial enzyme, possibly because of the acidic conditions in this organ. Nevertheless, broilers fed on diets containing Lic26-Cel5E and, to a greater extent, Lic26-Cel5E-CBM11 showed detectable β-glucanase activity in the gizzard, suggesting that the modular enzymes used in the present study have a higher resistance to the acidic conditions that are prevalent in this portion of the digestive tract when compared with the commercial enzyme mixture. In addition, the frequency of β-glucanase activity was higher especially in the crop, gizzard and duodenum of birds supplemented with modular enzymes. The results on the

Table 4. *Number of birds, out of 10 animals analysed, fed on a barley-based diet not supplemented (C0) or supplemented with a commercial cellulase mixture (Rov) or truncated derivatives of* C. thermocellum Ct*Lic26A-Cel5E β-glucanase containing (Lic26-Cel5E-CBM11) or not containing (Lic26-Cel5E) a family 11 CBM presenting β-glucanase activity in digesta samples collected from various gastrointestinal compartments*

	C0	ROV	Lic26-Cel5E	Lic26-Cel5E-CBM11	Chi-square value	*P*-value
Crop	2	6	9	9	14·5055	0·0023
Gizzard	0	0	2	4	8·6275	0·0347
Duodenum	0	5	7	6	11·7172	0·0084
Jejunum	0	0	1	0	3·0769	0·3799
Ileum	0	4	5	6	8·8533	0·0313
Caecum	10	10	10	10	–	–

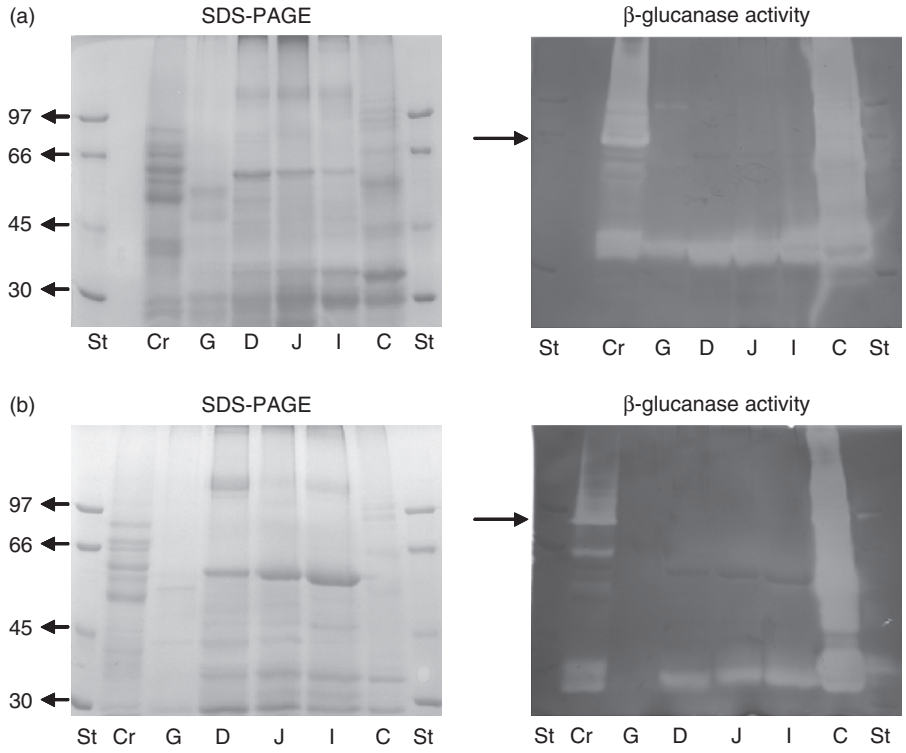

Figure 2. *Zymogram analysis of digesta samples collected from various regions of the GI tract of birds supplemented with the recombinant* β*-glucanases Lic26-Cel5 (Panel A) and Lic26-Cel5-CBM11 (Panel B). Proteins were fractionated through SDS-PAGE and stained for* β*-glucanase activity after enzyme renaturation. The arrows depict the location of the non-truncated versions of the two recombinant enzymes in the stained gels. Abbreviations: St, low molecular weight protein standards; Cr, crop; G, gizzard; D, duodenum; J, jejunum; I, ileum; C, caecum.*

chi-square analysis show a significant difference at the 0·05 level in the crop, gizzard, duodenum and ileum, indicating that in these compartments the type of enzyme added to the diet influenced the *β*-glucanase activity.

To analyse potential changes in the molecular architecture of the recombinant cellulases during passage through the GI tract, digestive samples of birds of treatments receiving Lic26-Cel5E-CBM11 and Lic26-Cel5E were subjected to zymogram analysis. The data, displayed in Figure 2, suggest that both Lic26-Cel5E-CBM11 and Lic26-Cel5 are prone to proteolytic cleavage in the birds' GI tract, which occurs initially but moderately in the crop and then completely in the gizzard and in the following GI compartments. Therefore, in agreement with the data reported by Guerreiro *et al*. (2008), it is suggested that both recombinant enzymes are proteolytically cleaved in the linker regions connecting the GH26, the GH5 and the CBM11 modules, which contributes to release the two 32 to 35 kDa catalytic domains that still retain significant catalytic activity in the digestive tract. Experiments performed *in vitro* revealed that the proteolytic cleaveage of *Ct*Lic26-Cel5 does not affect the biological capability of the resulting catalytic domains to degrade soluble *β*-glucans (Guerreiro, unpublished data), as it was previously demonstrated by Taylor *et al*. (2005).

Therefore, since both enzymes are subjected to proteolytic cleavage in the gizzard, it is suggested that the CBM must exert its effect mainly in the proximal section of the GI tract.

CONCLUSIONS

The results suggest that individual recombinant cellulases with a non-catalytic CBM module added at lower doses than a commercial enzyme mixture are equally effective in improving the nutritive value of barley-based diets for poultry. At the present recombinant enzyme doses the modular enzyme containing a family 11 CBM, which is β-glucan specific, was more effective in reducing the anti-nutritive properties of β-glucans when compared with its truncated counterpart lacking the CBM. In addition, both recombinant enzymes were prone to proteolysis in the birds' gizzard and subsequent GI compartments, leading to the conversion of the enzyme molecular architecture into two single-domain enzymes that are identical in birds of the two treatments. Therefore, considering the animal performance and the *in vivo* enzyme analysis reported here, it is suggested that *Ct*Lic26-Cel5E derivatives, and in general cellulases added to barley-based diets, may exert primarily their function at the initial portions of the GI tract.

ACKNOWLEDGEMENTS

We thank Sociedade Agrícola da Quinta da Freiria SA for supplying the 1-d-old chicks and Reagro for providing the feed ingredients used in these experiments. This work was supported by Fundação para a Ciência e a Tecnologia (POCI/CVT/69329/2006). T.R., P.P. and C.G. were supported by Fundação para a Ciência e a Tecnologia through grants SFRH/BD/32321/2006, SFRH/BD/17969/2004 and SFRH/BD/16731/2004, respectively.

REFERENCES

Apajalahti, J. & Bedford, M.R. (1999) Improve bird performance by feeding its microflora. *World Poultry Science*, **15**: 20–23.

Bedford, M.R. (2000) Exogenous enzymes in monogastric nutrition–their current value and future benefits. *Animal Feed Science and Technology*, **86**: 1–13.

Bedford, M.R. & Classen, A. (1992) Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected throughout changes in carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. *Journal of Nutrition*, **122**: 560–569.

Bedford, M.R. & Morgan, A.J. (1996) The use of enzymes in poultry diets. *World Poultry Science*, **52**: 61–68.

Bedford, M.R., Campbell, G.L. & Classen, H.L. (1991) The effect of pelleting, salt and pentosanase on the viscosity of intestinal contents and the performance of broiler fed rye. *Poultry Science*, **70**: 1571–1577.

Boraston, A.B., Bolam, D.N., Gilbert, H.J. & Davies, G.J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochemical Journal*, **382**: 769–781.

Brenes, A.M.S., Guener, W. & Marquardt, R.R. (1993) Effect of enzyme supplementation on the performance and digestive tract size of broiler chickens fed wheat- and barley-based diets. *Poultry Science*, **72**: 1731–1739.

Carvalho, A.L., Goyal, A., Prates, J.A., Bolam, D.N., Gilbert, H.J., Pires, V.M., Ferreira, L.M., Planas, A., Romao, M.J. & Fontes, C.M. (2004) The family 11 carbohydrate-binding module of Clostridium thermocellum Lic26A-Cel5E accommodates beta-1,4- and beta-1,3-1,4-mixed linked glucans at a single binding site. *Journal of Biological Chemistry*, **279**: 34785–34793.

Chesson, A. (1993) Feed enzymes. *Animal Feed Science and Technology*, **45**: 65–69.

Coutinho, P.M. & Henrissat, B. (2003) Subject: CAZy–Carbohydrate-Active EnZYmes. http://afmb.cnrs-mrs.fr/CAZY.

Dänicke, S., Jeroch, H., Bottcher, W. & Simon, O. (2000) Interactions between dietary fat type and enzyme supplementation in broiler diets with high pentosan contents: effects on precaecal and total tract digestibility of fatty acids, metabolizability of gross energy, digesta viscosity and weights of small intestine. *Animal Feed Science and Technology*, **84**: 279–294.

Fengler, A.I. & Marquardt, R.R. (1988) Water-soluble arabinoxylans from rye. II. Effects of rate of dialysis and on the retention of nutrient by the chick. *Cereal Chemistry*, **65**: 298–302.

Fernandes, A.C.G., Fontes, C.M.G.A., Gilbert, H.J., Hazelwood, G.P., Fernandes, T.H. & Ferreira, L.M.A. (1999) Homologous xylanases from Clostridium thermocellum: evidence for bi-functional activity, synergism between xylanases catalytic modules and the presence of xylan-binding domains in enzyme complexes. *Biochemical Journal*, **342**: 105–110.

Fontes, C.M.G.A., Ponte, P.I.P., Reis, T.C., Soares, M.C., Gama, L.T., Dias, F.M.V. & Ferreira, L.M.A. (2004) A family 6 carbohydrate-binding module potentiates the efficiency of a recombinant xylanase used to supplement cereal-based diets for poultry. *British Poultry Science*, **45**: 648–656.

Fuente, J.M., Perez de Ayala, P., Flores, A. & Villamide, M.J. (1998) Effect of storage time and dietary enzyme on the metabolizable energy and digesta viscosity of barley-based diets for poultry. *Poultry Science*, **77**: 90–97.

Gilbert, H.J., Bolam, D.N., Szabo, L., Xie, H., Williamson, M.P., Simpson, P.J., Jamal, S., Boraston, A.B., Kilburn, D.G. & Warren, R.A.J. (2002) An update on Carbohydrate Binding Modules, *Carbohydrate Bioengineering*, (London, Royal Society of Chemistry).

Guerreiro, C.I., Najmudin, S., Carvalho, A.L., Prates, J.A., Correia, M.A., Alves, V.D., Ferreira, L.M., Romão, M.J., Gilbert, H.J., Bolam, D.N. & Fontes, C.M. (2006) Xyloglucan is recognized by carbohydrate-binding modules that interact with beta-glucan chains. *Journal of Biological Chemistry*, **281**: 8815–8828.

Guerreiro, C.I.P.D., Ribeiro, T., Ponte, P.I.P., Lordelo, M.M.S., Falcão, L., Freire, J.P.B., Ferreira, L.M.A., Prates, J.A.M. & Fontes, C.M.G.A. (2008) Probing the role of a family 11 Carbohydrate Binding Module (CBM) in the function of a recombinant cellulase used to supplement a barley-based diet for broiler chicken. *British Poultry Science*, **49**: 446–454.

Hesselman, K. & Aman, P. (1986) The effect of β-glucanase on the utilisation of starch and nitrogen by broiler chickens

fed on barley of low and high viscosity. *Animal Feed Science and Technology*, **15**: 83–93.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.

Langhout, D.J. (1998) The role of the intestinal microflora as affected by non-starch polysaccharides in broiler chickens. Ph.D. Thesis, Agricultural University Wageningen, the Netherlands.

Misir, R. & Marquardt, R.R. (1978) Factors affecting rye (Secale cereale L.) utilization in growing chicks. I. The influence of rye level, ergot and penicillin supplementation. *Canadian Journal of Animal Science*, **58**: 691–701.

Mourão, J.L., Ponte, P.I.P., Prates, J.A.M., Centeno, M.S.J., Ferreira, L.M.A., Soares, M.A.C. & Fontes, C.M.G.A. (2006) Use of β-glucanases and b-1,4-xylanases to supplement diets containing alfalfa and rye for laying hens: effects on bird performance and egg quality. *Journal of Applied Poultry Research*, **15**: 256–265.

NRC (National Research Council) (1994) *Nutrient Requirements of Poultry*, 9th rev. edn, (Washington, DC, National Academy Press).

Petersen, S.T., Wiseman, J. & Bedford, M.R. (1993) The effect of age and diet on the viscosity of intestinal contents in broiler chicks. *Proceeding of the 10th Meeting of the British Society of Animal Production, Scarborough, UK*, 434.

Petterson, D. & Aman, P. (1989) Enzyme supplementation of a poultry diet containing rye and wheat. *British Journal of Nutrition*, **62**: 139–149.

Philip, J.S., Gilbert, H.J. & Smithard, R.R. (1995) Growth, viscosity and beta-glucanase activity of intestinal fluid in broiler chickens fed on barley-based diets with or without exogenous beta-glucanase. *British Poultry Science*, **36**: 599–603.

Ponte, P.I.P., Ferreira, L.M.A., Soares, M.A.C., Gama, L.T. & Fontes, C.M.G.A. (2004) Xylanase inhibitors affect the action of exogenous enzymes used to supplement Triticum durum-based diets for broiler chicks. *Journal of Applied Poultry Research*, **13**: 660–666.

Sas Institute (2004) edn, *SAS User's Guide: Statistics*, Version 8 edn, (Cary, NC, SAS Institute Inc).

Smith, C.H. & Annison, G. (1996) Non-starch plant polysaccharides in broiler nutrition towards a physiological valid approach to their determination. *World's Poultry Science Journal*, **52**: 203–221.

Taylor, E.J., Goyal, A., Guerreiro, C.I.P.D., Prates, J.A.M., Money, V.A., Ferry, N., Fontes, C.M.G.A. & Davies, G.J. (2005) How family 26 glycoside hydrolases orchestrate catalysis on different polysaccharides: structure and activity of a Clostridium thermocellum lichenase, CtLic26A. *Journal of Biological Chemistry*, **280**(38): 32761–32767.

van der Klis, J.D., van Voorst, A. & van Cruyningen, C. (1993) Effect of a soluble polysaccharide (carboxymethyl cellulose) on the physico-chemical conditions in the gastrointestinal tract of broilers. *British Poultry Science*, **34**: 971–983.

Viveros, A., Brenes, A., Pizarro, M. & Castaño, M. (1994) Effect of enzyme supplementation of a diet based on barley, and autoclave treatment, on apparent digestibility, growth performance and gut morphology of broiler diets. *Animal Feed Science and Technology*, **48**: 237–251.

White, W.B., Bird, H.R., Sunde, M.L., Burger, W.C. & Marlett, J.A. (1981) The viscosity interaction of barley beta-glucan with Trichoderma viride cellulase in the chick intestine. *Poultry Science*, **60**: 1043–1048.