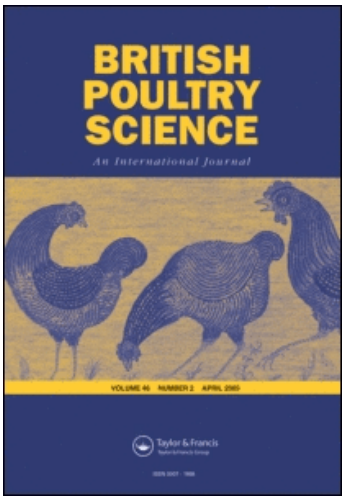

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Role of a family 11 carbohydrate-binding module in the function of a recombinant cellulase used to supplement a barley-based diet for broiler chickens

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Abstract 1. Cellulases and xylanases display a modular architecture that comprises a catalytic module linked to one or more non-catalytic carbohydrate-binding modules (CBMs). CBMs have been classified into 52 different families, based on primary structure similarity. These non-catalytic modules mediate a prolonged and intimate contact of the enzyme with the target substrate eliciting efficient hydrolysis of the target polysaccharides.

2. A study was undertaken to investigate the importance of a family 11 CBM, displaying high affinities for barley β-glucans, in the function of recombinant derivatives of cellulase *Ct*Lic26A-Cel5E of *Clostridium thermocellum* used to supplement a barley-based diet for broiler chicken.

3. The results showed that birds fed on diets containing the recombinant *Ct*Lic26A-Cel5E modular derivatives or the commercial enzyme mixture Rovabio^{TM} Excel AP displayed improved performance when compared with birds fed on diets not supplemented with exogenous enzymes.

4. It is suggested that the enzyme dosage used in this study (30 U/kg of basal diet), was probably too high for the efficacy of the family 11 CBM to be noticed. It remains to be established if the targeting effect resulting from the incorporation of CBMs in plant cell wall hydrolases may be effective at lower exogenous enzyme dosages.

INTRODUCTION

It is widely recognised that inclusion of exogenous β-1,3-1,4-glucanases in barley-based diets and β-1,4-xylanases in wheat- and rye-based diets for poultry improves the efficiency of feed utilisation, enhances growth and contributes to better use of low cost feed ingredients (Chesson, 1993; Bedford, 2000). It is usually agreed that plant cell wall hydrolases improve the nutritive value of cereal-based diets rich in soluble NSPs through a variety of mechanisms. Cellulases and hemicellulases efficiently contribute to reducing the digesta viscosity that is associated with the intake of soluble NSPs, therefore improving the rate of diffusion of substrates, digestive enzymes and nutrients (White *et al*., 1981; Fengler & Marquardt, 1988; Bedford *et al*., 1991; Bedford & Classen, 1992), while increasing the velocity of feed passage (van der Klis *et al*., 1993). In addition, exogenous polysaccharidases 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 & Morgan, 1996; Apajalahti & Bedford, 1999). Finally, plant cell wall hydrolases may release endosperm plant cell wall trapped nutrients that were otherwise unavailable for digestion (Hesselman & Aman, 1986). The action

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of one or a combination of the above-mentioned effects may depend on the type of animal, diet and exogenous enzyme used.

Glycoside hydrolases (EC 3·2·1) that degrade plant cell wall polysaccharides display a complex molecular architecture comprising both catalytic domains and non-catalytic carbohydrate-binding modules (CBMs). The close interaction established between CBMs and plant carbohydrates allows the appended catalytic domain to intimately contact its target substrate, therefore potentiating catalysis (Boraston *et al*., 2004). This proximity and targeting role of CBMs is of extreme importance since the complex interactions established between polysaccharides within the plant cell wall restrict their accessibility to enzyme attack (Guerreiro *et al*., 2006). In addition, some CBMs seem to play a more direct role during polysaccharide degradation by actively contributing to disrupting the carbohydrate structure (Vaaje-Kolstad *et al*., 2005). CBMs are currently grouped into 52 sequence-based families (Coutinho & 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, xylan, mannan, galactan, xyloglucan, arabinan and laminarin have been identified and the molecular determinates of binding specificity described. However, there is a paucity of information concerning the importance of non-catalytic CBMs in the function of exogenous β-1,3-1,4-glucanases used to supplement cereal-based diets for simple-stomached animals. Recently, a family 6 xylan-binding domain was shown to improve the efficacy of a microbial recombinant xylanase, *in vivo*, when used to supplement wheat- and rye-based diets for poultry (Fontes *et al*., 2004). Animals given diets supplemented with a bi-modular xylanase containing catalytic and xylan-binding domains grew significantly faster than animals fed on diets containing only the xylanase catalytic domain. However, the role of β-glucan and cellulose-binding domains in the function of exogenous cellulases used to supplement barley-based diets for poultry remains to be established.

The possibility of using individual recombinant enzymes for feed supplementation allows for the identification of the most efficient enzyme molecular architectures and biocatalyst combinations for the supplementation of various monogastric diets. The objective of this work was to compare the capacity of two truncated derivatives of *Clostridium thermocellum* bi-functional cellulase *Ct*Lic26A-Cel5E, consisting on the enzyme two catalytic modules expressed as an individual entity or fused to its endogenous family 11 CBM, to enhance the performance of poultry fed on barley-based diets. It was previously shown that the family 11 CBM from *Ct*Lic26A-Cel5E is a β-glucan-binding domain that presents significant affinities for both β-1,4- and β-1,3-1,4-mixed linked glucans (Carvalho *et al*., 2004).

MATERIALS AND METHODS

Enzyme preparation

*Clostridium thermocellum Ct*Lic26A-Cel5E is a thermostable bi-functional enzyme containing β-1,3-1,4-glucanase (GH26) and β-1,4-cellulase (GH5) catalytic domains and two non-catalytic modules. 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 GH26, followed by a second GH5 catalytic module, 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. 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).

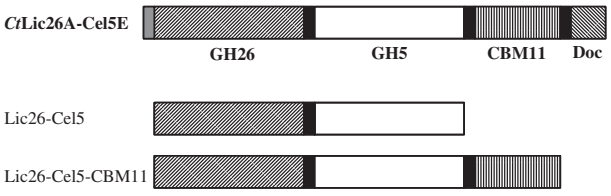

Figure 1. *Domain organization of CtLic26A-Cel5A and its truncated derivatives Lic26-Cel5 and Lic26-Cel5-CBM11 used in this study. The β-glucanase (GH26), cellulase (GH5), β-glucan-binding domain (CBM11) and the dockerin (Doc) are indicated. The grey and the black boxes represent the linker sequences and the signal peptide, respectively.*

Animals, diets and management

The composition of the barley-based diet used in this study (Table 1) was formulated to contain adequate nutrient levels as defined by the NRC (1994), with an energy content slightly below the recommended values. The basal diet was supplemented with no enzyme (C0) or with 30 U/kg of Lic26-Cel5E or Lic26-Cel5E-CBM11. In addition, a fourth treatment corresponded to the supplementation of the basal diet with a calculated 15 U/kg of the commercial enzyme cocktail Rovabio™ Excel AP (Adisseo, France; treatment termed Rov for simplification), which corresponds to an incorporation ratio of 50 g of enzyme per ton of feed as recommended by the fabricant. A total of 160 one-day-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 from automatic drinking nipples and a hanging feeder, respectively. The brooders were located in an environmentally controlled room, which was adjusted daily to the recommended temperature, 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 assigned to 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.

Analytical procedures

To standardise the number of enzyme units used to supplement the basal diet, the catalytic activity of the various exogenous enzymes, including the commercial mixture, was determined under identical experimental conditions. Catalytic activity was determined at 40°C by measuring reducing sugar released, following the method described by Taylor *et al*. (2005), using barley β-glucan (Megazyme®, Ireland) as the substrate. One unit of catalytic activity is defined as the amount of enzyme required to release one 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 Na–Hepes buffer, pH 7·5, which was followed by overnight incubation at 4°C with gentle agitation and a centrifugation at 13 000 × *g* for 5 min. 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®, Ireland) 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 with the Congo Red assay plate (Ponte *et al*., 2004; 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®, Ireland), 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 overnight 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.

Table 1. *Ingredient composition and estimated 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 kilogram 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, thiamin 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.

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) 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 (1994). Means with a significant F ratio were separated by the least significant difference test. The experimental unit was a cage of 4 birds. Unless otherwise stated, differences were considered significant when $P < 0{\cdot}05$.

RESULTS AND DISCUSSION

In order to evaluate the importance of a family 11 CBM in the function of a recombinant β-glucanase *in vivo*, truncated forms of the modular cellulase *Ct*Lic26A-Cel5E of *C. thermocellum*, with or without the enzyme's β-glucan-binding domain, were produced and used to supplement a barley-based diet for broiler chicks. The basal diets were prepared, supplemented with the required enzymes and used to feed broiler chicks *ad libitum* from *d* 1 to 28. During the experiment, the mortality was low (3·1%) and was not related to treatments (anatomopathological results not shown).

Bird performance

Results on body weight, weight gain, feed intake and gain : feed ratios throughout the experiment are summarised in Table 2. The data show that final body weight of birds fed on the barley-based diets supplemented with exogenous polysaccharidases was significantly higher than that of the control birds not supplemented with the microbial biocatalysts. Differences in body weight were visible as soon as *d* 7 and remained significant throughout the duration of the experiment. Differences in body weight and weight gain in birds receiving the recombinant cellulases, with or without the family 11 CBM, or the commercial enzyme mixture were not significant. In addition, feed intake was higher in birds fed on diets supplemented with β-glucanases, especially in the first 3 weeks of the experiment. During the last week of the trial, broiler chickens fed on the basal diet not supplemented with the exogenous

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 *CtLic26A-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	42·4	42·6	42·2	42·1	0·15	NS
7 d	142·8^{b}	154·9^{a}	154·1^{a}	148·1^{ab}	3·21	0·028
14 d	331·7^{b}	366·7^{a}	362·6^{a}	355·5^{a}	8·11	0·014
21 d	674·1^{b}	739·7^{a}	725·1^{a}	732·6^{a}	13·43	0·004
28 d	1158·0^{b}	1243·4^{a}	1240·4^{a}	1247·9^{a}	21·31	0·009
Weight gain (g)						
0–7 d	100·5^{b}	112·4^{a}	111·8^{a}	106·0^{ab}	3·20	0·030
7–14 d	188·9^{b}	211·6^{a}	208·6^{a}	207·5^{a}	5·75	0·023
14–21 d	342·5^{b}	373·0^{a}	362·4^{a}	377·1^{a}	7·59	0·008
21–28 d	484·0	503·8	515·4	512·4	10·54	NS
0–28 d	1115·7^{b}	1200·8^{a}	1198·2^{a}	1205·9^{a}	21·29	0·009
Feed intake[1] (g)						
0–7 d	120·6^{b}	138·6^{a}	138·2^{a}	128·8^{ab}	3·81	0·003
7–14 d	278·8^{b}	316·3^{a}	318·6^{a}	309·2^{a}	10·21	0·024
14–21 d	552·4b	609·9^{a}	587·8^{ab}	604·5^{a}	16·88	0·066
21–28 d	851·6	866·9	888·8	895·7	23·70	NS
0–28 d	1803·3^{b}	1931·4^{a}	1933·2^{a}	1937·7^{a}	42·10	0·063
Gain:feed ratio						
0–7 d	0·834	0·810	0·809	0·831	0·0173	NS
7–14 d	0·714	0·669	0·639	0·671	0·0337	NS
14–21 d	0·610	0·620	0·621	0·624	0·0148	NS
21–28 d	0·570	0·526	0·581	0·576	0·0239	NS
0–28 d	0·618	0·602	0·619	0·623	0·0131	NS

[a,b]Values in a row not sharing a common superscript are significantly different ($P < 0{\cdot}05$).
[1]For feed intake the p(F) values and significant differences were presented when $P < 0{\cdot}1$.

polysaccharidases had similar feed intakes when compared to birds from the other treatments. Since there were no differences between the gain : feed ratios of the 4 groups, the data suggest that an improvement in feed intake was responsible for the better performances displayed by birds fed on diets containing the microbial cellulases. Taken together, the results suggest that, at these levels of enzyme incorporation, the family 11 CBM of *Ct*Lic26A-Cel5E of *C. thermocellum* did not improve the biological effectiveness of the associated glycoside hydrolase catalytic modules, when the recombinant enzymes were used as a supplement for barley-based diets for poultry.

The effect of including exogenous cellulases in barley-based diets in the relative length and weight of the birds' organs and GI tract compartments were evaluated (Table 3). Enzyme supplementation had no effect on crop, gizzard or liver relative weights or on the lengths of duodenum, jejunum and caecum. In contrast, ileum relative length was significantly reduced ($P < 0{\cdot}05$) in birds receiving the commercial enzyme mixture. In addition, diet supplementation with exogenous β-glucanase activities significantly contributed to decrease the viscosity of small intestine contents (Table 3). It is well established that moderate and high concentrations of soluble NSPs in diets for broiler chicken contribute to increasing the size of specific compartments of the bird's GI tract (Brenes *et al*., 1993; Petersen *et al*., 1993). Solubilisation of structural polysaccharides contributes to increasing digesta viscosity, as observed in birds not receiving the exogenous enzymes, and this may contribute to a decreased rate of feed passage. Therefore, the lower feed intakes presented by birds given the control treatment may result from a higher digesta viscosity and a decreased feed passage rate. In addition, the increased viscosity and the decreased digesta passage rate contribute to increase the digesta bulk in the GI tract. As a consequence of this phenomenon the length of the small intestine is increased, which constitutes a potential physiological adaptation allowing for an improvement in feed consumption and nutrient uptake. However, although dietary cellulases and hemicellulases contribute to decreasing intestine length (Brenes *et al*., 2002), it is unknown why the recombinant enzymes used in the present study were not effective for decreasing ileum length such as was observed for the small intestine of birds receiving the commercial mixture.

Results presented here highlight the capacity of single recombinant cellulases to enhance 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) suggested 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 broiler chicken. It has been previously shown that 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 & Marquardt, 1988; Bedford & Morgan, 1996). Therefore, the data presented in this report indicates that single purified recombinant cellulases or enzyme

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 *CtLic26A-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	3·86	3·90	4·32	4·62	0·230	NS
Gizzard	14·59	14·60	12·83	13·85	0·924	NS
Liver	30·35	30·09	31·42	28·93	1·002	NS
Relative length (cm/kg BW)						
Duodenum	19·7	18·0	18·6	19·4	0·54	NS
Jejunum	55·7	50·9	51·8	52·2	1·51	NS
Ileum	55·9[a]	50·7[b]	54·5[a]	52·8[ab]	1·34	0·044
Caecum	13·7	13·8	13·4	14·5	0·32	NS
Contents viscosity (cpo)						
Duodenum + jejunum	11·30[a]	5·53[b]	7·10[b]	7·24[b]	1·40	0·039
Ileum	16·98[a]	8·95[b]	10·26[b]	9·61[b]	1·83	0·009

[a,b]Values in a row not sharing a common superscript are significantly different ($P < 0{\cdot}05$).

mixtures containing cellulases can perform this action equally. However, since the recombinant enzymes were incorporated in the barley-based diet at twice the concentration used by the commercial enzyme mixture it is presently unknown if at similar incorporation rates the exogenous enzymes could fulfil their roles with similar efficiencies. Only in that case could the presence of non-cellulase activities, such as xylanase, mannanase, galactanase or pectinase, in enzyme mixtures for supplementation of barley-based diets be considered redundant.

It is well established that CBMs 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 particularly 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. The family 11 CBM of *Ct*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 in the hydrolysis of insoluble substrates (Gilbert *et al*., 2002). We have previously shown that, although the molar activity of the recombinant cellulases Lic26-Cel5E-CBM11 and Lic26-Cel5E against barley β-glucan is similar, the presence of the family 11 CBM promotes the action of the modular cellulase against insoluble cellulose forms, such as Avicel (Taylor *et al*., 2005). Since barley-based diets are relatively poor in insoluble polysaccharides, we envisage that the major contribution of the family 11 CBM of *Ct*Lic26A-Cel5E would be related to the targeting of the associated catalytic domains to the anti-nutritive soluble β-glucans. However, data presented here revealed that the family 11 CBM was unable to affect the efficiency of the GH5 and GH26 catalytic domains *in vivo*, as reflected by the inability of the tri-modular enzyme to improve the nutritive value of a barley-based diet for poultry when compared with the recombinant bi-modular cellulase lacking a non-catalytic CBM. Nevertheless, it is possible that a lack of efficacy of the family 11 CBM is related to the relatively high dosage of recombinant enzyme used in this study. In fact, the level of enzyme used for feed supplementation could have been so high that there would always be enzyme available in the proximity of the anti-nutritive substrate. Therefore, it is possible that the ultimate effect of the family 11 CBM in directing the recombinant enzyme to its target β-glucans may be revealed only at lower levels of enzyme supplementation. In addition, it is also possible that a fraction of the recombinant enzyme containing the non-catalytic domain may be retained by the insoluble cellulose, therefore decreasing the effective concentration of the cellulase available for depolymerising the anti-nutritive β-glucans. If these two factors, individually or in conjunction, contributed for the lack of effectiveness of the family 11 CBM in the particular conditions of the present experiment, remains to be evaluated.

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 data, exemplified in Figure 2 and presented in Table 4, demonstrated that cellulase activity could be detected along the entire digestive tract of most animals fed on diets supplemented with the plant cell wall hydrolases. In addition, 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.

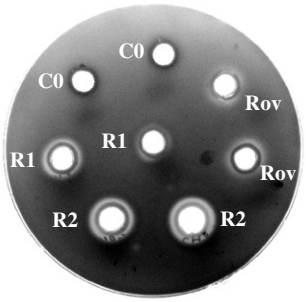

Figure 2. *Detection of β-glucanase activity in the crop contents of broilers fed on barley-based diet not supplemented (C0) or supplemented with a commercial cellulase mixture (Rov) or truncated derivatives of* C. thermocellum *CtLic26A-Cel5E β-glucanase containing (R2, enzyme Lic26-Cel5E-CBM11) or not containing (R1, enzyme Lic26-Cel5E) a family 11 CBM.*

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 *CtLic26A-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
Crop	2	7	8	7
Gizzard	0	3	3	4
Duodenum	0	3	3	3
Jejunum	0	0	3	−2
Caecum	10	10	10	10

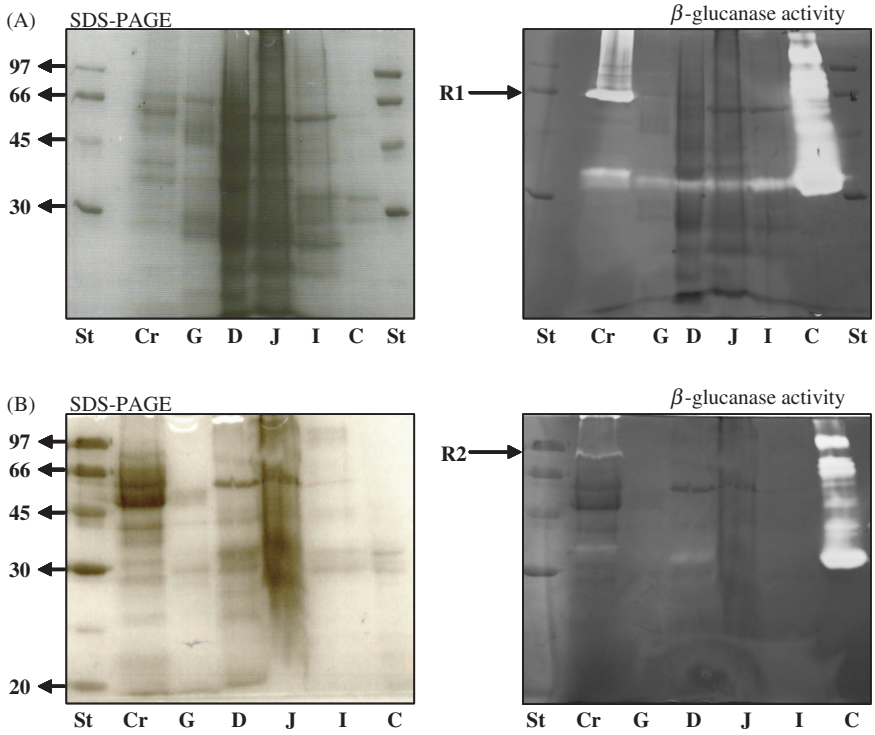

Figure 3. *Zymogram analysis of digesta samples collected from various regions of the GI tract of birds fed on a barley-based diet supplemented with the recombinant β-glucanases Lic26-Cel5 (Panel A) or Lic26-Cel5-CBM11 (Panel B). Proteins were fractionated through SDS-PAGE and stained for β-glucanase activity after enzyme renaturation. Abbreviations: St, low molecular weight protein standards; Cr, crop; G, Gizzard; D, duodenum; J, Jejunum; I, Ileum; C, Caecum. The location of the two exogenous recombinant enzymes is highlighted (R1, Lic26-Cel5 and R2, Lic26-Cel5-CBM11).*

However, two of the animals from this negative control group had low but detectable β-glucanase activity in the crop. Therefore, taken together, the results suggest that a considerable percentage of the exogenous enzymes resist to the acidic and proteolytic conditions that are prevalent in some portions of the digestive tract. 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 3, indicate that both Lic26-Cel5E-CBM11 and Lic26-Cel5 are prone to proteolytic cleavage in the GI tract, initially but moderately in the crop and then completely in the gizzard and in the following GI compartments. Therefore, it is suggested that both recombinant enzymes are proteolytically cleaved in the linker regions connecting the Lic26, the Cel5 and the CBM11 modules, which contributes to release the two 32–35-kDa catalytic domains that still retain significant catalytic activity. Experiments performed *in vitro*, using purified Lic26-Cel5E-CBM11 and Lic26-Cel5, demonstrated that incubation of both recombinant cellulases with a pancreatic extract of peptidases (EC 3·4) lead to a proteolytic cleavage of the recombinant enzymes at the linker sequences, as it was observed here *in vivo* (Guerreiro, unpublished data). The data confirmed that this phenomenon does not affect the biological capacity of the resulting catalytic domains to degrade purified soluble β-glucans *in vitro* (Guerreiro, unpublished data), as was previously demonstrated by Taylor *et al*. (2005). However, it is possible that the proteolytical removal of the β-glucan-binding domain from the Lic26-Cel5E-CBM11 could have contributed to reduce its capacity to interact with the antinutritive soluble polysaccharide in the duodenum and the subsequent GI compartments. If the similar efficacy presented by the two recombinant enzymes analysed in this study *in vivo*, results from the proteolytic transformation of both microbial recombinant enzymes in similar polypeptides after the crop, remains to be established. Notwithstanding this possibility, it was recently shown that the proteolytic release of a xylan-binding domain from a recombinant xylanase in the duodenum of broiler chicks have no effect on the capacity of the recombinant enzyme to improve the nutritive value of a wheat-base diet, when compared with the xylanase derivative consisting only on the enzyme catalytic domain (Fontes *et al*., 2004).

CONCLUSIONS

Results presented in this report suggest that individual recombinant cellulases could be as effective as complex mixtures of glycoside hydrolases in attenuating the detrimental effects of the soluble polysaccharides found in barley-based diets. In addition, the data suggest that, when incorporated at high dosage rates (30 U/kg of basal diet), a tri-modular cellulase containing a family 11 CBM and its double-domain counterpart consisting of the enzyme's catalytic modules have equal capacities to improve the nutritive value of a barley-based diet. Furthermore, both recombinant enzymes were prone to peptidolysis in the birds' GI tract. It is suggested that this process, although not affecting the capacity of the resulting enzymes to degrade the anti-nutritive β-glucans *in vitro*, may have influenced the capacity of Lic26-Cel5E-CBM11 to act *in vivo*. The capacity of the non-catalytic family 11 CBM to elicit the function of *Ct*Lic26A-Cel5E recombinant derivatives, when incorporated at lower dosages rates in barley-based diets for poultry, is currently under investigation.

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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's Poultry Science Journal*, **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 sue of broiler chickens fed wheat- and barley-based diets. *Poultry Science*, **72**: 1731–1739.

Brenes, A., Marquardt, R.R., Guenter, W. & Viveros, A. (2002) Effect of enzyme addition on the performance and gastrointestinal tract size of chicks fed lupin seed and their fractions. *Poultry Science*, **81**: 670–678.

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 – Carbo hydrate-Active EnZYmes. http://afmb.cnrs-mrs.fr/CAZY

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-funcional 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.

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, in: *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**: 8815–28.

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.

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 β-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. *Proceedings of the 10th Meeting of the British Society of Animal Production*, Scarborough, UK, p. 434.

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

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, *Ct*Lic26A. *Journal of Biological Chemistry*, **280**: 32761–32767.

VAAJE-KOLSTAD, G., HORN, S.J., VAN AALTEN, D.M., SYNSTAD, B. & EIJSINK, V.G. (2005) The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *Journal of Biological Chemistry*, **280**: 28492–28497.

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.

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.